The effect of metformin intervention on the programming of cardiometabolic health in offspring of obese pregnancy

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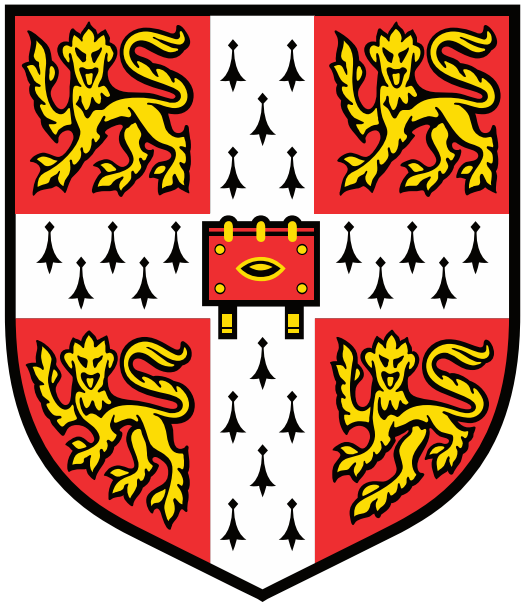

Department of Clinical Biochemistry

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This dissertation is submitted for the degree of Doctor of Philosophy.

This thesis is dedicated to my mother Vera, who has been the biggest support on my academic journey and would have been so proud to see this thesis

Declaration

The work presented in this thesis was carried out between October 2017 and March 2021 at the Department of Clinical Biochemistry. This thesis is the result of my own work and any work done in collaboration is specified in the text. It is not substantially the same as any work that has already been submitted before for any degree or other qualification. The thesis does not exceed the prescribed word limit of 60,000 words for the Clinical and Veterinary Medicine Degree Committee excluding tables, footnotes, bibliography, and appendices.

Signed Date

Josca Mariëtte Schoonejans **30 March 2021**

Publications

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Abstracts

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Summary

Obesity and/or diabetes during pregnancy is associated with obesity, metabolic and cardiovascular disease in the offspring. Clinically relevant interventions are required to break the transgenerational cycle of obesity. Metformin is used to treat GDM in the UK and RCTs are investigating metformin as a candidate for implementation in pregnancies complicated by obesity or polycystic ovary syndrome. However, metformin crosses the placenta and long-term offspring follow-up is sparse in both humans and animal models. Data throughout the life-course, investigation of potentially sexually dimorphic effects and cardiovascular outcomes are especially lacking. Using a mouse model, this thesis aimed to determine the short- and long-term effects on adiposity and metabolic health in male and female offspring in early life (neonatal growth trajectory, Chapter 3), young adulthood (8 weeks, Chapter 4) and older age (12 months, Chapter 5). It also aimed to investigate cardiovascular phenotypes in adult offspring at different points across the life-course (3, 6 and 12 months of age, Chapter 6).

Exposure to an obesogenic diet from pre-conception and during pregnancy and lactation increased maternal bodyweight, fat mass and food intake. Maternal obesity introduced hypertrophic adiposity in both male and female offspring at 8 weeks of age and this increased adiposity was maintained until 6 months. After 6 months, sex differences emerged: male offspring of obese dams were heavier and showed hypertrophic and hyperplastic adiposity with adipose tissue inflammation and insulin resistance, whereas adiposity in female offspring became less prominent with age.

Maternal metformin treatment decreased maternal fat mass and this difference was maintained until late pregnancy. The metformin intervention also increased gestation length and did not prevent the intrauterine growth restriction and catchup growth seen in offspring of obese pregnancy. In offspring, the metformin intervention did not correct the adiposity phenotype programmed by maternal obesity, but instead led to exaggerated adiposity and adipose tissue dysfunction in a sex- and a time-dependent manner. In both sexes, exposure to metformin during obese pregnancy caused adipocyte hypertrophy at 8 weeks of age prior to development of obesity. In males this was associated with adipocyte hyperplasia, inflammation and local insulin resistance. Although male offspring of control-fed and obese dams underwent age-related adipocyte hyperplasia, adipocyte number failed to increase in ageing metformin-exposed male offspring. Combined with more extensive macrophage infiltration and increased liver weight this leads to the hypothesis that adipose tissue expansion capacity is restricted in metformin-exposed males, resulting in adipose tissue dysfunction and ectopic lipid deposition. In female metformin-exposed offspring, adiposity in young adulthood was associated with alternate macrophage activation and an improvement in whole-body insulin sensitivity. However, after 6 months of age female metformin-exposed offspring diverged from other groups in

body weight and fat mass, showing inflammatory adiposity at 12 months of age associated with both peripheral and systemic insulin resistance as well as hyperinsulinaemia.

Maternal obesity also affected cardiovascular health of male and female offspring. In males, offspring of obese dams had diastolic dysfunction leading to pressure overload and hyperdynamic systolic function in absence of changes in blood pressure. In contrast, female offspring of obese dams had increased blood pressure throughout life associated with diastolic dysfunction at 3 months. The metformin intervention corrected some of the changes that were observed with maternal obesity. In male offspring, prenatal metformin halted progression of diastolic dysfunction with age but also introduced other independent cardiovascular changes. In female offspring, prenatal metformin corrected the hypertension seen in young offspring of obese dams, but by 12 months of age female metformin-exposed offspring had developed obesity-induced hypertension.

In conclusion, this thesis reports sex- and age-specific effects of maternal obesity and prenatal metformin intervention, illustrating the need for short- and long-term follow-up of both male and female offspring exposed to prenatal interventions.

Table of Contents

Abbreviations

AET	Aortic ejection time
AMPK	AMP-activated Protein Kinase
Atgl	Adipose triglyceride lipase
BAT	Brown Adipose Tissue
BMI	Body Mass Index
BP	Blood Pressure
Ccr2	C-C Motif Chemokine Receptor 2
Cd11c	Cluster of Differentiation 11c (Integrin Alpha X)
Con	Offspring of Control dams
CLS	Crown-Like Structures
CO	Cardiac Output
CRP	C-reactive Protein
CVD	Cardiovascular disease
DBP	Diastolic Blood Pressure
DIO	Diet-induced Obesity
DOHaD	Developmental Origins of Health and Disease
DT	Deceleration Time
E19	Embryonic day 19
EF	Ejection Fraction
EDV	End-Diastolic Volume
EMPOWaR	Efficacy of Metformin in Pregnant Obese Woman, a Randomised Controlled Trial
eWAT	epididymal White Adipose Tissue
FFA	Free Fatty Acid
FS	Fractional Shortening
GDM	Gestational Diabetes Mellitus
Glut4	Glucose Transporter type 4
GTT	Glucose Tolerance Test
gWAT	gonadal White Adipose Tissue
GWG	Gestational Weight Gain
HF	Heart Failure
HFD	High Fat Diet

HFHS	High Fat High Sugar diet
HFpEF	Heart Failure with Preserved Ejection Fraction
HFrEF	Heart Failure with Reduced Ejection Fraction
Hif1α	Hypoxia-Inducible Factor 1 alpha
HOMA	Homeostatic Model Assessment index
HR	Heart Rate
Hsl	Hormone Sensitive Lipase
IGT	Impaired Glucose Tolerance
Il	Interleukin
iNos	inducible Nitric Oxide Synthase
ipGTT	intraperitoneal Glucose Tolerance Test
IR	Insulin Resistance
IUGR	Intrauterine Growth Restriction
IVCT	Isovolumetric Contraction Time
IVRT	Isovolumetric Relaxation Time
LBW	Low Birth Weight
LGA	Large for Gestational Age
LAP	Left Atrial Pressure
LPS	Lipopolysaccharide
LV	Left Ventricle
LVEDP	Left Ventricular End-Diastolic Pressure
LVOT	Left Ventricular Outflow Tract
M1	classically activated Macrophage
M2	alternatively activated Macrophage
Mcp1	Monocyte chemoattractant protein-1 (C-C motif Chemokine Ligand 2)
MiG	Metformin In Gestational diabetes
Mip1a	Macrophage Inflammatory Protein 1 alpha (C-C motif Chemokine Ligand 3)
MOP	Metformin in Obese Pregnancy
MPAP	Mean Pulmonary Artery Pressure
MPI	Myocardial Performance Index
mSAX	modified Short-Axis
mTORC1	Mammalian Target of Rapamycin Complex 1

NFT	Non-Filling Time
NICE	National Institute for Health and Care Excellence
Ob	Offspring of Obese dams
Ob-Met	Offspring of Obese Metformin-treated dams
OE-NPY	NPY-overexpression in brain and peripheral central nervous system
oGTT	oral Glucose Tolerance Test
PAT	Pulmonary artery Acceleration Time
PCOS	Polycystic Ovary Syndrome
PET	Pulmonary artery Ejection Time
PN2	Postnatal day 2
Pparg	Peroxisome Proliferator-Activated Receptor Gamma
PR	Pulse Rate
PregMet	Metformin in Pregnant PCOS Women
PSLAX	Parasternal Long-Axis
PTB	Preterm Birth
PW	Pulsed Wave
RAS	Renin-Angiotensin System
RCT	Randomised Controlled Trial
ROS	Reactive Oxygen Species
RV	Right Ventricle
SBP	Systolic Blood Pressure
SGA	Small for Gestational Age
SV	Stroke Volume
T2DM	Type 2 Diabetes Mellitus
TD-NMR	Time-Domain Nuclear Magnetic Resonance
Tnfα	Tumour Necrosis Factor-alpha
TPR	Total Peripheral Resistance
Ucp1	Uncoupling Protein 1
VAT:SAT	Visceral to Subcutaneous Adipose Tissue ratio
VTI	Velocity Time Integral
WAT	White Adipose Tissue
WHO	World Health Organization

List of Tables

List of Figures

Appendices

1 General introduction

1.1 The obesity epidemic

The prevalence of obesity is rising at sufficiently rapid rates that the World Health Organisation (WHO) speaks of a global obesity epidemic (1). According to the WHO, the prevalence of adult obesity world-wide was 13% (Body Mass Index or BMI>30kg/m^2) in 2016, while a further 39% of adults were overweight (BMI 25-30kg/m^2)(2). This means that >50% of the adult population worldwide is currently overweight or obese. However, these rates differ between countries (Figure 1.1) and the prevalence of overweight and obesity in several countries including the UK is in fact even higher (Table 1.1)(3). Moreover, the problem is not limited to the current generation of adults since childhood obesity is also becoming increasingly common (4). The obesity epidemic is thus unlikely to improve, but instead become more substantial in the future.

% of UK population	1975	1990	2016
Obesity (BMI >30)	9.4	14.0	27.8
men	7.8	12.4	26.9
women	10.8	15.6	28.6
Overweight & obese (BMI >25)	41.9	50.2	67.2
men	43.8	53.3	68.6
women	36.5	44.3	58.9
Worldwide rates			52
Obese (BMI >30)			13
Overweight (BMI 25-30)			39

Table 1.1: UK prevalence of obesity and overweight in 2016, with world-wide reference point for 2016.
Source: https://ourworldindata.org/obesity. Data from WHO, available at http://apps.who.int/gho/data/view.main.REGION2480A

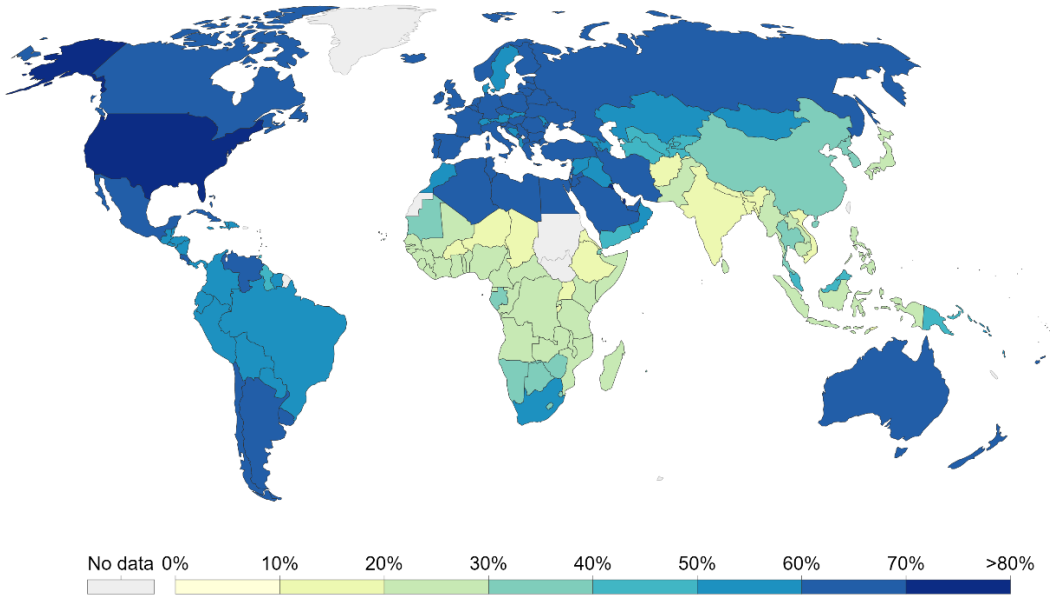

Figure 1.1: Share of adult individuals that were overweight or obese in 2016.
Reused with permission from (550)*, using data from WHO published previously* (551)

This rising prevalence is a substantial public health burden on a global scale because obesity is associated with an array of direct and indirect adverse health outcomes in the affected individual. Obesity is often accompanied by (and is a risk factor for) the metabolic syndrome: a syndrome of multiple pathologies encompassing abdominal obesity, dyslipidaemia, insulin resistance (IR), glucose intolerance and hypertension (5). Consequently, obese individuals are at higher risk of dying from ischaemic heart disease and complications arising from type 2 diabetes mellitus (T2DM). This is not only the case for adult obesity, but also childhood obesity which in itself is associated with increased risk of obesity in adulthood as well as chronic cardiovascular and metabolic disease (6–8). In addition, obesity can lead to problems with fertility, increase risk of certain cancers and even vulnerability to the detrimental effects of some infectious diseases as observed in the current COVID-19 pandemic (9). The increased mortality resulting from the comorbidities of obesity is therefore a substantial contribution to the global health burden. Thus, the WHO has included stopping the rise in obesity as a target in its Global Action Plan for non-communicable disease prevention (10).

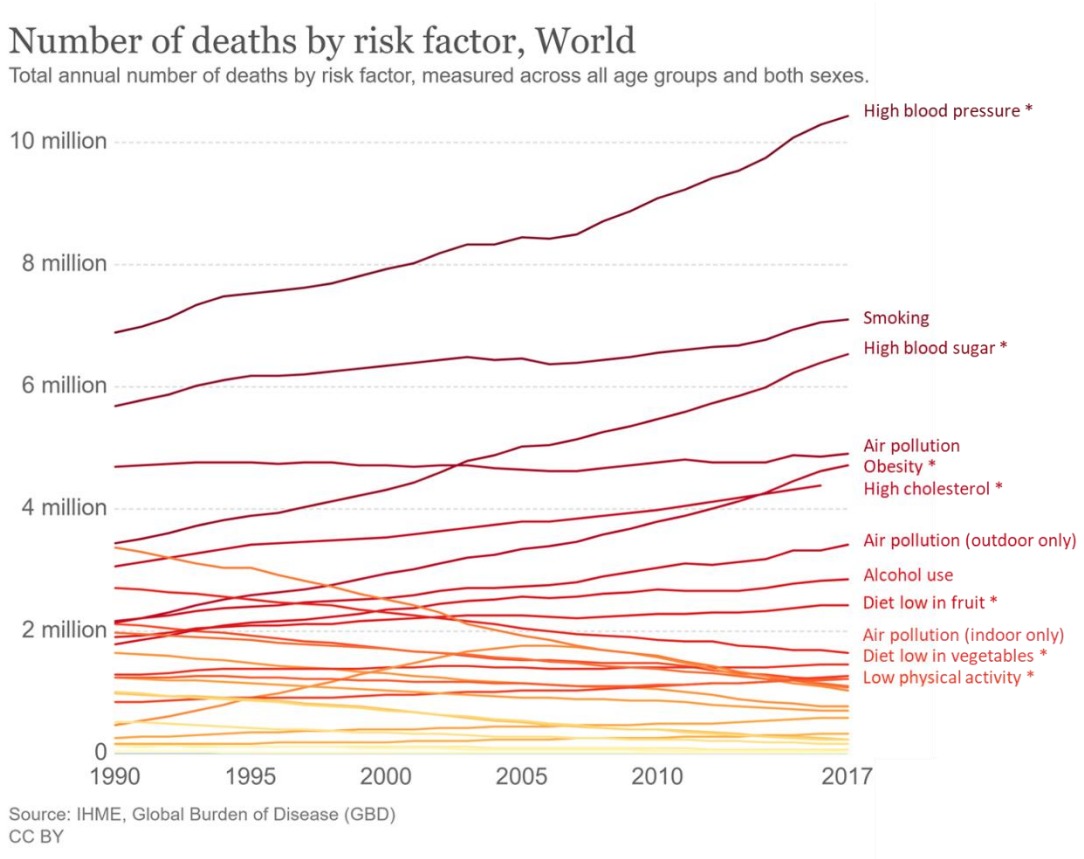

Figure 1.2: Number of world-wide deaths by risk factor.
The 10 risk factors with the highest numbers of deaths in 2017 are labelled. Risk factors with an asterisk are associated with obesity (either as comorbidity or as risk factor for obesity itself). Adapted with permission from* (550) *using data published previously* (490).

The cause of this rapid increase is likely multifactorial. Compared to only thirty years ago, the relative abundance of often calorie-dense food in combination with an increasingly sedentary lifestyle means we are currently living in an age of energy excess, facilitating the development of obesity. Moreover, diets rich in saturated fats, sugar or processed food can act as independent risk factors for cardiometabolic disease (11), thus also affecting public health independently of effects on BMI.

In addition to lifestyle factors, genetic background is known to influence risk of becoming obese. Monogenic disorders associated with extreme obesity, such as *MC4R* or leptin deficiency, although rare, provide insights into pathophysiology. GWAS studies to identify more common genetic variants related to obesity have identified many loci with variants associated with increased BMI, each conferring a small increase in obesity risk (0.1 BMI unit per locus, according to a recent meta-analysis of GWAS for obesity)(12). However, such common genetic variants are estimated to explain only 20% of the variation in BMI on a population level and thus cannot be wholly responsible for the obesity epidemic (12).

The rise in obesity and heart disease therefore cannot be explained by genetics and current lifestyle alone (13). In recent years, it has become widely accepted that the environment we are exposed to in the womb and during the first months of life may influence our risk of becoming obese as well as developing its comorbidities. This has been termed the Developmental Origins of Health and Disease (DOHaD, see section 1.4).

1.2 Maternal obesity

The obesity epidemic has resulted in high prevalence of obesity in women of reproductive age. In fact, over 50% of women of child-bearing age in the UK are currently overweight or obese (14). A proportion of these women will become pregnant, thus increasing the number of pregnancies complicated by maternal obesity. Indeed, the prevalence of maternal obesity during pregnancy tracks with the rate of obesity in the general population of England (15). This is not only associated with adverse metabolic and cardiovascular outcomes in the adult female (16), but also poses substantial risks to both mother and child should she become pregnant.

Obesity negatively affects fertility, indicated by ovulatory problems and increased time to conception in overweight and obese women (17–19). Once pregnant, obese women are at increased risk of miscarrying, especially during early pregnancy (20). Perinatal complications are also more common in obese pregnancy, with higher rates of premature delivery, post-partum haemorrhage and C-section (21–23). Stillbirth, perinatal or early infant death are also found to be increased (24). Moreover,

obesity during pregnancy predisposes to the development of several disorders of pregnancy, such as gestational diabetes mellitus (GDM) and preeclampsia (25). Although IR in late gestation is a normal physiological adaptation to ensure sufficient glucose delivery to the fetus, in some cases this develops into pathological IR and may lead to GDM (26,27). GDM is the most common metabolic complication in pregnancy and affects 1 in 7 pregnancies world-wide, although prevalence estimates vary with age, ethnicity and diagnostic criteria (28,29). GDM is normally diagnosed through an oral glucose tolerance test (oGTT) performed at an antenatal visit in at risk women, usually in the second or third trimester (30). In obese women, the 'Homeostatic Model Assessment for Insulin Resistance' (HOMA-IR) index is consistently higher during and after pregnancy compared to normal weight pregnant women, and increases more steeply with progressing gestation (31). Indeed, according to a meta-analysis of 20 studies, obese women are almost four times as likely to develop GDM (25), with risks even higher for severely obese women. Glucose intolerance is usually resolved soon after delivery, but GDM women remain at increased risk of developing T2DM in the future (27). Preeclampsia is an extreme form of gestational hypertension characterised by hypertension and proteinuria diagnosed after 20 weeks gestation (32). Risk of GDM and preeclampsia in a future pregnancy is especially high in women that fail to return to their pre-pregnancy weight after delivery (33). Furthermore, interpregnancy weight gain will also increase the number of women entering their second pregnancy with obesity and/or impaired glucose control, leading to knock-on effects of maternal obesity. Lastly, both GDM and preeclampsia are thought to contribute to the risk of prematurity and offspring death in obese pregnancy (3).

Maternal obesity during pregnancy can also have effects on the fetus. Babies born to overweight or obese mums have higher chance of being born macrosomic (>4500g) or large for gestational age (LGA)(34,35), which can lead to complications at delivery and admission to neonatal intensive care, especially in pregnancies complicated by diabetes (36,37). Paradoxically, obese pregnancies are also more likely to result in low birth weight (LBW) or small for gestational age (SGA) babies (23,35). Lastly, maternal obesity confers some increased risk of congenital abnormalities, including but not limited to neural tube defects, cleft palate and cardiac abnormalities (38). These observations highlight the potential impact that interventions to reduce obesity during pregnancy could have on both mother and baby.

In addition to these immediate detrimental effects on the baby, it has recently become more recognised that maternal obesity can also have long-lasting effects on offspring health.

1.3 Concept of developmental programming

1.3.1 The Thrifty Phenotype hypothesis

The notion that the intrauterine environment affects long-term offspring health was first described by David Barker and Nick Hales around thirty years ago. Population studies in England and Wales showed that death from ischaemic heart disease was common in poorer areas, and significantly correlated to both infant and maternal mortality fifty years prior (39,40). It was suggested that adverse development due to poor maternal health and nutrition could be the common denominator leading to this increased risk of cardiovascular mortality, which was previously associated with wealth and Western-style diets.

The work of David Barker provided further evidence for the relationship between intrauterine growth and cardiovascular disease (CVD) risk. In men born in Hertfordshire between 1911-1930, body weight at one year and (to a lesser extent) LBW were associated with increased risk of ischaemic heart disease mortality (41). Other cohorts also found an inverse relationship between LBW and systolic blood pressure (SBP) in children and/or adult men and women in the UK (42,43). Interestingly, some individuals of normal birth weight were hypertensive only if their placentas were large, and the highest SBP was observed when LBW coincided with high placental weight (42). This suggests that discrepancy between placental potential and birth weight, a proxy for placental efficiency, could be involved in the programming of cardiovascular risk factors. In contrast to LBW, current body weight positively correlated with SBP, and SBP was highest in people with the lowest birth weight and the highest current body weight (43).

Follow-up of the Hertfordshire cohort by Nick Hales showed a similar relationship with metabolic disease: men with the lowest birth weight had the highest chance of developing impaired glucose tolerance (IGT) or T2DM and showed higher glucose and insulin levels during an oGTT (44). As for SBP, 2-hour glucose was also highest in those with the lowest body weight at one year and highest adult BMI, suggesting an interaction between the pre- and postnatal environments. Follow-up studies confirmed the association with LBW also held true for the metabolic syndrome (45). The association between LBW and cardiometabolic disease risk was replicated in the Helsinki birth cohort, where indices of IR and CVD were related to low maternal body weight (sign of poor maternal nutrition) and LBW as well as rapid postnatal growth, suggesting 'catch-up growth' following suboptimal development *in utero* (46,47). Excessive postnatal growth may reflect a 'mismatch' between the intrauterine and the postnatal environment.

Barker and Hales hypothesised that LBW was a sign of suboptimal nutrition in the womb. In 1992, they formulated the 'Thrifty Phenotype hypothesis', which postulates that intrauterine exposure to poor

nutrition (whether through maternal malnutrition or placental insufficiency) leads to 'thrifty' metabolic adaptations by the fetus that would enhance their survival in a postnatal environment where resources are scarce (e.g. by promoting energy storage in adulthood)(48). If the child is instead born into an environment where food is plentiful, these changes may be 'maladaptive' and consequently predispose them to obesity, cardiovascular and metabolic disease in adulthood. Indeed, studies in various populations have since supported and expanded the hypothesis of Barker and Hales (reviewed in (49); see 1.3.2).

1.3.2 Evidence from historical famines

Another line of evidence linking maternal malnutrition to long-term health emerged from clinical records collected during World War II, when Nazi occupiers restricted food access to the Western part of the Netherlands. This resulted in a severe but short-lived famine (dropping to less than 800 kcals/day) in an otherwise well-supplied area, providing a unique opportunity to study the effects of undernutrition specifically during pregnancy. It was initially reported that those exposed to famine in early-mid gestation showed increased obesity rates as young men (50). Further studies in exposed adults from Amsterdam for whom birth and medical records were available showed that those exposed to the famine *in utero* were at increased risk of developing obesity, T2DM and CVD as adults compared to people whose mothers were pregnant outside of the famine (51–54). Moreover, data from this period indicated that the timing of the exposure also matters: people whose mothers were in their second and/or third trimester during the famine were more likely to develop IR and glucose intolerance (51), with those exposed in early gestation especially prone to developing obesity, dyslipidaemia and CVD (52–54). This likely reflects different critical periods of development of different organ systems.

Another famine occurred in rural areas in China (1959–1961), where it was found that those exposed to famine in early life showed higher prevalence of hyperglycaemia as well as the metabolic syndrome in adulthood, the latter especially if exposed in early gestation. This association was stronger when having a postnatal Western Diet compared to the traditional diet (55,56). People exposed *in utero* to the famine resulting from the Leningrad siege (1941-1944) showed increased risk of obesity-induced hypertension and endothelial dysfunction (57). In contrast, no association between intrauterine famine exposure and adult obesity was found. However, this famine was more prolonged than the Dutch famine (1944-1945) with poorer post-famine food supply. This study was thus confounded by postnatal undernutrition, and the absence of catch-up growth may have affected comparison between the studies (52,58).

1.3.3 Predictive Adaptive Responses

The evidence presented above suggests that maladaptation to maternal undernutrition *in utero* is more detrimental when placed into caloric excess after birth, suggesting that the mismatch in pre- and postnatal environments can influence disease risk. Furthermore, this phenomenon does not seem to be limited to maternal undernutrition. Rather than reflecting an immediate beneficial adaptation to the early environment, the Predictive Adaptive Response hypothesis stipulates that, when confronted with a particular environment *in utero*, the developmentally plastic fetus implements adaptative changes that will be beneficial later in life based on the predicted postnatal (reproductive) environment (59). If this prediction is incorrect, predisposition to disease may occur.

1.3.4 Developmental Origins of Health and Disease

Nowadays the Thrifty Phenotype and Predictive Adaptive Responses hypotheses have been expanded to a broader definition, which underpins the 'Developmental Origins of Health and Disease' (DOHaD). The overarching hypothesis is that insults during critical developmental windows (in pregnancy or the first few years of life) can have long-lasting effects on offspring health. By exposing the fetus to abnormal environments (such as but not limited to maternal under- or overnutrition), the fetus responds with changes in organ structure, function and/or molecular signature that may be necessary for their initial survival but could be maladaptive after birth. Therefore, insults in development can adversely 'program' predisposition to disease in later life.

Research has consequently expanded beyond maternal nutrition. For instance, exposure to prenatal stress leads to permanent hyperactivity of the hypothalamic-pituitary-adrenal axis, as well as increased risk of obesity, CVD, T2DM and mental health problems in affected offspring (60). Others have investigated the programming effects of prenatal androgen exposure, alcohol use, air pollution and maternal smoking (61–64). Exposures investigated for DOHaD are therefore incredibly varied and ever increasing in diversity. Interestingly, most programming effects converge on a phenotype of obesity, cardiovascular and metabolic abnormalities (65). This suggests programming by different stressors could be mediated by similar mechanisms, but equally programming factors could be exposure-specific but instead targeting the same systems in their critical window of development.

In addition to different exposures, the field is also expanding in terms of timing of programming. Research has illustrated that insults during both maternal and paternal gametogenesis as well as maternal health during the pre-implantation period are associated with altered embryonic/fetal growth and long-term offspring outcomes (66–68). Moreover, rather than just focusing on *in utero* development, the importance of the early postnatal period has also become more recognised. Furthermore, rapid postnatal growth was independently associated with metabolic indices in the

Helsinki birth cohort study (46). Hence, the postnatal environment is now seen as another important window of development during which programming can take place. This includes the first 1000 days of human life, but also early childhood and even adolescence.

1.4 Developmental programming by maternal obesity

1.4.1 Evidence from human cohorts

In addition to detrimental pregnancy and perinatal outcomes in mother and baby, maternal obesity has long-term effects on offspring health. Children born to obese mums are more likely to develop obesity, IR, T2DM, hypertension, CVD and the metabolic syndrome, and are at increased risk of cardiovascular mortality (69–75). This is not entirely explained by the shared postnatal environment, since adopted children subjected to the lifestyle of their adoptive parents still more closely resemble their biological parents in terms of BMI (76). Furthermore, several studies have shown that maternal obesity is a stronger predictor of offspring obesity/adiposity than paternal obesity, thereby suggesting not only parental genetics but also the intrauterine environment contributes to this increased risk (77,78). However, the strongest evidence that this association occurs independently of genetics and lifestyle is provided by the observation that siblings born after bariatric surgery have lower rates of obesity, IR and hypertension than their siblings born before maternal weight loss (79–81). The prenatal obesogenic environment can thus program offspring predisposition to metabolic and cardiovascular abnormalities. The current obesity epidemic in women of childbearing age therefore reflects an even larger public health burden through developmental programming by maternal obesity.

1.4.2 Animal models of maternal obesity

Human epidemiology studies are extremely useful in identifying associations. However, it is challenging to demonstrate causal relationships in such studies, and they are not suitable for elucidation of potential mechanisms as it is unethical to manipulate human pregnancy. In addition, many of the outcomes of *in utero* exposure to obesity, such as cardiometabolic disease, develop with age and the required life-course studies would take decades to complete. Animal models thus provide a valuable alternative. Species used to study developmental programming include rodents, sheep and to a lesser extent non-human primates. The latter are most closely related to humans, but their gestation period is long and they are expensive to maintain (65). Sheep are used for their similarities to humans with respect to maturity at birth, commonly singleton pregnancies as well as the ability to carry out complex fetal physiology studies (82,83). Nevertheless, rodent models are often preferred due to their relatively short gestation and lifespan thus allowing experiments across the life-course.

Moreover, their identical genetic background allows to specifically demonstrate non-genetic effects in a controlled environment. Animal models of maternal overfeeding or obesity have replicated epidemiological associations with cardiometabolic disease, converging on offspring adiposity, IR and hypertension (see 1.4.3 and Figure 1.3)(65).

There are several ways to study programming by maternal obesity or GDM in rodent models. Genetic models of obesity can be used, such as the $Lepr^{db/+}$ model, where dams heterozygous for the leptin receptor are bred with wild type males to generate offspring exposed to a GDM-like environment *in utero* (84). Even wild-type offspring of these dams have accelerated growth and adipocyte hypertrophy by 8 weeks of age, and adiposity and hepatic IR in adulthood (84,85). However, genetic models have limited translational impact due to confounding by their genetic background. Alternatively, models of diet-induced obesity (DIO) can be used. Maternal obesity can be induced by overfeeding on the same diet as the control group, such as in a sheep model of gestational overfeeding where sheep were provided with 150% of food as required by national guidelines, compared to 100% in the control group (86). Other models use experimental diets to reflect a human Western diet, either initiated before gestation to induce pre-conception obesity (87,88), or alternatively closer to conception to study the effects of the diet or its immediate metabolic consequences independent of maternal obesity (89,90). Experimental diets can also be introduced or replaced with control diets during lactation (91,92), or offspring can be cross-fostered to enable lactation by dams on a different diet (90), thereby targeting specific developmental windows for specific organ systems. Experimental diets include high fat diets (HFDs), usually ranging from 40-60% kcals from fat (93,94). Despite their ability to introduce metabolic alterations, HFD pellets are less palatable than chow and HFD-fed rodents often match chow-fed animals in caloric intake by suppressing the amount of pellet they eat. Therefore, although HFDs change maternal metabolism and sometimes body composition, this is often accompanied by little change in maternal weight or adiposity (87,90). Alternative diets include those high in both fat and sugar (HFHS) to cause maternal obesity (such as the model used in the Ozanne laboratory, see 1.4.4)(87,95) or diets consisting of highly palatable human foods termed 'junk food' or 'cafeteria diets' (96,97). Other models study specific diet components by using isocaloric diets with alterations in specific fatty acids (98) or sugar types (99).

Genetic or diet-induced maternal obesity models often lead to maternal IR and/or glucose intolerance (100) and can thus act as models for obese GDM as well. However, models specific for maternal hyperglycaemia/diabetes, but not necessarily obesity, are also informative and help address effects of exposure to increased glucose *in utero*. These include models of pre-gestational hyperglycaemia, either via genetic models such as the type 1 diabetes-like 'non-obese diabetic' mouse (101) or by injection of wild-type mice with streptozotocin, a toxin that preferentially attacks pancreatic β-cells

(102). Other models show normal or mildly altered glucose homeostasis before mating but develop into full-blown diabetes when pregnant. These include models of streptozotocin injections at a lower concentration or later timepoint in gestation (103,104), but also the Lepr$^{db/+}$ mouse (84).

1.4.3 Evidence from animal models

1.4.3.1 Obesity

Animal research clearly shows that body composition is adversely affected by exposure to an obese environment *in utero*. A recent meta-analysis looking at various animal models of maternal pre-conception obesity found significantly increased body weight [123 studies, n=5772], body fat percentage [32 studies, n=1284] and absolute fat mass [10 studies, n=205] in offspring of obese pregnancy. Importantly, all offspring were fed healthy chow postnatally, and the association held true when stratified by sex, species and offspring age (105). Notably, body weight in chow-fed male offspring exposed to maternal obesity was similar to body weight of control offspring given HFD from adulthood, indicating the independent effect of maternal obesity is of equal magnitude to that of postnatal diet, in relation to offspring obesity (106). Moreover, the effects of maternal and postnatal diet were additive, with highest adiposity in the 'double-exposed' group. Furthermore, adiposity in animal offspring is often observed as early as weaning (106–108). Considering the hypercaloric environment of many populations in modern society and the impact of childhood obesity discussed in section 1.1, these data highlight the importance of the association between maternal and offspring obesity.

Maternal obesity may introduce permanent changes in white adipose tissue (WAT) function as well as fat mass. Rat offspring of dams fed a HFD before and during pregnancy (but not lactation) developed adiposity by 8 weeks of age, as well as alterations in both the plasma and WAT lipidome characterised by significant elevation of triglycerides and induction of *de novo* lipogenesis (92). These results suggest that the WAT lipid-storing capacity had been reached and WAT expansion was initiated but not yet effective in maintaining lipid homeostasis. Offspring adiposity and WAT dysfunction may not be programmed in the same critical developmental window. For instance, male offspring of mice fed a HFD pre-pregnancy showed exaggerated adiposity when challenged with a HFD in adulthood, whereas age-matched offspring from dams where the HFD was continued in pregnancy showed WAT inflammation and IGT without differences in fat mass (109), demonstrating that exposure to an insult at different stages of developmental vulnerability can have diverging effects. WAT inflammation was observed in 3-week-old offspring of obese mice fed HFD throughout lactation as well (110).

The development of adipose tissue in offspring may be influenced by the intrauterine environment and thus affect offspring predisposition to adult obesity. Adipocyte hypertrophy is an important

feature of the development of obesity (111). Additionally, adipocyte hyperplasia (especially in early development) may predispose offspring to developing obesity later in life by increasing the amount of adipocytes available for lipid storage, consequently increasing life-long lipid-storage capacity of WAT (111). Both have been implicated in the developmental programming of later obesity. Indeed, adipocyte hypertrophy has been seen in 8-week-old offspring of obese HFHS-fed dams in the Ozanne laboratory (112). Additionally, overfeeding during late gestation significantly increased expression of the adipogenic *PPARg* in fetal sheep, suggesting early upregulation of adipocyte differentiation (113). Adipogenic markers were also elevated in 2-week-old rat offspring in a maternal HFHS-induced obesity model. This adipogenic transcription profile was still seen at two months of age, potentially indicating continued adipose tissue development and lineage commitment later in life (114).

Altered energy homeostasis in offspring, for example through increased energy intake, is also likely to contribute to changes in offspring adiposity. A rat study of maternal HFHS-induced obesity in pregnancy and lactation by Kirk *et al.* showed obesity, adiposity and basal hyperphagia when fed a chow diet in both male and female offspring (115). Basal hyperphagia preceding obesity was also reported in a similar model in mice (87), although other studies require a 'stressor' such as a postnatal unhealthy diet or fasting and refeeding to observe offspring hyperphagia (96,116). These effects on food intake and obesity may be related to programmed changes in the hypothalamic circuits regulating appetite and energy expenditure. Accordingly, upregulation of the orexigenic peptide *Npy* and downregulation of the anorexigenic peptide *Pomc* mRNA was observed in the hypothalamus of female rat weanlings of HFD-induced obese dams suggesting an early programmed effect (108). Similarly, offspring of lean diabetic rats showed increased hypothalamic orexigenic NPY and AgRP immunopositivity as well as impaired POMC processing to anorexigenic αMSH (104). Additionally, offspring in the Kirk *et al.* study had reduced response to the appetite-suppressing effect of leptin administration, indicating central leptin resistance associated with changes in hypothalamic peptides that may partly underpin the development of hyperphagia and obesity in offspring of maternal obesity (115). Interestingly, this was seen both before and after the development of obesity and hyperleptinaemia in offspring, suggesting that programmed early leptin resistance could contribute to the development of offspring obesity. In addition, it has been reported that offspring of obese dams may have higher 'feeding efficiency' meaning they gain more weight per amount of energy ingested (106). Reduced energy expenditure is also suggested as a contributing mechanism (87).

1.4.3.2 Type 2 Diabetes

Hyperglycaemia (87,93,117–119) and hyperinsulinaemia (85,87,90,108,119,120) are common outcomes in animal studies looking at adult offspring of obese or HFD-fed dams. This was confirmed in a meta-analysis of animal models of maternal pre-conception obesity, which found that exposed

offspring have significantly higher glucose [68 studies, n=1980] and insulin levels [70 studies, n=1975] as well as increased HOMA-IR [13 studies, n=554 animals](105). Changes in insulin sensitivity may precede alterations in glycaemia. Rat offspring from obese Western diet-fed dams had high serum insulin concentrations compared to controls already at birth, which was maintained at 2 weeks and 2 months of age, at which point they developed glucose intolerance (114). Similarly, despite normal GTT results for the first three months of life, both male and female mouse offspring of dams fed a HFD from two weeks pre-conception developed IGT by 36 weeks, which persisted until 52 weeks of age with males being more severely affected (120,121). This was accompanied by hyperinsulinaemia at 52 weeks, as well as elevated glucose levels during an insulin tolerance test in both sexes, indicating whole-body IR (121). Moreover, hepatocytes isolated from female offspring at 6, 12 and 24 weeks of age were resistant to the inhibitory effect of insulin on glucose production, indicating that peripheral IR may precede and contribute to changes in whole-body IR observed later in life (120). Indeed, tissue-specific IR in liver (85,122), muscle (95,123) and WAT (92,112,124) has been described in offspring exposed to maternal obesity, HFD-feeding or GDM by our laboratory as well as others. Systemic IR was also demonstrated using the gold standard technique, the euglycaemic-hyperinsulinaemic clamp, in aged female offspring of rats fed a chow diet supplemented with animal lard (125).

Evidence for the relationship between maternal obesity and offspring IR is plentiful, but its effect on insulin secretion remains less well studied, despite impaired insulin secretion being required for progression from IGT to T2DM. Samuelsson *et al.* found that male offspring of HFHS-fed obese mice were insulin resistant at 3 months of age, evidenced by elevated serum insulin and pancreatic insulin content without differences in glucose. By 6 months of age, they had developed a T2DM-like phenotype with decreased serum and pancreatic insulin resulting in fasting hyperglycaemia, indicative of β-cell exhaustion and insulin secretory impairment (87). Defects in insulin secretory capacity may be programmed directly by maternal obesity. A study of maternal HFHS-induced obesity from the Ozanne laboratory found signs of islet malfunction in 8-week-old male offspring. These mice maintained normal glucose-stimulated insulin secretion at this age, but these underlying defects may predispose to future β-cell failure (126). Interestingly, an improvement in islet function was observed in female offspring, suggesting that exposure to an intrauterine obesogenic environment may have prepared females, at least in the short-term, for a nutrient-rich postnatal life (126). This is consistent with the Samuelsson *et al.* study, where unlike males, 6-month-old female offspring had increased pancreatic insulin content and a comparable intraperitoneal GTT (ipGTT) to controls (87). However, whether this protection is long-lived remains unknown, since a decrease in glucose-stimulated insulin secretion was seen in 9-month-old female offspring of HFD-fed rats (125). Therefore, maternal obesity seems to increase T2DM risk by affecting both insulin resistance and insulin secretion.

1.4.3.3 Cardiovascular disease

The aforementioned meta-analysis of pre-conception maternal obesity models also found a significant increase in offspring SBP by tail cuff plethysmography [9 studies, n=251] as well as dyslipidaemia (elevated triglycerides [46 studies, n=1337], total cholesterol [27 studies, n=795] and LDL-cholesterol [4 studies, n=158 animals]) independent of species, sex or age (105). Since hypertension and dyslipidaemia are both independent risk factors for CVD (127), this suggests maternal obesity can program predisposition not only to T2DM, but CVD as well. Using radio-telemetry, 3- and 6-month-old mouse offspring exposed to maternal HFHS-induced obesity showed elevated SBP, diastolic blood pressure (DBP) and altered heart rate (HR) in the dark active phase, with increased DBP also present in the light phase at 6 months (87). The mice in this study already showed increased adiposity at this age. Although obesity is a risk factor for hypertension, changes in offspring BP often precede offspring obesity and thus seem independently programmed. Indeed, studies from the Ozanne laboratory have shown elevated SBP in male offspring exposed to maternal obesity at 8 weeks of age, when body weight remained similar to controls (128,129). Similarly, maternal HFHS-induced obesity programmed both basal and stress-induced BP present before onset of obesity in rat offspring, proposed to result from increased sympathetic tone (130). Accordingly, male and female offspring of obese glucose intolerant dams showed hyperreactivity of mesenteric arteries to noradrenaline stimulation as well as impaired endothelium-dependent relaxation to acetylcholine (87). Programmed changes in offspring vascular endothelial function by maternal obesity could thus contribute to their risk of hypertension.

The maternal environment also affects the heart of exposed offspring. Maternal HFHS-induced obesity increased left ventricle (LV) weight in 8-week-old male and female offspring when assessed by echocardiography (131). Interestingly, it was found that the relative thickness of the LV wall was increased in males but decreased in females, suggesting sex-specific programming of cardiac structure. Studies from the Ozanne laboratory using the same model found increased heart weight at 3 weeks of age in male offspring, accompanied by cardiomyocyte hypertrophy and re-expression of fetal cardiac genes, suggesting pathological rather than physiological hypertrophy (132). These changes may persist in later life as this phenotype was also observed at 8 weeks (132,133). Indeed, structural changes may be an early phenotype since gestational overfeeding in sheep led to cardiac morphological alterations in exposed fetuses (86).

In addition to structural changes, exposure to maternal HFD-feeding and/or diabetes has been associated with adverse effects on cardiac function. Indeed, *ex vivo* assessment of heart function using the isolated Langendorff heart perfusion preparation showed a blunted decrease in HR and LV pressure after parasympathetic stimulation and an increased contractile response to sympathetic stimulation by isoprenaline, indicating systolic and diastolic dysfunction with sympathetic dominance

(132). Similarly, impaired systolic and diastolic function have been shown *in vivo* using echocardiography in rodent offspring (by the Ozanne laboratory as well as others)(94,128) and in human fetuses of obese or diabetic mothers (134,135).

1.4.4 Our mouse model of diet-induced obesity

The Ozanne laboratory has used a well-established mouse model of maternal DIO first described by Samuelsson *et al.* (87) for many years. In this model, dams are fed an obesogenic diet high in both fat and sugar as this more accurately mimics a typical Western diet than HFD alone. Dams are fed the diet before mating and are consequently obese, hyperphagic, hyperleptinaemic and hyperinsulinaemic at conception, and develop glucose intolerance in pregnancy (87,136,137). Our model may thus be a model of GDM as well as maternal obesity. In contrast to dams, offspring are fed a healthy diet from weaning, thereby separating the effects of a maternal and post-weaning obesogenic diet. Using this model, the Ozanne lab found that male offspring of obese dams are hyperphagic and develop obesity from 12 weeks of age (87,138). They also present with hyperinsulinaemia, whole-body and tissue-specific IR, dyslipidaemia and fatty liver (112,122,133,138,139) and develop cardiovascular alterations and hypertension from a young age (128,129,132,133)(see section 1.4.3.3 and Chapter 6 for more details). Importantly, this occurs before the development of obesity, indicating direct cardiovascular programming by maternal obesity independently of postnatal adiposity (132,133).

1.5 Factors mediating effects of maternal obesity

Intrauterine exposures can program offspring outcomes in various ways. Firstly, the concentration of signalling molecules at critical timepoints can alter the developmental program of the organ, leading to altered structure and cell number. These organisational changes cannot be reversed post-exposure. Secondly, factors associated with the intrauterine environment can lead to epigenetic alterations including altered DNA methylation patterns and miRNA expression, with the potential to change the gene expression profile of the affected cell long-term. Several factors may mediate developmental programming by maternal obesity, including diet, lipids, glucose homeostasis, adipokines, inflammation, placental dysfunction and oxidative stress (Figure 1.3).

1.5.1 Maternal diet

Feeding pregnant rats a 'junk food' diet from conception (thus avoiding pre-conception obesity) to weaning induced obesity and altered food preferences in 10-week-old offspring, illustrating the influence of maternal diet (96). Deficiency of particular vitamins and nutrients in some Western-style diets may have programming effects (96), although animal models tend to control for that by providing diets containing nutritional supplements (132). However, a study in rats given 'junk food' from

conception resulted in growth restricted fetuses, which was partly attributed to the decrease in voluntary protein intake (96), suggesting this could represent common effects of both maternal undernutrition and overnutrition. To avoid the well-studied programming effects of low protein intake (140) most studies therefore aim for comparable protein contents in experimental diets.

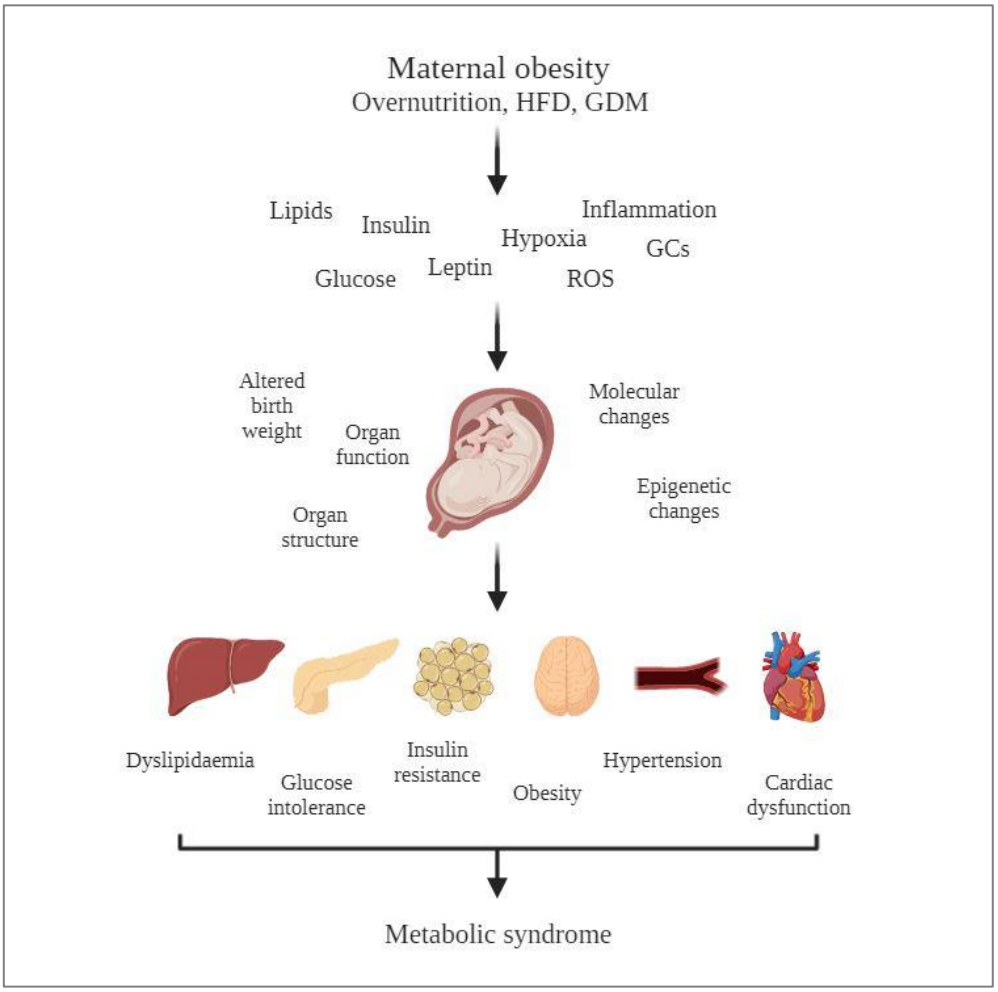

Figure 1.3: Developmental programming by maternal obesity.
Schematic overview of programming factors (see 1.5) and offspring outcomes (see 1.4). GCs: glucocorticoids. ROS: reactive oxygen species. Image created with BioRender.com.

Dietary lipid may also play an important role, since maternal HFD initiated at conception is sufficient to program obesity, IR and IGT in mouse offspring (141). Although lipoproteins don't readily cross the placenta, fatty acids and cholesterol are efficiently transferred to the fetal circulation and maternal lipidaemia can have considerable effects on fetal and neonatal lipid profiles (142). Interestingly, a rat study comparing offspring of dams fed HFD without obesity (HFD in pregnancy and lactation only) to offspring of pre-conception obese HFD-fed dams found comparable degrees of obesity and hyperinsulinaemia in adult offspring from both groups, suggesting the maternal lipid intake as a crucial factor (118). In contrast, another study found increased body weight and fat mass in offspring when dams were fed a HFD *ad libitum,* but not when isocaloric to control dams, indicating that the obese

intrauterine environment itself is also important for the programming of offspring adiposity (106). Interestingly, lipids may be involved in mediating the epigenetic reprogramming in offspring. HFD-feeding during lactation in rats changed the specific fatty acid ratio but not overall breast milk composition (or dam weight), which in adult offspring led to DNA hypo-methylation of the stearoyl-CoA desaturase 1 promoter and consequent upregulation of its expression in epididymal WAT (which had expanded in size compared to offspring from chow-fed dams)(91). Similarly, feeding dams a diet with altered fatty acid composition (high in ω6-polyunsaturated fatty acids) in gestation resulted in global DNA hypermethylation and changes in chromatin accessibility in offspring brains (98). Additionally, feeding rat dams a slow- compared to rapid-digesting isocaloric diet prevented adiposity, dyslipidaemia and programming effects on skeletal muscle, indicating that in addition to the type and amount of nutrients, the kinetics of their metabolism also plays a role (92,123).

1.5.2 Glucose homeostasis

As mentioned above, altered glucose homeostasis during pregnancy can be a feature of maternal obesity. Metabolic function is different in siblings discordant for maternal GDM (143), and mouse models of GDM without pre-pregnancy obesity show obesity, IR (females) or IGT (males) in adult offspring (85,144), providing evidence for the involvement of maternal glucose intolerance and/hyperinsulinaemia.

Maternal glycaemia may be one contributing programming factor. Enhanced placental transfer of glucose in hyperglycaemic pregnancies stimulates fetal insulin secretion, leading to fat accretion and consequent adiposity. Indeed, increased thoracic adipose tissue deposits were observed in fetal rhesus monkeys chronically infused with insulin in late gestation at a concentration similar to that expected in human diabetic pregnancy (145). Moreover, a positive relationship between maternal glycaemia, cord blood insulin and neonatal adiposity was demonstrated in human pregnancies where women were hyperglycaemic but below the GDM threshold, indicating that this programming effect also occurs in humans and across a gradient of maternal glucose levels (146).

In addition to adiposity, offspring from treated GDM and T1DM women were four and eight times more likely to develop IGT/T2DM by age 22 compared to a background population, indicating that an intrauterine hyperglycaemic environment can affect offspring glucose control (147). Hyperstimulation of the fetal pancreas in response to hyperglycaemia may adversely affect pancreatic development. Indeed, mouse offspring of dams treated with streptozotocin at conception to induce maternal diabetes showed IGT and altered insulin levels at weaning and at 8 weeks. This was accompanied by perturbed glucose-stimulated insulin secretion, pancreatic endoplasmic reticulum disorganisation and decreased expression of *Igf2* and *H19* due to DNA hypermethylation of the loci, effects that were

transmitted to the next generation (148). The fact that stimulation of fetal islets with glucose *in vitro* also decreased *Igf2* and *H19* expression suggests that glucose can modulate the epigenetic signature of developing islets (148). Moreover, since female offspring of diabetic dams are unable to sufficiently increase their β-cell recruitment during their own pregnancies (149), this may lead to a transgenerational vicious cycle of GDM-complicated pregnancies.

Maternal hyperglycaemia may also contribute to CVD risk in offspring. Neonatal rat offspring of both HFD-fed and streptozotocin-injected dams displayed cardiac dysfunction, but offspring exposed to the combination of maternal HFD-feeding and diabetes had the most severe impairment, indicating a role for both diet and glycaemia (94). This was associated with altered histone modifications in newborn hearts, potentiating the activation of gene pathways relating to cholesterol, BP, body weight and CVD (103). Interestingly, cardiac glycolytic and fatty acid oxidative capacity was only affected in offspring of streptozotocin-injected dams (irrespective of diet), suggesting maternal hyperglycaemia in late gestation may be specifically involved in programming the metabolic signature of developing hearts (94).

In addition to glucose, maternal fasting insulin (but not lipids or adiposity) directly correlates with offspring insulin at 8 weeks of age in the HFHS-induced maternal obesity mouse model used in the Ozanne laboratory, pointing toward a key role for maternal hyperinsulinaemia in the programming of offspring T2DM risk (136). Accordingly, intrauterine exposure to maternal genetic insulin resistance (haploinsufficiency for *Irs-1*; characterised by hyperinsulinaemia without maternal obesity or IGT) causes wild-type offspring to develop hyperinsulinaemia, IR and IGT as well as altered hepatic lipid metabolism and fatty liver (150). These offspring did not show changes in body weight or adiposity up to 6 months of age, suggesting that factors other than maternal hyperinsulinaemia or IR were responsible for the programming of obesity (150).

Although maternal insulin likely plays a role in mediating the long-term outcomes of obese pregnancy, direct effects on the developing fetus are unlikely as maternal insulin does not cross the placenta. Programming effects may instead occur secondary to maternal hyperlipidaemia induced by IR, leading to enhanced placental lipid transfer to the fetus (150). The placenta also expresses insulin receptors and increased insulin signalling could thus promote placental lipid deposition as well as directly alter placental function (136). Moreover, as glucose freely crosses the placenta and stimulates the fetal pancreas to produce insulin, fetal hyperinsulinaemia may mediate some of the long-term outcomes of obese or GDM pregnancies (including macrosomia as mentioned above). Moreover, insulin is a neurotrophic factor and elevated fetal insulin levels could therefore impact on the hypothalamic circuits regulating energy balance mentioned in section 1.4.3.1. Indeed, morphological changes in the

ventromedial nucleus involved in the regulation of appetite have been observed in rats exposed to neonatal insulin injections (151). Furthermore, alterations in hypothalamic projections in offspring of streptozotocin-treated rats were attenuated by maternal islet transplantation on embryonic day 15 (E15), suggesting a direct effect of maternal hyperglycaemia (104). Whether alterations in the fetal hypothalamus are a result of maternal IR, fetal hyperinsulinaemia or glycaemia, together these results indicate that hyperphagia may be mediated by perturbed hypothalamic signalling in fetuses from glucose intolerant mothers.

1.5.3 Leptin and other adipokines

Catalano *et al.* found that pre-pregnancy BMI but not maternal glucose was the strongest predictor for childhood adiposity in human offspring (77), suggesting programming potential of the obese intrauterine environment independent of maternal glycaemia. Obesity is accompanied by chronic imbalance in the production of adipokines, WAT-produced signalling molecules whose importance for whole-body energy balance and insulin sensitivity is increasingly recognised (152,153). Maternal obesity is associated with excess circulating levels of the adipokine leptin, as commonly seen with obese pregnancy in human studies (31) and animal models (87,106,154). Few studies have directly assessed the programming effects of prenatal leptin. Protection against DIO was observed in offspring from rats injected with leptin during late gestation (155). However, this study was carried out in non-obese mothers and the results are therefore not comparable to obese pregnancies that are already hyperleptinaemic. Interestingly, late gestation leptin infusion in fetal sheep led to alterations in WAT morphology and transcriptome, indicating effects of leptin on developing WAT (156). Leptin administration in mid-gestation was recently shown to affect placental size and transcriptome, suggesting leptin may influence fetal development through actions on the placenta (157).

Maternal obesity also influences leptin levels in offspring, as indicated by elevated leptin in cord blood from human obese pregnancies (158) as well as in rodent neonatal plasma (114). In adults, leptin suppresses food intake and promotes energy expenditure by acting on the hypothalamus (153). However, these central effects of leptin on energy balance are not yet present in the first weeks of life (159). Leptin levels in rodents rise sharply in the second week of life, giving rise to a 'neonatal leptin surge' that occurs independently of neonatal adiposity and does not impact on food intake (160). During this time leptin regulates the development and organisation of hypothalamic circuits involved in food intake (161). Indeed, both acute and chronic neonatal leptin treatment induced changes in hypothalamic expression of *Npy* and *Pomc* mRNA, suggesting that alterations in neonatal leptin exposure can influence development of hypothalamic feeding circuits (162). Abnormal leptin levels in offspring exposed to maternal obesity, such as the prolonged and exaggerated leptin surge observed in offspring of HFHS-fed obese dams, may thus lead to permanent changes in hypothalamic control of

energy balance (115,163). Leptin also regulates BP in adulthood: leptin injection or blockade increases or decreases SBP, respectively, in adult humans and mice (164). Like maternal obesity, neonatal leptin injections also increased offspring BP in adulthood (163), suggesting alterations in the leptin surge may program both hyperphagia and hypertension. In fact, neonatal hyperleptinaemia may program CVD by driving sympathetic hyperactivation in the brain independent of its effects on food intake and obesity (165).

Research has also turned to adipokines other than leptin. Adiponectin is an insulin sensitising and anti-inflammatory adipokine produced in quantities negatively correlated to the amount of WAT (5). Levels are decreased in obese and GDM-complicated pregnancies (31,166), and women with low pregnancy adiponectin levels are more likely to deliver LGA infants independent of BMI or insulin sensitivity (167). Moreover, cord blood adiponectin was a significant predictor of adiposity at 3 years of age in a prospective study of pregnant women (168). Adiponectin receptors are downregulated in human obese placentas and the adiponectin receptor promoter is hypermethylated, suggesting disrupted adiponectin signalling in obese pregnancies (169). Other adipokines investigated include resistin, visfatin and ghrelin, although their relationships with fetal development remain controversial (142).

1.5.4 Inflammation

WAT produces adipokines with inflammatory properties. Indeed, obesity confers a state of subclinical inflammation, as evidenced by the increased circulating levels of inflammatory cytokines and adipose tissue immune cell infiltration in obese individuals (170). Since maternal inflammation induced by lipopolysaccharide (LPS) injection in pregnancy leads to adiposity, IR and hypertension in offspring similar to the phenotype in offspring of obese dams (171), maternal inflammation could also facilitate some of the programming effects of maternal obesity.

Obese pregnancy is associated with elevated circulating levels of inflammatory markers such as interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and C-reactive protein (CRP)(172,173). Obese dams in our model show elevated plasma levels of IL-6, tumour necrosis factor-alpha (TNFα), IL-10 and MCP-1 at mating as well as increased macrophage infiltration in WAT, indicating dams enter pregnancy in a pro-inflammatory state (137). Accordingly, TNFα levels were increased in plasma and gonadal adipose tissue (gWAT) of obese HFD-fed pregnant mice during late gestation, and similar increases in inflammatory markers as well as immune cell infiltration were seen in fetal WAT (124). This suggests that the (inflammatory) maternal environment might influence the inflammatory signature of offspring WAT as well (124). Another study of HFD-induced obesity in a mouse model found elevated production of IL-6 by maternal gWAT in late pregnancy, leading to significant elevation in maternal serum (174). Moreover, increased placental IL-6 signalling was

proposed to be directly responsible for the decreased fetoplacental vasculature and endothelial cell damage observed in this model (174).

Certain maternal cytokines like IL-6 (but not TNFα or IL-1α) can cross the placenta (175), and so can maternal monocytes, T cells and B cells (171). In addition, the obese placenta can contribute to an inflammatory intrauterine environment itself by producing cytokines such as IL-6, TNFα and leptin (114,171). Upregulation of cytokine transcription (TNFα, IL-6, IL-8 and IL-18) and macrophage maturation was observed in mid-gestation placentas of DIO sheep (176), as well as pro-inflammatory signalling and macrophage infiltration in a mouse HFD model (88). Increased markers of inflammation have also been found in human GDM placentas and in both placental tissue and placental macrophages from obese humans at term (172,177). In the latter study, increased inflammatory marker mRNA levels were found in monocytes derived from maternal but not umbilical blood at delivery, suggesting obesity-induced inflammatory monocytes may not transfer to the fetus at every point in gestation (172), although placental transfer of cytokines themselves was not assessed in this study. Alternatively, cytokines such as IL-1β, IL-6 and TNFα have been reported to influence placental transfer of glucose and fatty acids (171), and placental inflammation has been linked to placental hypoxia (88). Placental inflammation may thus also affect the fetus indirectly via altered nutrient and oxygen delivery (see 1.5.5).

Inflammatory markers are found in several tissues of rodent offspring exposed to maternal obesity including WAT (110,124,138), liver (178) and brain (110). Moreover, in humans circulating CRP levels were increased in children born to obese mothers even when corrected for offspring BMI (179). Importantly, the observations of increased CRP and DNA methylation changes in the promoters of genes involved in inflammation in siblings born before versus after bariatric surgery strongly implicates the maternal obesogenic environment in generating this phenotype (79,80). Lastly, obesity-related inflammation can also induce oxidative stress (see 1.5.6)(180)

1.5.5 Placental function and hypoxia

Damage to the placental vasculature has been described in mouse models of maternal obesity, including our own (174,181). Increased presence of vascular lesions in obese women was observed even in uncomplicated pregnancies, which may explain the increased risk of pregnancy complications with maternal obesity. Moreover, these changes were accompanied by some indicators of maternal mal-perfusion and decreased cord blood pH, an indicator of fetal hypoxia (182). Oxygen saturation in cord blood also tended to be decreased in a small human study of GDM-exposed newborns (183). Placental abnormalities may thus lead to decreased nutrient and oxygen delivery to the fetus, consequently leading to intrauterine growth restriction (IUGR)(181). Accordingly, levels of hypoxia-

inducible factor 1 alpha (HIF1α) are elevated in late gestation placentas from obese pregnancies in our mouse model (136). Interestingly, HIF1α levels correlated with maternal insulin suggesting a direct effect of the maternal metabolic state on placental function. Moreover, hypoxia in placentas of obese HFD-fed mouse dams in mid-gestation was associated with relative vascular immaturity despite increased density of the placental vasculature, processes that may both alter nutrient transport capacity (88). Spiral artery remodelling, fetal capillary density and overall area of the labyrinthine zone of the placenta (responsible for oxygen and nutrient exchange) were decreased in our mouse model of maternal DIO (181), further indicating that maternal obesity likely impairs oxygen delivery to the fetus. Interestingly, increased *Hif1α* expression has also been found in pre-gravid uteruses of obese rodents, suggesting that a hypoxic intrauterine environment may also be present independent of effects of obesity on placental function (97).

In addition to fetal growth, intrauterine hypoxia also contributes to adverse outcomes in adult offspring, as shown by experimental models of maternal and fetal hypoxia using hypoxic chambers which have demonstrated cardiovascular dysfunction in adult rat and sheep offspring (184,185). Paradoxically, hypoxia can also lead to oxidative stress (186). Indeed, supplementation of antioxidants during maternal hypoxia ameliorates fetal and adult offspring outcomes, indicating a role for oxidative stress in mediating programming effects of hypoxia (184,186).

1.5.6 Oxidative stress

Oxidative stress results from the inappropriate generation of Reactive Oxygen Species (ROS) and/or inadequate antioxidant defence capacity. ROS act as signalling molecules in small quantities but induce damage when production is imbalanced (65). Several properties of maternal obesity have the possibility to induce oxidative stress and consequently programming. These include obesity per se, which is associated with systemic oxidative stress driven by increased ROS production and impaired antioxidant defence in WAT (187), but also hypoxia and mitochondrial dysfunction, as is observed in obese placentas and oocytes (188,189). In addition, hyperlipidaemia in maternal and placental tissue may predispose to oxidative stress through increased lipid peroxidation (142). Accordingly, increased lipid droplets and markers of lipid peroxidation were found in isolated cardiomyocytes from newborn rat offspring of HFD-fed (but not of diabetic) dams (94). In our model, excess lipid deposition was also found in the placenta, increasing the risk of placental oxidative stress with maternal obesity (136). Increased markers of oxidative stress have indeed been found in placentas from obese humans (189–191). Furthermore, both maternal and placental redox balance were strong predictors of fetal oxidative stress in obese (but not lean) pregnancies, indicating that maternal obesity-induced oxidative stress directly affects the fetus (190).

Signs of oxidative stress were also found in hearts, pancreatic islets and livers from rodent offspring exposed to maternal obesity (126,133,192). This may be an early and persistent effect as evidence of oxidative stress was already found in pre-implantation embryos from Western diet-fed dams, which led to oxidative stress and inflammatory markers in fetal and newborn serum (114). Gestational dietary supplementation with the antioxidant Quercetin in a murine maternal DIO model attenuated the hyperinsulinaemia, hyperglycaemia, obesity and hypertension observed in female offspring (93), supporting a direct role for the involvement of ROS in programming by maternal obesity. In rats, antioxidant supplementation to Western (but not control) diet-fed dams decreased offspring adiposity, leptin and insulin levels, and improved glucose tolerance (114). Interestingly, the intervention prevented maternal hyperinsulinaemia but did not affect maternal body composition, potentially pointing toward an interaction between insulin and oxidative stress, while also indicating that programming effects can be prevented without changes in maternal adiposity (114). Interestingly, developmental programming by oxidative stress may include epigenetic effects, supported by the prevention of fetal cardiac *Pkcε* promotor DNA methylation by antioxidant treatment to hypoxic mothers (186). Antioxidant supplementation also corrected cardiovascular outcomes in rat models of neonatal glucocorticoid exposure (193,194). Oxidative stress could thus be a common mechanism underlying developmental programming by different exposures.

1.5.7 Glucocorticoids

In addition to insulin and leptin, other endocrine changes are associated with obesity. Extensive research has shown developmental programming of obesity and CVD as a consequence of fetal or early life over-exposure to glucocorticoids [reviewed in (195)], leading to phenotypes that mimic those seen with maternal obesity. Non-pregnant obesity is associated with elevated glucocorticoids (196), but the relationship between glucocorticoids and obese pregnancy is unclear. In humans, both elevated and decreased glucocorticoid levels have been described with maternal obesity (196,197), as well as altered circadian rhythm and pulsatility of cortisol release (197,198) and dysregulated glucocorticoid signalling in the placenta (199). Increased corticosterone levels have been described in some but not all rodent models of maternal obesity (200–202). However, elevated glucocorticoids were reported in fetuses of obese ewes (200) as well as altered glucocorticoid levels and signalling in adult offspring (203). Alternatively, since the metabolic actions of glucocorticoids oppose those of insulin, IR in obese pregnancy may explain the similarities between these models.

1.6 Intervention strategies

The concept of the DOHaD offers a new and early intervention window for the prevention of non-communicable diseases (49). In order to prevent the negative effects of the obesity epidemic being transmitted to the next generation, we need interventions that can be easily implemented in pregnancy. It is especially important to break the vicious cycle of maternal obesity, referring to the feed-forward effect that daughters of obese mothers are at increased risk of being obese in pregnancy themselves as a result of developmental programming. The studies comparing siblings born before and after bariatric surgery, although a drastic intervention, clearly indicated that changing the maternal obesogenic environment has the potential to improve offspring outcome (79–81). Glycaemic management in pregnancy is also key, as supported by the ACHOIS trial where randomising pregnant women with mild glucose intolerance (but not overt GDM) to specialist monitoring and treatment decreased perinatal morbidity and cord hyperleptinaemia compared to routine care (204–206). Moreover, offspring from women treated for diagnosed GDM are at lower risk of macrosomia or childhood obesity compared to offspring from women with milder glucose abnormalities who did not receive treatment (204).

Clinically relevant interventions aim to target the abovementioned programming factors (see section 1.5), with most human studies focusing on improving maternal glycaemia, insulin sensitivity and/or weight. These can be classified into two groups: lifestyle and pharmaceutical interventions.

1.6.1 Lifestyle interventions

Lifestyle interventions often involve dietary advice. Animal models of maternal diet manipulation show beneficial effects of dietary change in obese pregnancy and/or lactation. In rats, switching dams back from a junk food to a control diet after birth prevented hyperphagia and obesity when offspring were weaned onto a junk food diet (96). Similarly, after 2-4 years of HFD in non-human primates, placing mothers on a healthy diet for a few months before pregnancy attenuated the maternal insulin response to GTT, in absence of changes in body weight (154). More importantly, this prevented fetal growth restriction and significantly attenuated the increase in fetal hepatic triglycerides and gluconeogenic gene expression (154). These data demonstrate that the pre-conception and early postnatal periods (in addition to pregnancy itself) are promising time windows for early life intervention. However, although pair-feeding hyperphagic Lepr$^{db/+}$ mice to wild-type dams improved maternal and fetal outcomes, restricting intake to 70% of control levels led to IUGR (85). Therefore, improving diet quality rather than caloric content may be a more successful approach. Indeed, restrictive dieting in pregnancy is not recommended due to risk of ketosis, which can be harmful to both mother and fetus (23).

Several ongoing studies in humans investigate lifestyle interventions involving diet and moderate exercise. The LIMIT randomised controlled trial (RCT) randomised obese pregnant women to a lifestyle intervention comprising dietary advice (limit saturated fat and refined sugar intake, and increase dietary fibre, vegetable and fruit intake) as well as encouragement to adopt an active lifestyle (207). Although total caloric intake, maternal body weight and weight gain were unaffected, the intervention was successful in increasing physical activity by 15-20 minutes per day and women in the lifestyle group were significantly less likely to deliver babies over 4000 or 4500 grams compared to women receiving standard prenatal care (208–210). There was no improvement in offspring adiposity at 6 and 18 months (211,212), but longer-term outcomes have not yet been assessed.

UPBEAT (UK Pregnancies Better Eating and Activities Trial) studied the effect of a combined dietary and exercise intervention in non-diabetic obese women in the UK (213). By providing extensive healthy eating advice, weekly personal training sessions and information about activity in pregnancy, they significantly improved maternal diet and activity levels (214). The change in activity (12-13 minutes more per day) was similar to the LIMIT trial, demonstrating that clinically relevant interventions are able to increase activity in human pregnancy and that the magnitude of the effect is consistent across studies. Although the primary outcome of LGA was unaffected, the intervention decreased maternal food intake, weight gain and adiposity, as well as adiposity indices in 6-month-old offspring (214,215). Moreover, causal analysis revealed the decrease in infant adiposity was directly related to improved maternal adiposity and saturated fat intake, indicating beneficial effect of targeting maternal characteristics (215).

Exercise interventions for maternal obesity or GDM have also sparked interest from basic researchers, including our laboratory. Previous work in mice from our group has demonstrated that moderate exercise during obese pregnancy can correct placental hypoxia, maternal insulin levels and glucose tolerance in pregnancy without affecting maternal weight, adiposity or hyperleptinaemia (128,136). These maternal improvements corrected the circulating insulin levels and adipose tissue IR in 8-week-old offspring (136), and prevented the cardiac hypertrophy and LV dysfunction observed in offspring of untreated obese dams through improved calcium homeostasis and cardiac contractility (128). Interestingly, the exercise intervention had no effect on offspring SBP. Cardiac function and BP thus seem to be programmed through different mechanisms and will therefore likely need different intervention strategies to correct them.

Lifestyle interventions are extremely useful since they are affordable and theoretically easy to implement. However, in humans they often lead to only modest improvement in maternal body

weight or metabolic health (208,214,216), and differences in offspring obesity risk can disappear with time (211,212). Therefore, research has also addressed potential pharmacological interventions.

1.6.2 Pharmacological interventions

Controlling maternal glucose homeostasis is key to prevent programming (see section 1.5.2). Insulin can be used to control glucose levels in GDM, but this is expensive, involves daily injections, requires refrigerated storage, is associated with neonatal hypoglycaemia and may increase risk of hypertensive disorders of pregnancy (28). Affordable, non-invasive oral hypoglycaemic agents may be more suitable alternatives. Sulfonylureas such as glibenclamide (glyburide) have been used in pregnancy for many years (especially in the USA) and have been found effective in controlling maternal glycaemia (27). However, a large retrospective study of health insurance records in the USA found increased risk of LGA, neonatal hypoglycaemia, respiratory distress and admission to the neonatal intensive care unit with glyburide compared to insulin treatment of GDM (217). Moreover, a recent meta-analysis from our lab found that glyburide use in GDM was associated with increased birth weight, LGA, macrosomia and neonatal fat mass compared to insulin, confirming the increased risk of adverse offspring outcomes (218). Therefore, glyburide may not be an appropriate intervention to prevent programming by maternal obesity and/or GDM and these detrimental outcomes likely explain why it is not used standardly in the UK to treat GDM. Instead, the oral glucose-lowering drug with the most potential is metformin.

1.7 Metformin intervention

1.7.1 Metformin in clinical practice

The biguanide drug metformin, derived from the *Galega officinalis* plant, is an insulin sensitising agent. In non-pregnant individuals, metformin is thought to work by decreasing hepatic glucose output, promoting glucose uptake by skeletal muscle and intestinal tissue and increasing fat oxidation (219). In the UK metformin is currently the first-line treatment for T2DM- and GDM-complicated pregnancies where glucose levels cannot be controlled by lifestyle alone, according to The National Institute for Health and Care Excellence (NICE) guidelines (30). Several organisations in the USA now consider metformin an acceptable second-line alternative to insulin in GDM, including the American Diabetes Association and the American College of Obstetricians and Gynecologists (220,221). Furthermore, the Society of Maternal-Fetal Medicine even recommends that metformin replaces insulin as first-line treatment for GDM after nutrition advice fails (222). Although caution for the off-label use of metformin in GDM pregnancy was urged in an earlier version of the guidelines, as of 2018 the Diabetes Canada Clinical Practice Guidelines Expert Committee accepts both insulin and metformin as first-line

pharmacological treatments after lifestyle interventions (223,224). As the obesity epidemic is associated with rapidly increasing incidence of GDM (15), metformin is a relevant intervention to investigate.

In addition to GDM, metformin may be used in pregnancies complicated by T2DM. NICE guidelines recommend that women with a pre-gestational diagnosis of T2DM may continue metformin or switch from insulin to metformin while becoming pregnant, if this is not detrimental to their glycaemic control (30). Metformin is also used in women with PolyCystic Ovary Syndrome (PCOS) to ameliorate the IR associated with the disease and to improve ovulation. Consequently, some PCOS patients will take metformin in the first trimester and some may choose to continue using it throughout gestation (225). NICE does not currently advise the routine use of metformin in pregnancies complicated with PCOS, but states that metformin can be prescribed by a specialist (226). The Royal College of Obstetricians and Gynaecologists also advise against the use of metformin to manage glycaemic control in PCOS women, except for those with IGT (227). Consequently, a proportion of women with T2DM or PCOS will enter pregnancy whilst on metformin, thereby increasing the number of women taking the drug in pregnancy. In contrast to GDM where metformin is introduced later in gestation, this group will be taking metformin from the pre-conception period onwards. The NICE guidelines also state that T2DM patients who have not taken metformin in gestation may (re)start metformin treatment after delivery, thus potentially exposing their infants to metformin while breastfeeding (30).

Metformin use in pregnancy has become increasingly popular in recent years. An international utilisation study by Cesta *et al.* looking at pregnancy data from the USA, Australia and Nordic countries reported that between 2006-2016 metformin use in pregnancy increased in almost all countries investigated, even rising sufficiently to become equivalent to insulin by the end of the study period in Finland, Iceland and the USA (228). These data partly reflect the increased prevalence of GDM with time. However, in a sub-study of pregnancies where antidiabetic medication was initiated in late gestation (a proxy for GDM) a proportional increase in metformin use was also seen, indicating that treating GDM with metformin rather than insulin is becoming more common practice world-wide (228). Similarly, increased metformin use for GDM was observed in Wales and the rest of the UK from 2008 after the NICE guidelines were updated to recommend metformin (229).

Rates of biguanide use in pregnancy in the 2010s reportedly ranged from <0.5% in Sweden to over 2% in Australia, with recent rates in most countries hovering around 1-1.5% (228). Similarly, using data from Addenbrooke's hospital in Cambridge, it was extrapolated that an estimated 1-2% of children born in the UK today will have been exposed to metformin *in utero* (personal correspondence with Dr

C. Aiken, unpublished). Metformin use in pregnancy is therefore becoming increasingly common and warrants investigation.

1.7.2 Why metformin?

Metformin has the potential to counteract many of the programming factors discussed in section 1.5. As mentioned, its use as first-line pharmacological treatment for T2DM and GDM in the UK demonstrates the potential to improve glucose homeostasis in obese pregnancy (30). Additionally, metformin administration is shown to promote weight loss in non-pregnant patients with T2DM or PCOS (230,231). In pregnancy, metformin improves glucose tolerance in women with GDM to the same extent as insulin, and its use is associated with lower gestational weight gain (GWG) compared to insulin or placebo in GDM or obese glucose tolerant women, respectively (232,233). This suggests that metformin could prevent programming by maternal hyperglycaemia or hyperinsulinaemia, as well as decreasing the effect of being obese by preventing excessive GWG which is in itself associated with adverse pregnancy outcomes and programming effects (234,235). Furthermore, two months of metformin treatment decreased circulating leptin levels in non-pregnant women with PCOS (236), suggesting the intervention may be able to attenuate hyperleptinaemia in obese pregnancy too.

Metformin may also improve the inflammatory obesogenic environment. *Ex vivo* treatment with metformin of placental lobules obtained at C-section resulted in lower levels of inflammatory markers (237), and pre-treatment with metformin attenuated *in vitro* IL-6 production in a human trophoblast cell line (238). Metformin also has the potential to decrease inflammatory markers *in vivo*, as evidenced by the decreased serum CRP in non-pregnant PCOS patients following 2-6 months metformin treatment (239). Accordingly, the EMPOWaR trial (Efficacy of Metformin in Pregnant Obese Women, a Randomised Controlled Trial) investigating metformin treatment in obese glucose tolerant women showed a reduction in circulating markers such as IL-6 and CRP (240). In addition to effects on the mother, maternal metformin treatment also prevented fetal inflammation in a rat model of maternal obesity (238). Interestingly, the *ex vivo* placenta study found that the anti-inflammatory effect of metformin was enhanced in presence of hyperglycaemia (237), suggesting metformin may be able to attenuate the inflammatory environment of both obese and diabetic pregnancy.

Metformin is described as having vasculo-protective effects. Metformin use is associated with lower incidence of stroke, coronary flow rate and measures of endothelial dysfunction in T2DM patients and/or non-pregnant women with PCOS (241–244). Similarly, metformin administration restores vascular impairment in obese rats, partly by ameliorating ROS production and increasing nitric oxide sensitivity (245). These beneficial effects in non-pregnant individuals suggest the intervention may also be protective for maternal and placental vasculature in obese and/or diabetic pregnancy. Indeed,

in vitro metformin administration to cultured HUVECs, primary placental cells and placental explants from preeclampsia patients decreased soluble Flt-1 and endoglin, factors implicated in vascular dysfunction and the pathogenesis of preeclampsia (246). Metformin also prevented the upregulation of VCAM-1 (a marker of endothelial dysfunction) in HUVECs and rescued the impaired vasorelaxation and angiogenesis in arteries exposed to placental factors (246). In accordance with these *in vitro* data, low dose metformin treatment *in vivo* prevented impaired placental vascularisation in rat dams fed a fructose-rich diet (247). Hence, metformin has the potential to improve placental function in obese pregnancy.

In addition to its anti-inflammatory and angiogenic properties, metformin may exert beneficial effects on placental vasculature and other organs by reducing oxidative stress. In mice, metformin administration ameliorates the ovarian oxidative stress associated with hyperandrogenisation (248), and prenatal metformin treatment attenuated the increased lipid peroxidation in embryos from HFD-induced diabetic mice (249). Metformin has also been shown to decrease indicators of oxidative stress (including ROS) in blood of T2DM and non-pregnant PCOS patients (250,251), suggesting it may correct maternal obesity-related oxidative stress in humans as well.

1.7.3 Current data on metformin use in pregnancy

Information on the safety and efficacy of metformin use during pregnancy has originated from several sources. The earliest reports come from Coetzee *et al.* investigating the use of metformin in pregnancies complicated by non-insulin dependent (gestational) diabetes in South Africa in the 1970-80s. In their pioneering cohort studies they observed no additional risk of birth defects, LGA, neonatal hypoglycaemia or other adverse maternal and fetal outcomes with metformin use in diabetic pregnancy (252–255). Similarly, Glueck *et al.* published several cohort studies investigating the effect of continued metformin use post-conception in women with PCOS in the US. They describe a lack of teratogenicity, improved insulin sensitivity and insulin secretion and decreased early pregnancy loss with continued use of metformin (256,257). They also report no difference in preeclampsia rate between metformin-treated PCOS and the general population, suggesting a protective effect on preeclampsia pathogenesis as baseline risk for PCOS women is increased (258). Although the work of Coetzee and Glueck is extremely informative, retrospective studies are inherently biased and RCTs are required to properly elucidate treatment effect as well as demonstrate causation.

Several RCTs have been carried out to investigate the effects of metformin interventions in pregnancy. These studies focus on women with GDM (e.g. the Metformin in Gestational Diabetes [MiG] trial in Australia and New Zealand), women with PCOS (e.g. Metformin in Pregnant PCOS Women [PregMet]

trial in Norway), and obese glucose tolerant women (e.g. EMPOWaR and Metformin in Obese Pregnancy [MOP] trials in the UK).

1.7.3.1 Maternal outcomes

Data on maternal outcomes following metformin intervention in pregnancies are generally positive. Metformin treatment results in similar HbA1c [4 studies, n=1094] and fasting blood glucose [3 studies, n=956] in pregnant women with GDM compared to insulin, according to a meta-analysis of RCTs (259). Moreover, data from the MiG trial, the largest RCT in GDM women to date, reported that metformin-treated women reach glycaemic targets sooner than those treated with insulin (260), which was confirmed by several meta-analyses of RCTs of metformin versus insulin in GDM (261,262). This could indicate increased effectiveness compared to insulin, but the difference has also been attributed to difficulties with injecting the correct dose when first starting insulin treatment (261). Additionally, two studies in diabetic women (GDM and T2DM combined) found decreased risk of perceived maternal hypoglycaemia with metformin compared to insulin alone (263,264). Moreover, women in the MiG trial preferred metformin tablets over insulin injections and were more likely to start this treatment in a future GDM-complicated pregnancy (260). Although a proportion (14-46%) of GDM women fail to achieve glucose control on metformin and require supplemental insulin (260,265–272), the amount of insulin required is lower (260). Hence, these findings show metformin is useful for maintaining glucose control in diabetic pregnancy. Furthermore, both the EMPOWaR and MOP trials in obese women describe improved HOMA-IR at 28 weeks indicating metformin may also improve insulin sensitivity in non-diabetic pregnancy (273,274).

Metformin is associated with lower GWG, a commonly reported outcome in RCTs (260,268,269) and cohort studies (275) for GDM as well as the PregMet RCT for PCOS (276). Several meta-analyses have confirmed this association for GDM-complicated pregnancies, with the difference ranging from 0.47 to 2.07kg less weight gain in the metformin group (259,261,277–280). In contrast, in obese glucose tolerant women, GWG was unaffected by metformin compared to placebo in the EMPOWaR trial (274). The MOP trial, however, observed decreased GWG in metformin-treated women compared to placebo at all points of gestation (233). In a study in GDM women, a lower degree of metformin exposure was associated with lower GWG (281), and it has been suggested that the effect of metformin on gestational/post-partum weight gain is more prominent in obese compared to overweight GDM populations (282). Since the MOP trial recruited heavier women (BMI >35.0 versus BMI >30.0 in EMPOWaR) and used higher metformin doses with better adherence, this may explain the discrepancy between the studies.

The potential for metformin to improve vascular health (mentioned in section 1.7.2) led to the suggestion that metformin may prevent preeclampsia. Although a protective effect on preeclampsia was found in the MOP trial for obese women (233), meta-analyses published at the time this study was designed (in 2017) did not find a significant protective effect in diabetic women (261,277,278,283). However, pregnancy-induced hypertension [4 studies, n=1260] was found to be significantly decreased (278). Therefore, larger meta-analyses including more studies may find a protective effect of metformin on preeclampsia development in the future. Indeed, a larger meta-analysis has since been published showing a protective effect of metformin in preeclampsia prevention compared to insulin-treated GDM (284). Interestingly, in the MOP trial preeclampsia incidence was positively associated with GWG, suggesting metformin may partly exert protection by preventing excessive GWG (233).

The MiG trial found an increased rate of preterm birth (PTB) with metformin, which could not be explained (260). Although other studies failed to replicate this result (268,269,271,272,285,286), some early meta-analyses of up to 5 GDM RCTs corroborated the findings (261,277,283). However, the largest and most up to date meta-analysis for metformin versus insulin in diabetic pregnancies published at the time of study design found no significantly increased risk of PTB with metformin [7 studies, n=1520](278), suggesting this increased risk was mostly driven by the MiG data. In contrast, a meta-analysis of PCOS-complicated pregnancy outcomes where metformin was continued throughout gestation found a protective effect on PTB when assessing RCTs [3 studies, n=367], cohort studies [3 studies, n=223) or both types of studies together [6 studies, n=590](287). Other adverse pregnancy outcomes such as C-section are also not altered by metformin, according to meta-analyses and RCTs (278). Metformin treatment in pregnancy therefore seems safe and beneficial for maternal health.

1.7.3.2 Short-term offspring outcomes

Metformin readily crosses the placenta, and the fetus is consequently exposed to concentrations of metformin similar to the mother (288). Nevertheless, results from studies investigating neonatal outcome are reassuring. Metformin treatment does not increase risk of neonatal mortality or congenital abnormalities compared to placebo or insulin when given to obese or GDM women, respectively (280,289). This was also found in a meta-analysis [9 studies, n=351] investigating first trimester metformin exposure in PCOS women who took metformin pre-conception (290). There was also no difference in birth defect rate between those who continued or stopped metformin after the first trimester (290). The MiG trial found no difference in the composite outcome of neonatal complications (including prematurity) despite increased PTB rates with metformin (260). The finding that metformin does not induce perinatal morbidity or mortality was confirmed in a large meta-

analysis that also found decreased risk of neonatal hypoglycaemia [14 studies, n=2165] and neonatal intensive care unit admission [10 studies, n=1822] compared to offspring of insulin-treated diabetic pregnancy (278). Results from the PregMet trial and a meta-analysis of two RCTs investigating metformin versus placebo in obese glucose tolerant women (the EMPOWaR and MOP trials) also confirmed safety with respect to adverse neonatal outcomes for metformin-exposed offspring of non-diabetic pregnancy (276,289). Therefore, metformin does not pose additional risk for the neonate and may even be associated with improved outcomes.

Treatment of GDM is mostly aimed at improving maternal hyperglycaemia and reducing the risk of macrosomia, which are therefore the primary outcomes of most RCTs. Lower birth weight following metformin compared to insulin treatment for GDM is reported in some but not all studies, but individual RCTs often fail to report a reduction in macrosomia (260,268,271,272,291,292). At the time of study design (in 2017) the role of metformin in macrosomia prevention was unclear, with only two meta-analyses reporting a decrease in macrosomia and/or LGA (278,293) whereas others found no effect (259,261,277,280,283). At the time of writing (in 2020), however, the relationship between metformin use and decreased birth weight, incidence of macrosomia and of LGA in GDM-complicated pregnancies has been confirmed (218,284,294). However, an underlying increased risk of macrosomia may be required for metformin to affect birth weight. Accordingly, neither EMPOWaR or MOP found an effect of the metformin intervention on birth weight in offspring of obese non-diabetic women (233,274), and the same was found in trials focusing on PCOS-complicated pregnancy (276,295,296)

1.7.3.3 Long-term offspring outcomes

Although the data mentioned above are reassuring, long-term offspring follow-up is lacking and therefore any beneficial effects of the metformin intervention on longitudinal offspring outcomes remain uncertain. Since metformin readily crosses the placenta (288), research into long-term safety of intrauterine metformin exposure is warranted. At the time this study was designed, only a few studies in humans (297–300) or animal models (301–304) investigating offspring outcomes following gestational metformin past the neonatal period were published. Consistent with results from a cohort study looking at offspring of metformin-treated PCOS mothers (305), follow-up from the RCT by Ijäs *et al.* (271) reported that there were no differences in motor, social or linguistic skills in offspring of metformin- or insulin-treated GDM pregnancy (297). However, body weight of metformin-exposed children was increased at 12 months of age, and by 18 months of age they were heavier and taller than children of insulin-treated mothers (297). Follow-up from the MiG trial showed that although no changes in body composition were seen at birth, children in the metformin-exposed group showed increased arm circumference as well as subscapular and biceps skin-fold thickness at 2 years of age (298). Since there were no significant differences in total body fat mass, the authors concluded that

the intervention may have led to redistribution of fat mass to the subcutaneous rather than the visceral adipose tissue depots, which was proposed to be metabolically beneficial (298). The PregMet trial also showed increased body weight at the 12 month follow-up of children from metformin-treated PCOS patients (300). Consistent with these human studies, pilot data from our lab (for more information on the model see Chapters 2 and 3) showed increased weight of the epididymal adipose tissue (eWAT) depot in offspring of obese metformin-treated dams (unpublished, Figure 1.4). At present it is difficult to predict the long-term effects of a potential increase in offspring body weight and subcutaneous fat following exposure to metformin *in utero*. One small study (n=25) followed up 8-year-old offspring from metformin- and placebo-treated PCOS pregnancies. The authors reported no difference in body weight, composition or insulin sensitivity, but saw an increase in fasting blood glucose and SBP (299). More research is therefore needed to determine the long-term offspring effects of a metformin intervention in obese pregnancy.

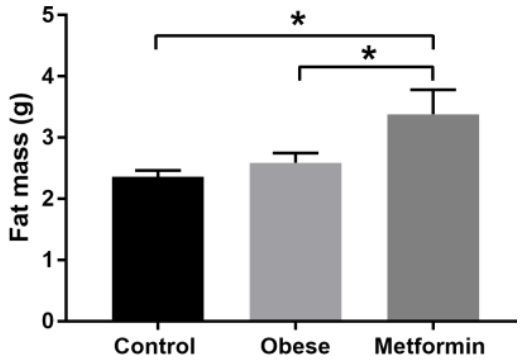

Figure 1.4: Absolute fat mass (TD-NMR) in 8-week-old male offspring.
Preliminary data from the Ozanne lab obtained by Dr H. Blackmore (unpublished).

1.8 Aims and hypotheses

The global obesity epidemic (and consequent increase in obesity during pregnancy and GDM) poses a significant public health burden through developmental programming of obesity and cardiometabolic disease in the offspring. Suitable interventions are therefore urgently needed to stop the adverse effects being transmitted to the next generation, and to prevent transgenerational vicious circles of maternal obesity and GDM. The existing literature suggests that metformin may be a beneficial intervention to improve maternal and short-term offspring outcomes in obese and diabetic pregnancy. However, there are gaps in the literature with regards to long-term offspring outcomes. Short-term follow-up of published trials suggest safety of the intervention, but also include interesting findings of offspring adiposity consistent with what was found in preliminary studies by our laboratory. Whether these are transient, permanent, protective or detrimental effects remains to be elucidated. Moreover, offspring from the abovementioned human RCTs remain young at present. It will therefore

take years before conclusions can be drawn about the effectiveness of a metformin intervention to prevent metabolic and cardiovascular abnormalities in adult human offspring exposed to obese or diabetic pregnancy. Long-term animal studies looking at offspring phenotype are thus urgently needed. Lastly, human follow-up studies largely fail to differentiate between male and female offspring. Since developmental programming (by maternal obesity or other) is increasingly recognised to be sex-specific (306), it is important to follow up both male and female offspring separately to see if sex differences exist in response to a metformin intervention.

This thesis addresses these gaps in knowledge through the following 4 aims:

1. To describe the effect of metformin intervention on body weight/composition and food intake of obese dams and neonatal growth trajectory of their offspring in a mouse model of DIO
2. To establish the effect of metformin intervention on white adipose tissue from male and female offspring of obese dams and potential consequences for metabolic health
3. To determine long-term consequences of obese pregnancy and metformin intervention on male and female offspring body composition and metabolic health by performing a longitudinal offspring study
4. To determine long-term consequences of obese pregnancy and metformin intervention on male and female offspring cardiovascular health *in vivo* by assessing blood pressure and heart function at 3, 6 and 12 months

The outcomes of my PhD project will expand our knowledge of how prenatal metformin affects metabolic and cardiovascular health in exposed offspring. By contributing to this body of novel research, the PhD may contribute to policies regarding the prescription of metformin in pregnancy.

2 General materials & methods

2.1 Mouse model

2.1.1 Maternal obesity model

All mouse work was performed according to the Home Office Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012, after ethical review by the University of Cambridge Animal and Welfare Ethical Review Board (AWERB). Female C57BL/6J mice were fed standard laboratory chow (RM1, 7% sugars, 3% fat) or a HFD (10% sugars, 20% fat) supplemented with sweetened condensed milk (55% sugar, 8% fat) and vitamin and mineral pre-mix (AIN-93G-MX, Special Diets Services, UK) from weaning (Table 2.1). After 6 weeks they were mated for a primary pregnancy to ensure breeding effectiveness. Obese dams were mated for the experimental pregnancy when they reached a critical threshold of 12g absolute fat mass as measured by non-invasive Time-Domain Nuclear Magnetic Resonance (TD-NMR, Bruker Minispec LF series, Bruker Optik GmbG, Germany), whereas control dams remained below 5g absolute fat mass at mating. Dams were fed their respective diets *ad libitum* throughout pregnancy and lactation[1] (Figure 2.1).

	RM1	45% HFD	Condensed milk
AFE (kcal/g)	3.3	4.5	3.26
Energy (% kcal)			
Fat	7.4	44.7	-
Protein	17.5	20.3	-
Carbohydrate	75.1	35.0	-
Composition [%w/w]			
Fat	2.7	22.6	8.0
Protein	14.4	23.0	7.5
Carbohydrate	61.7	39.8	55.0
Simple sugars	4.05	10.5	55.0

Table 2.1: Composition of the experimental diets used in this study.
AFE = Atwater free energy.

During the second pregnancy, weights of dams, food and milk intake were recorded once or twice weekly[2]. Dams also underwent TD-NMR at E16 to assess body composition[3]. Litter size was standardised by culling back to six pups per litter on postnatal day 2 (PN2). Male and female offspring were weaned onto RM1 at PN21 and housed in littermate pairs of the same sex until culling at 8 weeks or 12 months of age.

[1] Dam husbandry by C. Custance
[2] Collection of body weight and food intake data by C. Custance, analysis by JMS
[3] TD-NMR on dams by C. Custance, analysis by JMS

2.1.2 Metformin intervention

Half of the obese dams were treated with 300mg/kg/d metformin-hydrochloride for one week before mating until E19, after which they were given the normal obesogenic diet without metformin. Metformin was dissolved in the milk and doses were adjusted twice-weekly based on calculations using dam body weight and milk intake. More details about the dosing protocol can be found in Chapter 3.

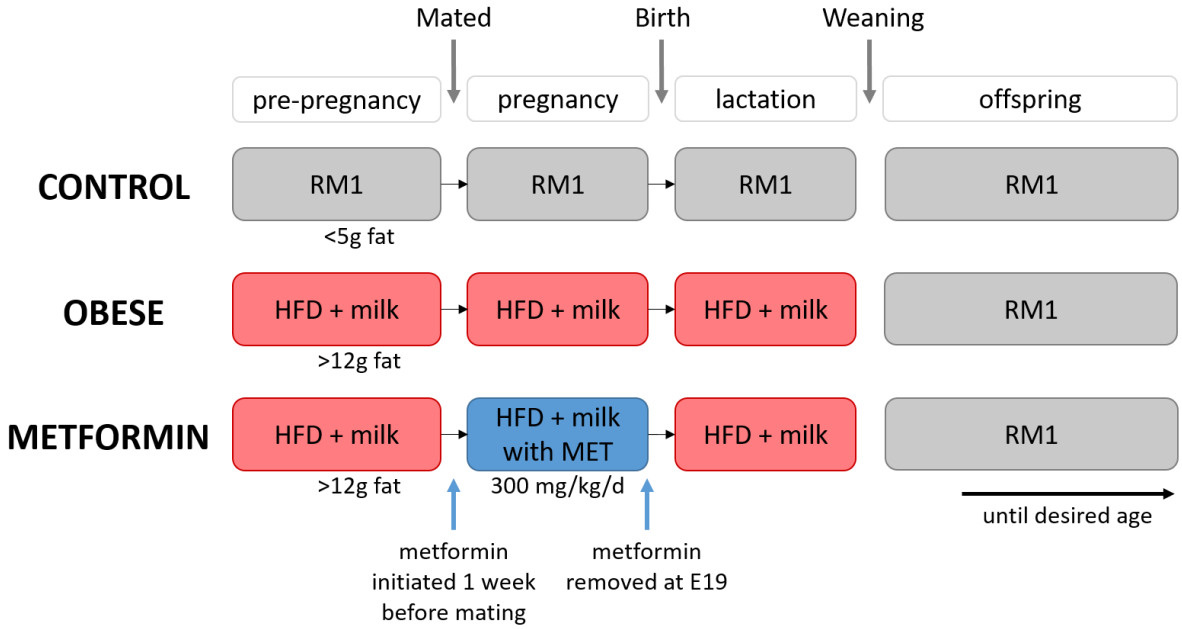

Figure 2.1: Model of developmental programming by maternal diet-induced obesity with metformin intervention. *C57BL/6J dams were fed a chow (RM1, 7% sugars, 3% fat) or high fat diet (HFD, 10% simple sugars, 20% fat) with sweetened condensed milk (55% simple sugars, 8% fat) from 6 weeks of age, and were mated for a second pregnancy or treated with metformin once obese dams exceeded 12 grams fat mass (TD-NMR). Metformin (MET, approx. 300mg/kg/day) was dissolved in the milk and given 1 week before mating until E19. Offspring were weaned onto a chow diet at 3 weeks of age until tissue collection at 8 weeks or 12 months of age. A total of n=90 dams (n=31 Control, n=33 Obese, n=26 Obese Metformin) were used to generate n=263 offspring (n=61 males and n=63 females at 8 weeks, n=68 males and n=71 females at 12 months, for more details see relevant Results chapters 4, 5 and 6).*

2.2 *In vivo* metabolic phenotyping of offspring

2.2.1 Body composition

Whole litter weights were recorded at PN2, 7, 14 and 21. After weaning, body weight and food intake were recorded weekly[4]. Offspring body composition was assessed in the afternoon between 1-4pm using TD-NMR either once at 8 weeks (Chapter 4), or weekly from 4 to 12 weeks of age and monthly until 6 months of age (Chapter 5).

[4] Collection of body weight and food intake data by C. Custance, analysis by JMS

2.2.2 Intraperitoneal glucose tolerance test

Following an overnight fast in a clean cage, offspring were injected intraperitoneally with 1g/kg glucose[5]. Blood glucose was measured from the tail vein at baseline, 15, 30, 60 and 120 minutes post-injection (AlphaTRAK2, Zoetis, USA).

Changes in glucose levels during the ipGTT were analysed using repeated measures two-way ANOVA in Prism 8.0 (GraphPad, USA). Area under the curve (AUC) was calculated for each animal using the trapezoid rule and compared between groups using one-way or two-way ANOVA where applicable.

2.3 Tissue collection

Body weight and tail blood glucose (AlphaTRAK2, Zoetis, USA) were recorded prior to culling via Schedule 1. Mice that had undergone metabolic and cardiovascular phenotyping were euthanised (rising CO_2 concentration or cervical dislocation[6]) in the fed state, whereas their untouched siblings were euthanised (rising CO_2 concentration) following a 16h fast. Blood was obtained through cardiac puncture in fasted animals and left to clot for a minimum of thirty minutes. Serum was collected following centrifugation for 2-3min at 3000g and stored at −80°C. Excised tissues were weighed and formalin-fixed (fed tissues) or snap-frozen on dry ice (fasted tissues).

2.4 Molecular analysis

2.4.1 Serum analysis

Measurement of serum cholesterol and triglycerides was performed at the Core Biochemical Assay Laboratory at the University of Cambridge[7]. Serum insulin was measured using the Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, USA). HOMA indices of IR and β-cell action were calculated using Equations 1 and 2.

Equation 1: HOMA-IR = (fasted glucose in mmol/l · fasted serum insulin in mU/L) / 22.5

Equation 2: HOMA-%B = (20 · fasted insulin in mU/L) / (fasted glucose in mmol/l − 3.5)

2.4.2 Gene expression

Epididymal or gonadal adipose tissue was lysed on ice in 700µl QIAzol Lysis Reagent (Qiagen, USA) using the TissueRuptor II (Qiagen). RNA was extracted using the miRNeasy MiniKit (Qiagen) with DNase digestion (RNase-Free DNase Set, Qiagen) according to the manufacturer's protocols, and RNA was eluted in 25-40µl nuclease-free water. RNA concentration was determined by the NanoDrop 1000

[5] Intraperitoneal injection by Dr D. Fernandez-Twinn or C. Custance, blood collection and glucose measurement by JMS
[6] Cervical dislocation performed by T. Ashmore or P. Wilsmore
[7] Assay performed by K. Burling, analysis by JMS

Spectrophotometer (ThermoScientific, UK) and RNA was stored at −80°C until used for gel electrophoresis (1% agarose gel run at 80V for 45'-1h). All samples showed clear 18S and 28S rRNA bands (Figure 2.2). cDNA was generated on the same day using the HiCapacity RT kit (Applied Biosystems, USA) with addition of RNAse inhibitor (Applied Biosystems) using the Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Table 2.2). cDNA was diluted based on starting RNA concentration and stored at −20°C until used for qPCR.

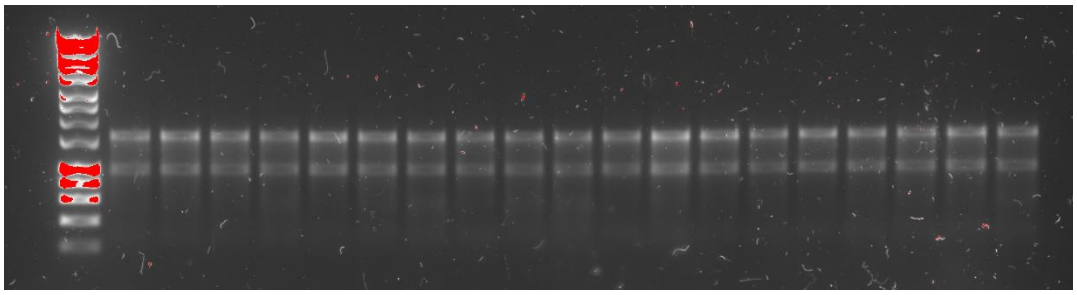

Figure 2.2: Example of good RNA quality on 1% agarose gel

Reagent	**For 1 reaction (µl)**	**Thermal cycler protocol**	
10x RT buffer	2.0	Step 1	10' at 25°C
25x dNTP mix	0.8	Step 2	120' at 37°C
10x RT primer	2.0	Step 3	5' at 85°C
MultiScribe™ RT enzyme	1.0	Step 4	4°C until stored
RNase inhibitor	1.0		
Nuclease-free H_2O	3.2		
Total 2x master mix	**10**		
RNA	10		
End reaction volume	**20**		

Table 2.2: cDNA synthesis reaction protocol

In order to test the efficiency and linearity of qPCR primers, the diluted cDNA was pooled to generate standards through a two-fold serial dilution. Primers were considered validated if they showed single-peak melt curves and primer efficiency of between 80-120%. cDNA samples were then further diluted to the optimal concentration to measure all target genes. Real Time quantitative PCR (qPCR) was performed in duplicate on 1:40 (12-month samples) or 1:80 (8-week samples) diluted cDNA using SYBR™ Select Master Mix (Applied Biosystems) and the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems)[8]. Primer sequences are shown in Table 2.3. qPCR data was analysed using the comparative CT method normalised against suitable housekeepers for which expression did not change with experimental group or sex.

[8] For primer efficiency tests, StepOnePlus™ Real-Time PCR System (Applied Biosystems) was used instead

Gene name	Forward 5'-3'	Reverse 5'-3'
Adiponectin	CAAGGCCGTTCTCTTCACCT	CCCCATACACTTGGAGCCAG
Atgl	GGAGGAATGGCCTACTGAACC	ATCCTCTTCCTGGGGGACAA
Ccl2/Mcp1	CAGATGCAGTTAACGCCCCA	TGAGCTTGGTGACAAAAACTACAG
Ccl3/Mip1a	CAGCCAGGTGTCATTTTCCTGA	AGGCATTCAGTTCCAGGTCA
Ccr2	AGGAGCCATACCTGTAAATGCC	TGTGGTGAATCCAATGCCCT
Cd11c	TGCTGTTGGGGTTTGTTTCTTG	CGAACTCAGCACCGTCCAT
F4/80	CACTTCCAAGATGGGTTAACATCC	CTGCCATCAACTCATGATACCCT
Glut4	GGCCGGGACACTATACCCTA	AGAGCCGATCTGCTGGAAAC
Hif1a	TCAGTTGTCACCATTAGAGAGCAAT	GGGTCTGCTGGAATCCTGTAAC
Hprt	GAGAGCGTTGGGCTTACCT	ATCGCTAATCACGACGCTG
Hsl	CACACCTACTACACAAATCC	GGCATAGTAGGCCATAGCA
Il1b	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
Il6	GGAGTCACAGAAGGAGTGGC	AGGTTTGCCGAGTAGATCTCAA
Leptin	CAAAACGTGCTGCAGATAGC	CCAGCAGATGGAGGAGGTC
Nos2/iNos	TGCGAAAGGTCATGGCTTCA	GTCCCTGGCTAGTGCTTCAG
Pparg	CTCCTGTTGACCCAGAGCAT	CCATGGTAATTTCTTGTGAAGTGCT
Ppia	GTCCAGGAATGGCAAGACCA	GGGTAAATGCCCGCAAGTC
Sdha	TTACAAAGTGCGGGTCGATGA	TGTTCCCCAAACGGCTTCTT
Tnf	AAGTTCCCAAATGGCCTCCC	CACTTGGTGGTTTGCTACGA
Ucp1	ACTGCCACACCTCCAGTCATT	CTTTGCCTCACTCAGGATTGG

Table 2.3: Primer sequences for qPCR

2.5 Histological analysis

2.5.1 Preparation of adipose tissue sections

Formalin-fixed epididymal or gonadal adipose tissue was processed, embedded, sectioned and stained with haematoxylin and eosin (H&E). Whole sections were scanned using the Axioscan digital slide scanner (Zeiss, Germany)[9]. Images were then analysed using HALO analysis software (Indica labs, USA) software as outlined below.

2.5.2 Cell size analysis

A classifier was generated differentiating between background (green), adipose tissue (red), or 'other' (yellow) which included processing artefacts, blood vessels and other structures that were not adipose tissue. The classifier was trained using artificial intelligence (DenseNet AI Plugin) overnight and adjusted based on a selection of sections from each experimental group until satisfactory. Adipocyte size analysis was then performed on areas classified as adipose tissue using the Vacuole Quantification tool adapted to the H&E-stained WAT sections (Figure 2.3). Data for each adipocyte was exported for all animals and analysed using Microsoft Excel and Prism 8.0 (GraphPad) to determine frequency distribution, median cell size and proportion of large cells (>90th centile of controls) present in the section.

[9] Histological processing expertise provided by T. Ashmore

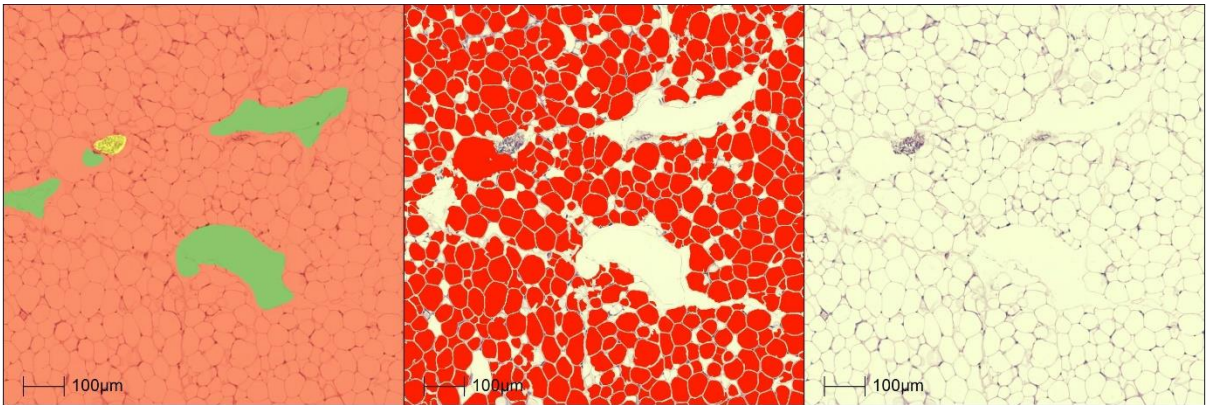

Figure 2.3: Cell size analysis using Indica Labs HALO image analysis software.
Left: classifier showing adipose tissue (red), background (green) and artefacts (yellow). Middle: Vacuole Quantification tool adjusted to adipose tissue. Right: representative haematoxylin and eosin-stained adipose tissue section from an 8-week-old animal.

Estimated adipocyte number was calculated based on methods previously described (307–309). Briefly, cell number can be calculated using Equation 3 below. Under the assumption that adipose tissue density is equal between groups, an estimate of total epididymal or gonadal adipose tissue volume can be obtained based on the density of adipose tissue [0.9029g/cm^3 reported for rodent internal adipose tissue(310)] and the weight of the collected tissue (Equation 4). Under the assumptions of cell sphericity and cell area representing mid-line cross-sections, mean adipocyte volume can be calculated from mean adipocyte area based on the equations for the cross-sectional area (Equation 5) and volume of a sphere (Equation 6). Together, this provides an equation for estimated mean adipocyte volume (Equation 7).

Equation 3: Estimated cell number = total volume of tissue / mean volume of one adipocyte

Equation 4: Total tissue volume in µm^3 = adipose tissue weight in mg · (10^{12} / 902.9)

Equation 5: Cell cross-section area in µm^2 = π · r^2 → r = √(area/π)

Equation 6: Cell volume in µm^3 = 4/3 · π · r^3

Equation 7: Mean cell volume in µm^3 = 4/3 · π · (√(mean area/π))^3

2.5.3 Crown-like structures

A second classifier was generated to detect aggregates of macrophage infiltration termed crown-like structures (CLS, see Chapter 4.1.4). This classifier differentiates between background (yellow), adipose tissue (red), CLS (blue), or 'other' (green) which included processing artefacts, blood vessels and other structures that were not adipose tissue (Figure 2.4). In order to ensure that the CLS classifier only detected aggregated and not individual macrophages, only areas >99µm^2 were included. The classifier was trained using artificial intelligence and adjusted based on a selection of sections from each

experimental group until satisfactory, which in this case required overnight training following adjustment. The classifier was generated using 8 week adipose tissue samples and adjusted and retrained for the 12m cohorts. Data for each classified object per animal was exported, and Microsoft Excel was used to determine the number and median size of CLS, as well as the proportion of adipose tissue area taken up by CLS using Equation 8. To correct for variation in section sizes, CLS number was expressed relative to the area classified as WAT.

Equation 8: CLS% = CLS area / (CLS area + WAT area)

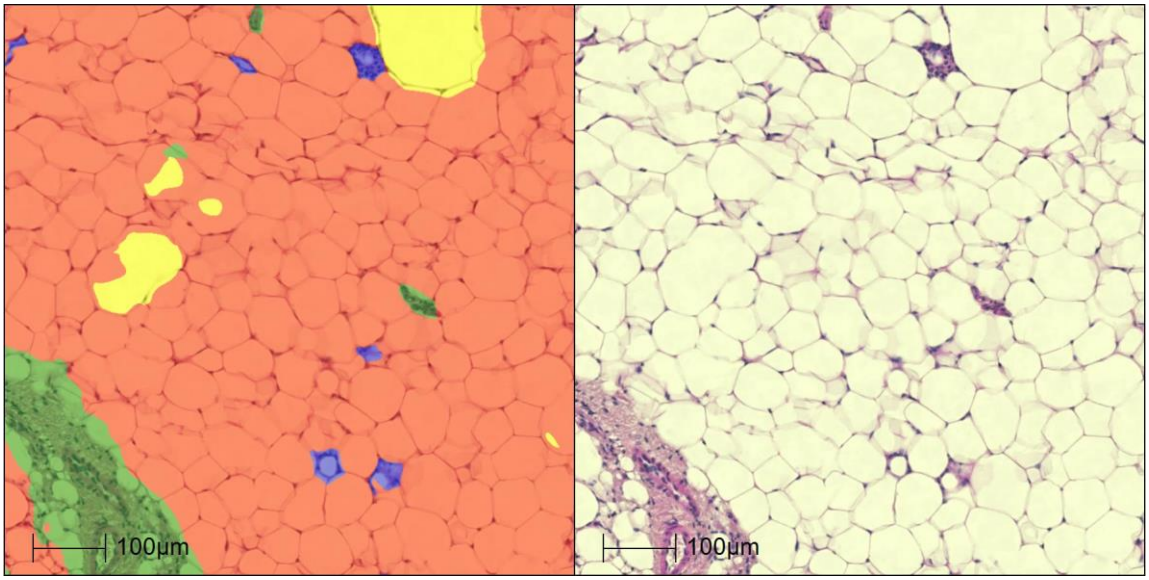

Figure 2.4: Crown-like structure analysis using Indica Labs HALO image analysis software.
Left: classifier showing adipose tissue (red), background (yellow), crown-like structures (blue) and artefacts (green). Right: representative haematoxylin and eosin-stained adipose tissue section from an 8-week-old animal.

2.5.4 Adipose tissue browning

Upon visual inspection of WAT sections, areas resembling brown adipose tissue (BAT, characterised by small multilocular adipocytes) were observed in some sections. Therefore, a third classifier was generated differentiating between background (yellow), adipose tissue (red), BAT-like tissue (green), or 'other' (blue) which included processing artefacts, blood vessels and other structures that were not adipose tissue (Figure 2.5). The classifier was trained using artificial intelligence overnight and adjusted based on a selection of sections from each experimental group until satisfactory. Data was exported and analysed using Microsoft Excel to determine the proportion of adipose tissue that showed evidence of browning using Equation 9.

Equation 9: % Browning = BAT-like area / (BAT-like area + WAT area) · 100%

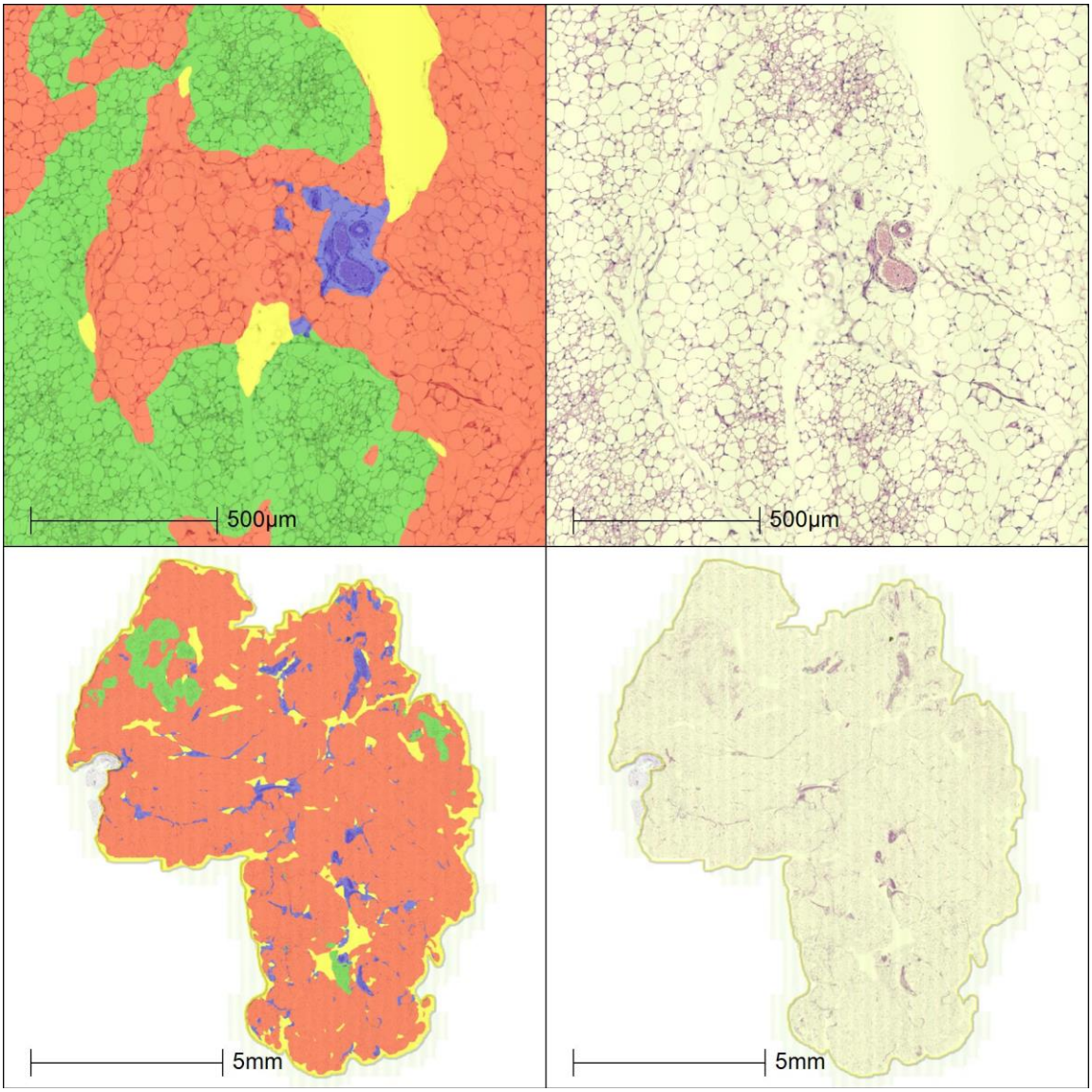

Figure 2.5: Analysis of adipose tissue browning using Indica Labs HALO image analysis software.
Left: classifier showing adipose tissue (red), background (yellow), brown adipose tissue-like areas (green) and artefacts (blue). Right: representative haematoxylin and eosin-stained adipose tissue section from an 8-week-old animal.

2.6 Statistical analysis

Data were analysed using Prism 8.0 software (GraphPad). For time-course data and comparison across ages, repeated measures two-way ANOVA (complete dataset) or mixed effects model analysis (incomplete dataset) was performed with Tukey's post-hoc test for multiple comparison where appropriate. Data taken at a single time point were analysed using t-test and one-way ANOVA or relevant non-parametric alternatives (Mann-Whitney U and Kruskal-Wallis) with Tukey's post-hoc test for multiple comparison where appropriate.

Data was tested for normality using the normality tests provided by Prism (Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov test): if at least three of these indicated a normal distribution then parametric testing was warranted. When data was not normally

distributed, lognormality was tested and relevant parametric tests were performed on log-transformed data instead. When data were not normally or lognormally distributed, non-parametric testing was performed as described above.

Data are shown as mean ± SEM or median [interquartile range] where appropriate. N-numbers refer to independent litters from which maximum one male and/or one female was used. Data are considered statistically significant if $p<0.05$ unless specified otherwise. Significant and trending ($0.10<p<0.05$) results in tables and textboxes (two-way ANOVA) are depicted by **bold** or <u>underlined</u> text, respectively. In figures, significant results are depicted as follows: *Con vs Ob, $^{\#}$Con vs Ob-Met and †Ob vs Ob-Met, with *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

3 Dam phenotype

3.1 Introduction

3.1.1 Metformin in human pregnancy

In the UK, metformin is the first-line pharmacological treatment for GDM if diet and lifestyle changes are insufficient to achieve glycaemic control, with the exception of women for whom metformin use is contraindicated, who prefer insulin or who have fasting glucose levels higher than 7.0mmol/l (30). As GDM is usually diagnosed in the second or third trimester, many fetuses in the UK will be exposed to metformin use from mid to late gestation. In addition, women with T2DM or PCOS may continue pre-gestation metformin use when becoming pregnant, thereby exposing the fetus to the drug from early gestation (30,225). Hence, an estimated 1-2% of live births in developed countries will be exposed to metformin *in utero* (228).

Metformin treatment for GDM in the USA usually comprises 500-1000mg twice daily in the form of oral tablets, although the American College of Obstetricians and Gynecologists states the dose may increase up to 3500mg if glycaemic control is not achieved (221). The British National Formulary does not provide advice on metformin use in pregnancy, but recommends T2DM patients start with 500mg metformin once daily to be increased to a maximum of 2000mg, with a maximum of 1700mg for non-pregnant PCOS patients (311). Doses in RCTs range between starting dose of 500mg and maxima of 1700-3000mg for GDM, obese and PCOS pregnancies (233,240,260,271,272,295,312,313). Metformin use in pregnancy is well-tolerated despite reported side effects of diarrhoea and bloating (260,314). However, some women fail to maintain glucose control on metformin and require supplemental insulin. These women tend to be older and have higher BMI and/or glycaemia at enrolment (265,271,272).

3.1.2 Pharmacodynamics and pharmacokinetics

Metformin is an insulin sensitising agent. The main target organ is the liver, where metformin improves glycaemia by improving insulin sensitivity and consequently suppressing hepatic glucose output (219). In addition, metformin enhances glucose uptake by the intestine and skeletal muscle, and is shown to have anti-inflammatory and cardioprotective effects (225). The molecular mechanism of action of metformin is not entirely understood, but it is agreed that metformin exposure leads to activation of AMP-activated protein kinase (AMPK)(225), a global nutrient sensor often dysregulated in metabolic disease. Hence, phosphorylation of AMPK is often used as a marker of metformin action in tissues (315). This may, in part, be mediated by inhibition of complex 1 of the mitochondrial electron transport chain, which has been shown especially with high doses of metformin, although metformin may activate AMPK through electron transport chain-independent mechanisms as well (225). Other

molecular effects of metformin include altered mammalian target of rapamycin complex 1 (mTORC1) signalling and increased levels of GDF15, the latter of which was recently suggested to be essential for metformin-induced weight loss (225,316).

As metformin is taken orally, it enters the body through the intestinal tract, reaches the liver via the portal circulation and it is ultimately actively excreted in the urine. Metformin largely circulates in an unbound form and is not metabolised by the body (317). In accordance with increased renal excretory capacity in pregnant individuals, renal clearance of metformin is increased in mid- and late pregnancy (288,318,319). Its circulating half-life is reported at 4-5h during mid-late pregnancy leading to almost complete elimination from the maternal circulation within 24h (288,319,320). However, metformin bioavailability following intestinal absorption is increased during pregnancy thereby preventing any significant changes in systemic metformin exposure compared to non-pregnant women with oral doses of >1000mg, although circulating metformin levels may be lower in pregnancy when receiving a 500mg dose (319). The pharmacokinetics of metformin are thus dependent on the dose taken and the stage of pregnancy, but are not known to be affected by pregnancy complications such as GDM or PCOS (288,321).

Metformin is hydrophilic and ionised at physiological pH and requires transporters to enter target cells. Transporters include those from the organic cation and multidrug and toxic compound extrusion families, the serotonin transporter and plasma membrane monoamine transporter (322). These transporters are predominantly expressed in the liver, intestine and kidney, but are also found in the brain, skeletal muscle, heart and reproductive tissues of adult humans and rodents (322). Relevant transporter expression has also been found in the human (first trimester and term) and rodent (mid-late gestation) placenta (323–325). Furthermore, although expression is close to undetectable in embryos (315), higher expression levels have been detected in fetal tissues including brain, kidney, liver and intestine (325) in mid-late gestation. However, detailed reports on fetal transporter expression are lacking.

Metformin readily crosses the placenta. Human studies using the *ex vivo* dually perfused placenta report dose-dependent bidirectional transfer of metformin, with transport from the fetal to maternal compartment being higher thus theoretically protecting the fetus from excessive metformin exposure (326–329). The bidirectional placental passage was also confirmed in *ex vivo* rodent studies, indicating conservation across species (324). However, human trials showed that cord metformin concentrations are similar (281,288,319,320) or even higher (330) than in the maternal circulation, indicating that *in vivo* placental transfer is likely higher than in the perfused placenta model. This was also observed in a mouse model of maternal metformin treatment in pregnancy (301). Moreover, the maternal-to-fetal

half-life was measured at 5 minutes in women during the second or third trimester, indicating maternal and fetal exposure is almost concurrent (320). The rate of transplacental passage does not differ between normal and GDM-complicated pregnancies (328). In accordance with its short half-life, studies in rodents have shown that metformin concentrations in offspring decline rapidly after birth (331).

3.1.3 Human RCTs

The main indication of metformin during pregnancy is GDM and most RCTs investigating pregnancy outcomes have compared metformin to insulin in a GDM context. As described in detail in Chapter 1.7.3, the largest RCT was the MiG trial in Australia and New Zealand (260). In addition, two Finnish trials by Ijäs *et al.* (271) and Tertti *et al.* (272) have been carried out, and there are plans for combined offspring follow-up from these RCTs (NCT02417090 registered on www.clinicaltrials.gov). Trials for pre-existing T2DM are now also underway: the MiTy trial investigates the potential use of metformin as an adjuvant to insulin in pregnant T2DM patients (332). In the context of PCOS, initial studies focused on the safety of continued metformin use in the first trimester after using the drug for ovulation induction (290). The largest RCTs investigating metformin initiated in the first trimester and continued throughout pregnancy are the Norwegian PregMet and PregMet2 trials (276,313). Interestingly, some studies in women with GDM or PCOS have reported different outcomes in lean and obese pregnancies (313,333–336). Indeed, research is ongoing into the specific effect of the metformin intervention in pregnancies complicated by obesity. Three large RCTs investigating the use of metformin in obese glucose tolerant pregnancy have been published to date: the EMPOWaR and MOP trials in the UK (233,274) and the more recent GRoW RCT investigating obese and overweight women in New Zealand (337).

As outlined in Chapter 1.7.3, metformin use in pregnancy is associated with improved glycaemic control (in GDM women), decreased GWG and a reduction in pregnancy-induced hypertension and preeclampsia (259,278,284). Metformin also decreases birth weight, has protective effects on macrosomia/LGA risk and leads to decreased rates of neonatal hypoglycaemia and intensive care admission compared to insulin treatment (278,294). However, studies in humans rarely investigate maternal body composition and food intake. Moreover, metformin crosses the placenta and long-term effects of fetal exposure remain unknown. Animal models investigating metformin intervention are therefore crucial to elucidate short- and long-term effects on both mother and offspring.

3.1.4 Animal models of metformin in gestation

As mentioned in Chapter 1, rodent models can provide a useful tool to study the safety and efficacy of interventions during obese pregnancy due to their short generation time and the ability to control

external factors. In addition to practicality, it is more ethical to investigate gestational treatments in small animals before directly testing them in humans. When this project was designed, few published studies investigated the effects of early metformin exposure. The earliest reports focused on safety of metformin to the developing embryo, by culturing whole embryos in media containing metformin (338). In day 9 mouse embryos, metformin did not affect embryo growth or facial development and was not teratogenic, although delayed neural tube closure was observed in metformin-exposed embryos (338). Another study found no adverse effects on glucose uptake or apoptosis in mouse embryos cultured with metformin, and implantation rates and crown-rump-length were unaffected after embryo transfer to pseudo-pregnant mice (339). Similarly, metformin did not impair blastocyst formation rate in porcine oocytes or embryos *in vitro* when given before or after fertilisation (340). The absence of teratogenicity may be related to the lack of metformin transporters at early developmental stages equivalent to the first trimester in humans, as evidenced by the lack of embryonic AMPK activation and transporter expression at E7.5 (315). Moreover, metformin co-administration prevented aberrant embryonic metabolism and improved successful implantation rate in mouse embryos exposed to high levels of IGF-1 *in vitro* (339). Furthermore, metformin treatment to blastocysts from obese T2DM-like TallyHo mice decreased the incidence of metabolic abnormalities compared to untreated blastocysts, suggesting metformin may actually prevent abnormal embryonic development in a diabetic setting (341).

The first *in vivo* studies published in 2006-2008 focused on the effect of metformin intervention on reproductive function in androgenised dams, modelling a PCOS phenotype (248,342). In contrast, studies published between 2011-2017 highlighted the increased use of metformin in GDM-complicated or obese pregnancy (Table 3.1). These *in vivo* studies include models of chow-fed animals to assess safety and effects of metformin per se without confounding by maternal phenotype or effect of metformin on maternal glycaemia. In absence of obesity or diabetes, most studies report no effect on GWG, body weight in pregnancy or in lactation (247,301,331), although one study reported lower weight gain in rat dams exposed to daily gavage with metformin (343). Similarly, despite its glucose-lowering properties, metformin did not affect maternal glucose levels at mid-gestation or at E14 (247,303,331), although one of these studies observed a reduction in area under the curve following an ipGTT at E14 in absence of any changes in insulin secretion, indicating improved insulin sensitivity (247). Maternal intake of the chow diet was not affected by the metformin intervention in any of these studies (247,301,343).

Study	Strain	Model	Dose	Method	Period
Chow-fed models					
Alzamendi 2012 (247)	Sprague-Dawley rats	chow	50mg/kg/d	drinking water	E1-culling
Tartarin 2012 (348)	NMRI mice	chow	300mg/kg/d	oral gavage	E0.5-13.5* E0.5-culling
Salomäki 2013 [a] (301)	C57BL/6NHsD	chow	300mg/kg/d	oral gavage	E1-E17.5*
Lee 2014 (315)	ICR mice	chow	120mg/kg/d	oral gavage	E1-culling
Gregg 2014 (331)	C57BL/6 mice	chow	5mg/ml	drinking water	E0.5-culling
Novi 2017 (343)	Wistar rats	chow	293mg/kg/d	oral gavage	E1-E21 E1-PN21
Overnutrition/obesity/insulin resistance models					
Tong 2011 (304)	C56BL/6J mice	HFD pre-conception	350mg/kg/d	drinking water	E1-PN21*
Alzamendi 2012 (247)	Sprague-Dawley rats	FRD from conception	50mg/kg/d	drinking water	E1-culling
Desai 2013 [b] (238)	Wistar rats	HFHS pre-conception	300mg/kg/d	oral	E1-culling
Salomäki 2014 [a] (303)	C57BL/6NHsD mice	HFD pre-conception	300mg/kg/d	oral gavage	E1-E17.5*
Harris 2016 [b] (178)	Wistar rats	HFHS pre-conception	300mg/kg/d	oral	E1-culling
Albaghdadi 2017 (344)	NONcNZO (genetic IR)	HFD pre-conception	200mg/mL	drinking water	weaning-E18
Salomäki-Myftari 2016 [a] (302)	OE-NPY mice (genetic OB)	chow	300mg/kg/d	oral gavage	E0.5-E17.5*
Liang 2016 (345)	C56BL/6J	HFD in lactation	200mg/kg/d	oral to pup	PN1-PN21*

Table 3.1: Rodent models investigating metformin use in pregnancy and/or lactation published by September 2017.
*FRD = fructose-rich diet, HFD = high fat diet, HFHS = high fat high sugar diet, IR = insulin resistance, OB = obesity. *dams were allowed to litter and generate offspring. [abc] letters identify studies that were carried out by the same research group.*

Some groups have used more clinically relevant models of maternal overnutrition, DIO or genetic susceptibility to adiposity or IR to mimic the environment in GDM pregnancy. Alzamendi *et al.* provided rat dams with high fructose-containing drinking water alongside a chow diet from conception, increasing maternal caloric intake and altering maternal glucose homeostasis by E14 without changes in body weight. When given to high fructose-fed dams, metformin attenuated the hyperinsulinaemia upon ipGTT as well as the observed defects in fetoplacental vasculature that were seen in placentas from untreated dams (247), consistent with aforementioned protective effects on preeclampsia development. Other studies introduce their diet before mating to induce an obese and/or glucose intolerant phenotype. Salomäki *et al.* fed mice a HFD from one month before pregnancy, and found metformin treatment during pregnancy decreased maternal glycaemia while no effect was seen in chow-fed dams (303). Other studies used maternal preconception HFHS to induce maternal obesity, resulting in maternal hyperleptinaemia, hyperlipidaemia, hyperinsulinaemia and placental and fetal inflammation (238). Maternal metformin treatment prevented placental and

fetal inflammation and improved fatty acid metabolism in the fetal liver without altering maternal metabolic state (178,238). In addition to overfeeding, studies have used strains that are genetically predisposed to obesity or diabetes, either with (344) or without the additional challenge of HFD feeding (302). In a model of genetic diabetes, administering metformin from weaning had beneficial effects on maternal glycaemia, weight, placentation and reproductive outcome in HFD-fed New Zealand Obese mice (344). In contrast, genetically obese NPY-overexpressing mice showed decreased weight gain and chow intake in pregnancy after metformin treatment but glucose levels were unaffected (302). Lastly, one study exposed dams to HFD during lactation, and administered metformin directly to pups to investigate effects of the intervention in lactation (345).

3.1.5 Our metformin intervention mouse model

The Ozanne laboratory has published the results from our well-established maternal DIO model widely (87,112,122,128,129,132,133,136–139). Since metformin is being investigated for use in obese pregnancy (233,240,346) this seems an appropriate model for a metformin intervention study. Moreover, this model is especially clinically relevant since dams do not develop glucose intolerance until pregnancy and the model is thus reflective of obese GDM (136). Moreover, most trials investigating metformin use in GDM also have an overweight or obese mean BMI at baseline (271,272,347). Lastly, although ours is not a model of PCOS, IR is a hallmark feature of both our mouse model and PCOS (276) and therefore our results may be informative in a PCOS context as well.

Although some use lower doses (247,248), most rodent studies use around 300mg/kg metformin per day (Table 3.1). As reported by Salomäki *et al.*, allometric scaling based on body surface area revealed that this dose equates to 1700mg in an adult man weighing 70kg (301). This is comparable to the maximum doses aimed for by the pregnancy RCTs mentioned above, which vary between 1700-3000mg (233,260,271,272,274,276,295,313,337).

Metformin can be administered through oral gavage once (301–303,343,348) or up to three times daily (315). Oral gavage is an invasive procedure which can be stressful for the dam (349). With the established role of maternal stress in developmental programming in mind (60), gavage may not be the most suitable method of metformin administration for this study. In humans, metformin tablets are taken several times per day and therefore daily gavage would not be reflective of the human dosing protocol. Placing metformin in the diet or (as often published, see Table 3.1) in the drinking water is a stress-free method of ensuring continuous dosing throughout the active phase. Unfortunately, our animal facility was not equipped to accurately measure water intake and therefore the amount of metformin ingested could not be measured if placed in the drinking water. Instead, metformin can be dissolved into the sweetened condensed milk that provides the sugar component

of the experimental diet used in our model. Data regarding milk intake during pregnancy and lactation is routinely collected by our animal technicians and can be used to calculate both the amount of metformin ingested and what dose should be placed in the new milk pot. Therefore, in our model metformin is administered via the condensed milk.

Most studies commence metformin administration at conception (178,238,247,301–304,315,343,345,348), although some initiate metformin earlier in life which may be more reflective of pre-pregnancy metformin use that is continued after conception (344). However, to avoid confounding by potential stress related to a dietary change in early pregnancy, metformin in this study was administered from one week before mating. This ensures acclimatisation to the metformin intervention pre-gestation as well as intervention effectiveness by increasing the administration time. Moreover, in the exercise intervention model used by our laboratory previously, the dams are also trained for one week before mating (136). By introducing the metformin intervention at the same time as the exercise, comparisons between the two interventions can be made. As with the exercise intervention, metformin is removed at E19, shortly before parturition usually takes place in the mouse (E20-21). This minimises the potential stress during delivery caused by metformin exposure. Additionally, because the milk pot and diet are replaced on E19, the cage does not need to be opened again until PN2 therefore avoiding additional stress during the first days of postnatal life. During lactation, dams are provided with regular condensed milk without metformin. This duration of dosing reflects the human situation where metformin is also discontinued at delivery (350).

Since the start of this study, more study designs for metformin exposure in early life have been published. For instance, instead of diet-induced GDM, animal models of streptozotocin-induced non-obese GDM have now been published (351–354). Also of note are models that initiate metformin in mid-late gestation (351,354–357), or those that introduce or continue the metformin intervention during lactation (307,358,359), thereby targeting a different intervention window. Interventions implemented during lactation are particularly interesting with respect to offspring outcomes in organ systems that undergo certain developmental processes postnatally [e.g. β-cell proliferation in pancreatic islets and adipocyte maturation (307)] and hence may be influenced by changes in maternal health during this time. In contrast to the placenta, transmission through the breast milk is negligible in both humans and rodents (288,307). Like our model, one other study also initiated metformin treatment one week pre-mating (359). Other studies investigate metformin intervention combined with other treatments, such as insulin or swimming exercise (351,360). Most models use rodents but studies in Iberian sows and chinchillas have also been published (361–363). Lastly, animal models of maternal metformin treatment for other conditions have been designed, such as IUGR, PTB and preeclampsia (357,362,364–366).

3.2 Methods

3.2.1 Metformin dosing protocol

Upon reaching the critical threshold of fat mass (12g by TD-NMR), half of the obese dams were single housed and provided with metformin in the condensed milk. The amount of metformin aimed for was 300mg/kg/day. The dose to be added to a 75g milk pot was calculated throughout the dosing period using the average milk intake over the days prior and current body weight (calculations in Appendix A). This dose was dissolved in 1ml distilled water which was stirred into the milk pot. The water was previously shown not to affect the palatability of the milk, although it did decrease the caloric content of the milk from 3.26 (un-dosed milk) to 3.22 kcal/gram (dosed milk). The dose was adjusted twice weekly. The estimated dose ingested was calculated (see Appendix A) for the following four periods: the pre-mating week, week 1 of gestation (E1-E7), week 2 of gestation (E7-E14) and week 3 of gestation (E14-E19). Some metformin-treated dams did not show a copulatory plug on the date of conception despite being pregnant. For these dams with 'missed plugs' an estimated plug date based on average gestation length of obese metformin-treated dams was used to calculate the ingested amount. This did not significantly alter the amount of metformin ingested each week (not shown).

3.2.2 Body composition assessment

Dams underwent TD-NMR before mating, after 1 week of metformin dosing (if applicable) and on E16[10]. Unfortunately, issues with the TD-NMR machine meant it was out of action from mid-January until June 2019. Therefore, TD-NMR data is unavailable for dams mated in 2019 and offspring during this period.

3.2.3 Routine data collection

During gestation dams were weighed on E1, E7, and E14, after which they were left alone to deliver without handling. During both pregnancy and lactation, the control diet was topped up on a weekly basis, and the obesogenic diet was topped up once or twice weekly coinciding with the replacement of a fresh milk pot. Body weights and start and end weights of the diet and milk pots were recorded[11]. After parturition, pups were left unhandled for the first two days of postnatal life. Litters were standardised by culling back to 6 per litter and only used if they contained more than 4 pups. Dams and whole litters were weighed on PN2, PN7, PN14 and PN21[12]. Gestation length was computed from the recorded plug and litter dates. Fractional growth rate was calculated using Equation 10.

Equation 10: Fractional growth rate (g/d) = ((PN21 weight – PN2 weight) / 19 days

[10] TD-NMR performed by C. Custance or T. Ashmore, data analysis by JMS
[11] Weights recorded by C. Custance and T. Ashmore, data analysis by JMS
[12] Weights recorded by C. Custance and T. Ashmore, data analysis by JMS

3.2.4 Analysis of food intake

Using routinely recorded weights of the pellet (chow or HFD) and condensed milk, average intake in grams was calculated for each day of pregnancy and lactation. Because the HFD pellet is crumbly, average intake in grams was plotted for each dam in the Obese and Obese Metformin group to determine what values represented accurate intake. There was clear separation between the data and a daily intake of below 5 grams (pregnancy) and 11.5 grams (lactation) was considered correctly measured. Using the caloric content of the diet shown in Table 2.1, intake in grams was converted to kcals. As described above, caloric content of the metformin-treated milk was 3.22 rather than 3.26kcal/g. Intake of the HFD pellet and condensed milk were combined to provide total caloric intake of the obesogenic diet. Average intake for week 1 (E1-E6), week 2 (E7-E13) and week 3 (E14-E19) was calculated to assess changes in caloric intake with advancing gestation, while total gestational intake in pregnancy comprised cumulative intake between E1-E19. For lactation, average intake was calculated for week 1 (PN2-7), week 2 (PN8-14), week 3 (PN15-21) and for all of lactation (PN2-PN21). Caloric intake data from both pregnancy and lactation are shown normalised to control pregnancy intake as fold change. Caloric intake data in time-courses was normalised to the caloric intake of control dams during week one of pregnancy.

3.2.5 Statistical analysis

Data were analysed using Prism 8.0 (GraphPad) as described in section 2.6. Data are presented as mean ± SEM or median [interquartile range] where appropriate. Time-courses are analysed using repeated measures two-way ANOVA (complete dataset) or mixed effects model analysis (missing datapoints) with factors (gestational) age and maternal environment with appropriate post-hoc analyses. A p-value <0.05 is considered statistically significant.

3.3 Results

3.3.1 Metformin dosing

3.3.1.1 Metformin ingested per week

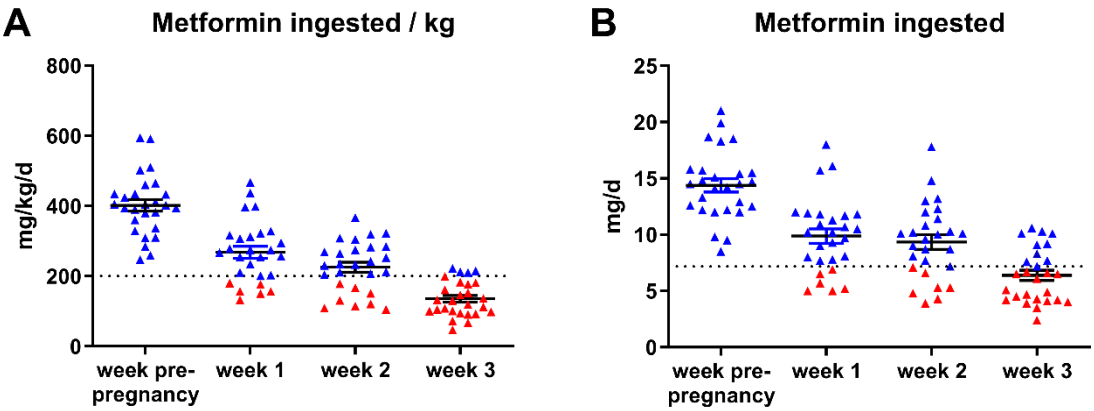

Figure 3.1: Estimated amount of metformin ingested during the pre-pregnancy week and in gestation.
Doses are shown A) corrected for dam body weight in mg/kg/d and B) uncorrected in mg/d (n=27 dams). Each symbol represents a dam that has either reached (blue) or not reached (red) the lower limit of 200mg/kg/day metformin for that week (A), or the dose equivalent of 200mg/kg/d at day 1 of gestation (7.17mg, B).

We aimed for an average dose of 200-300mg/kg/d metformin. The highest dose was achieved in the pre-pregnancy week. The estimated dose of metformin ingested decreased with progressing gestation, but only in the third week of gestation did this drop below the lower limit of 200mg/kg/day (Figure 3.1A). At the start of pregnancy mean body weight was 35.9 ± 0.5g, therefore the amount of metformin ingested to achieve 200mg/kg/d was 7.17mg. The absolute amount of metformin ingested also decreased with gestation, but fewer dams ingested less than 7.17mg metformin (Figure 3.1B), suggesting the decreased concentration of metformin in week 3 was partly related to changes in body weight with advancing gestation. It should be noted that, especially in late gestation, much of the change in body weight will be related to increased fetal weight.

3.3.1.2 Litter losses

We experienced some litter losses during breeding, especially in dams that were exposed to the obesogenic diet ($p<0.01$ compared to Control dams, Table 3.2). No difference was found in the rate of litter losses between Obese and Obese Metformin-treated dams (Table 3.2), indicating litter survival was unaffected by metformin treatment. There was no difference in the amount of metformin ingested ($p>0.05$) between dams that had generated live litters and those that lost their litter (Figure 3.2), suggesting exposure to higher doses of metformin did not increase the risk of losing litters after birth.

Maternal environment	Surviving litters	Lost litters	Rate of loss	*p*-value vs Obese
Control	41	2	5%	***<0.01***
Obese	32	11-16	26-33%	-
Obese Metformin	27	10	27%	*>0.05*

Table 3.2: Litter survival rates.
Data is shown for all dams bred in the laboratory between late January 2018 (earliest plug date of metformin-treated dams) and early June 2019 (latest litter date of metformin-treated dams). The p-values reflect outcomes of two-tailed chi-squared testing. Data is missing for 5 obese untreated dams, therefore the minimum and maximum rate of litter losses are shown for this group (this did not change the outcome of statistical testing).

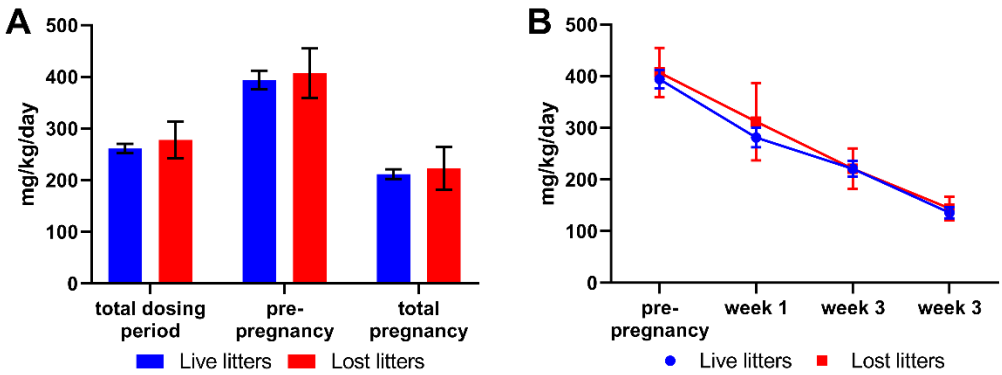

Figure 3.2: The amount of metformin ingested in dams with surviving ('live', n=22) and lost litters (n=5).

3.3.2 Body weight and composition at mating

Upon reaching 12g fat mass measured by TD-NMR, Obese Metformin dams were treated with metformin for one week prior to mating. After this week, TD-NMR was repeated in Obese Metformin dams to allow comparison of baseline body composition between the groups at time of randomisation (when Obese and Obese Metformin groups are identical) and at time of mating (when the Obese Metformin group have been administered metformin for one week).

At randomisation to metformin, there was no significant difference between Obese and Obese Metformin dams in body weight (Obese 38.6 ± 0.4, Obese Metformin 37.5 ± 0.4g) or fat mass (Figure 3.3A). At mating, dams fed the obesogenic diet were significantly heavier (Control 26.5 ± 0.3, Obese 38.6 ± 0.4, Obese Metformin 35.7 ± 0.6g, $p<0.0001$) and fatter (Figure 3.3B) compared to Control dams while lean mass was unchanged (Control 17.1 ± 0.3, Obese 17.1 ± 0.4, Obese Metformin 17.0 ± 0.3g). After one week of metformin treatment (i.e. at the time of mating) Obese Metformin dams were significantly lighter ($p<0.0001$) and less fat (Figure 3.3B) than Obese dams.

All control dams remained below the 5g fat mass limit at the time of mating. Similarly, all females on the obesogenic diet had reached the critical threshold of 12g fat mass before randomisation to metformin (Figure 3.3A). However, some metformin-treated females had lost sufficient fat mass during the pre-mating week of metformin treatment that their fat mass was below 12g at the time of mating (Figure 3.3B). Indeed, Obese Metformin females lost on average 1.8g body weight during the

metformin pre-mating treatment week. This was due to a mean loss of 1.7g fat mass as there was no difference in lean mass before and after treatment (Figure 3.3C).

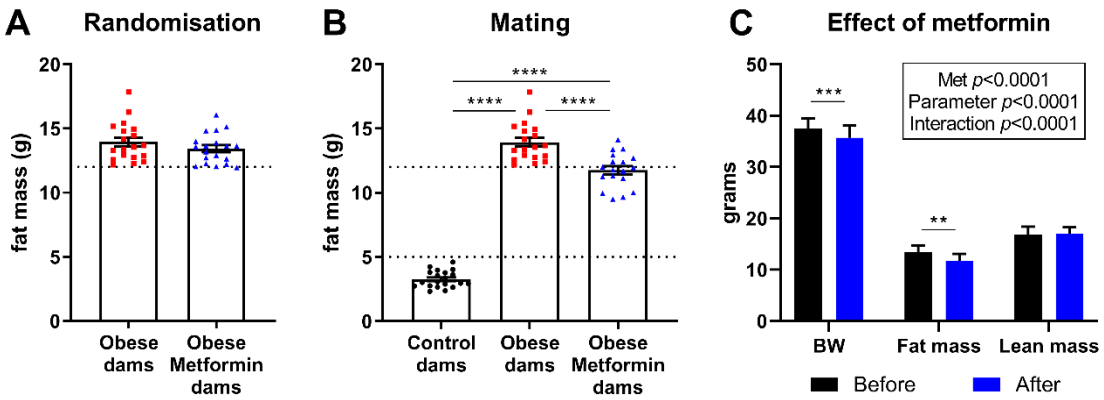

Figure 3.3: Fat mass at randomisation, at mating and following one week of metformin treatment.
*A-B) The line at 12g fat mass denotes the critical threshold of fat mass that obese dams should exceed before being mated or randomised to metformin. The line at 5g fat mass denotes the critical threshold of fat mass that control dams should remain under before mating. The data for Obese dams is the same in panels A and B because these dams were mated immediately at the time of randomisation to the untreated group. ****p<0.0001, one-way ANOVA with Tukey's multiple comparison test. C) The effect of one week metformin treatment on body composition (n=18). Box: results from repeated measures two-way ANOVA for the effect of metformin treatment (Met), the TD-NMR parameter and the interaction between them. **p<0.01, ***p<0.001 using Sidak's multiple comparison test. Numbers are n=20 Control, n=19 Obese, n=18-19 Obese Metformin.*

3.3.3 Body weight and composition during pregnancy

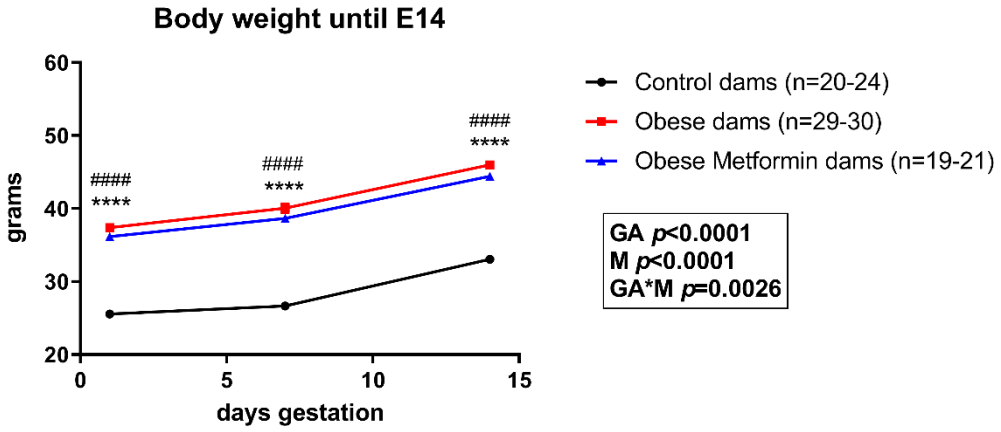

Figure 3.4: Body weight in pregnancy.
*Box: results from mixed-effects model analysis for the effect of gestational age (GA), the maternal environment (M), and the interaction between them (GA*M). ****p<0.0001 control vs obese, ####p<0.0001 control vs metformin using Tukey's multiple comparison test.*

As at the time of mating, dams fed the obesogenic diet were heavier than Control dams on E1 irrespective of metformin treatment, and this difference was maintained throughout pregnancy (Figure 3.4). There was no significant difference in GWG until E14 between Obese and Obese Metformin-treated dams in both absolute (Obese 8.6 ± 0.4, Obese Metformin 7.8 ± 0.4g, p=0.1723) or relative terms (Obese 23.1 ± 6.3, Obese Metformin 21.7 ± 1.1%, p=0.4019).

At E16, dams on the obesogenic diet (with or without metformin) were still heavier and fatter than controls. However, Obese Metformin dams had significantly lower body weight, absolute and relative fat mass compared to untreated Obese dams (Table 3.3).

	Control (n=15)	Obese (n=13)	Obese Metformin (n=6-7)	*p*-value
Body weight (g)	36.0 ± 0.7	49.0 ± 0.8 [a]	44.9 ± 1.2 [a,b]	***<0.0001***
Lean mass (g)	23.9 ± 0.7	24.7 ± 0.6	23.9 ± 0.6	*0.5955*
Fat mass (g)	4.5 ± 0.3	15.6 ± 0.6 [a]	12.4± 0.6 [a,c]	***<0.0001***
Fat mass (%)	12.5 ± 0.7	31.7 ± 0.9 [a]	27.6 ± 0.9 [a,c]	***<0.0001***

Table 3.3: Body weight and composition at embryonic day 16 (TD-NMR).
[a]p<0.0001 vs control, [b]p<0.05, [c]p<0.001 vs obese, one-way ANOVA with Tukey's multiple comparison test.

3.3.4 Food intake during gestation

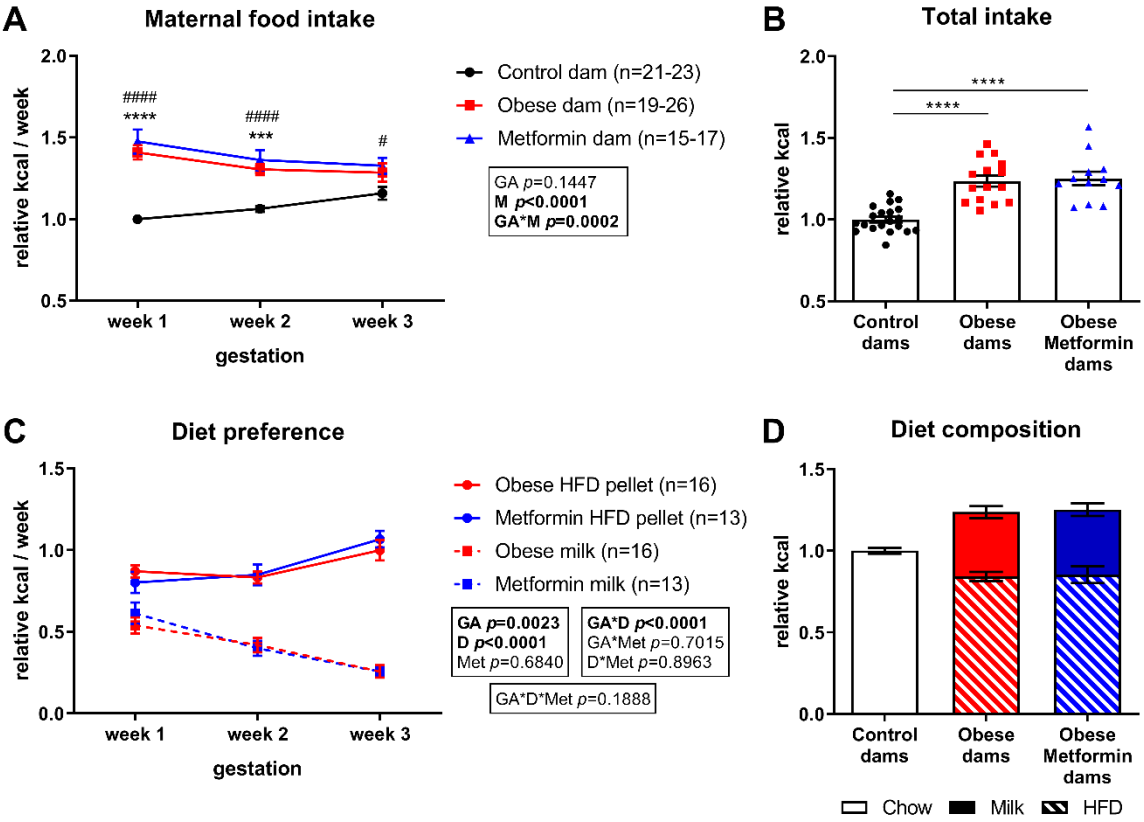

Figure 3.5: Caloric intake in pregnancy.
*A) Mean caloric intake per week gestation relative to week 1 of control dams. Box: results from repeated measures mixed-effects model analysis for the effect of gestational age (GA), the maternal environment (M) and the interaction between them (GA*M). ****p<0.0001, ***p<0.001 control vs obese, #p<0.05, ####p<0.0001 control vs metformin using Tukey's multiple comparison test. B) Total caloric intake by embryonic day 19, relative to control. ****p<0.0001 vs control, one-way ANOVA. Numbers are n=20 Control, n=15 Obese, n=12 Obese Metformin. C) Caloric intake from the HFD pellet (solid line) and condensed milk (dashed line) components of the obesogenic diet. Boxes: results from three-way ANOVA analysis for the effect of GA, diet type (D) and Metformin (Met), and the interactions between them. D) Composition of the calories ingested in the three experimental groups. Numbers are n=20 Control, n=15 Obese, n=12 Obese Metformin.*

Average weekly caloric intake (relative to week 1 in control dams) is plotted in Figure 3.5A. Obese and Obese Metformin dams showed significantly increased caloric intake compared to Control dams

during each gestational week (Figure 3.5A) and in pregnancy as a whole (Figure 3.5B). The interaction between gestational age and the maternal environment suggests gestation affected caloric intake differently between groups: whereas Control dams increased their caloric intake throughout gestation, dams fed the obesogenic diet (with or without metformin) decreased their intake with progressing gestation, almost reaching control levels by week 3 of gestation. Dams ate significantly more of the HFD pellet than the condensed milk during gestation (Figure 3.5C-D). HFD pellet intake increased with gestation in a pattern similar to chow pellet intake in Control dams. Intake of the condensed milk, however, decreased with advancing gestation (Figure 3.5C).

3.3.5 Dam phenotype during lactation

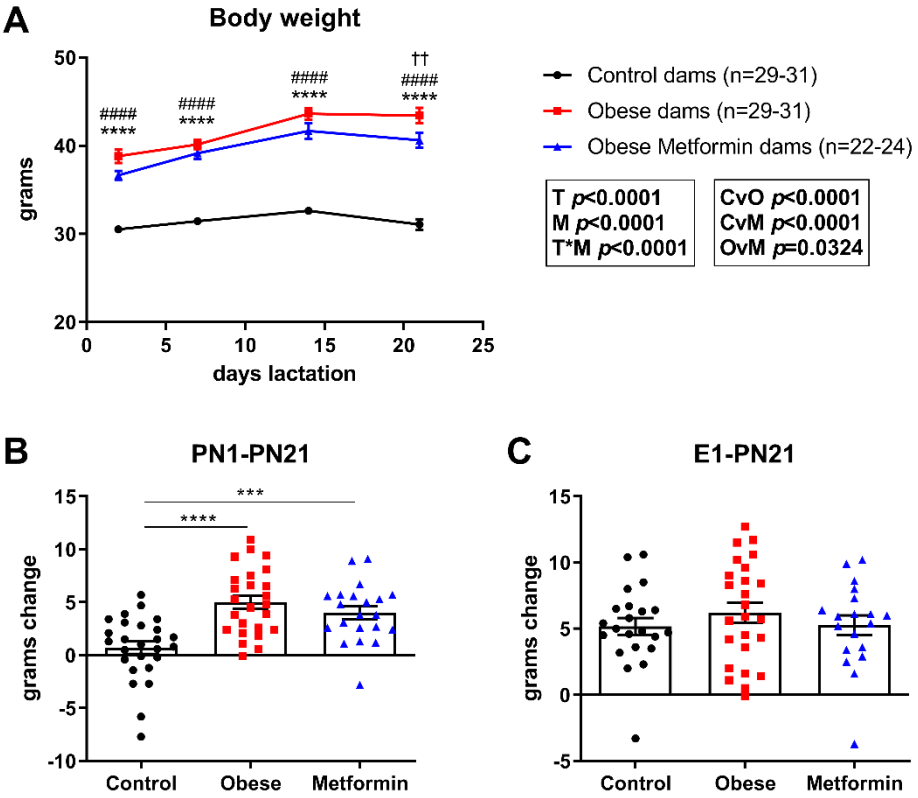

Figure 3.6: Lactation body weight and lactational weight gain.
*A) Body weight trajectory in lactation. Box: results from repeated measures mixed-effects model analysis for the effect of time (T), the maternal environment (M), and the interaction between them (T*M). C = control dams, O = obese dams, M = obese metformin-treated dams. ****p<0.0001 control vs obese, ####p<0.0001 control vs metformin, ††p<0.01 obese vs metformin using Tukey's multiple comparison test. B) Lactational weight gain between postnatal days 2 and 21 (numbers are n=26 Control, n=25 Obese, n=21 Obese Metformin), C) change in body weight from pregnancy to weaning (between embryonic day 1 and postnatal day 21, numbers are n=22 Control, n=25 Obese, n=19 Obese Metformin). ***p<0.001, ****p<0.0001 vs control, one-way ANOVA with Tukey's multiple comparison test.*

Dams fed the obesogenic diet were significantly heavier than control dams throughout lactation, irrespective of metformin treatment. Obese Metformin dams were significantly lighter than Obese dams at postnatal week 3 (p<0.01) as well as over the entire lactation period (Figure 3.6A, p=0.0324). Control dams on average did not gain or lose weight during lactation (Figure 3.6B) whereas Obese and

Metformin dams gained weight (5.0 and 4.0 grams, respectively, an effect that was similar in both groups). No group had returned to their pre-pregnancy weight by weaning (Figure 3.6C).

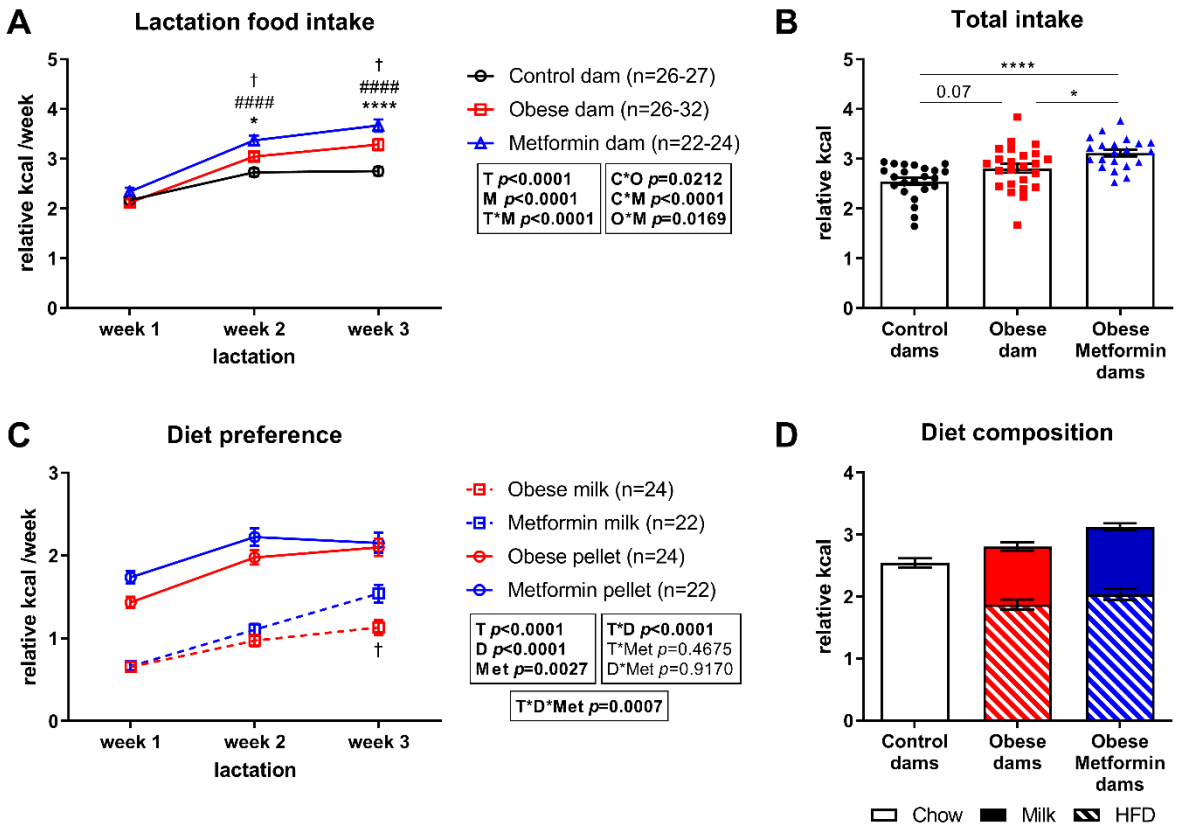

Figure 3.7: Caloric intake in lactation.
*A) Mean caloric intake per week lactation relative to week 1 gestation intake of Control dams. Box: results from repeated measures mixed-effects model analysis for the effect of time (T), the maternal environment (M) and the interaction between them (T*M).*p<0.05, ****p<0.0001 control vs obese, ####p<0.0001 control vs metformin, †p<0.05 obese vs metformin using Tukey's multiple comparison test. B) Total caloric intake by postnatal day 21, relative to total Control intake in pregnancy. *p<0.05, ****p<0.0001, one-way ANOVA with Tukey's multiple comparison test. Numbers are n=23 Control, n=24 Obese, n=21 Obese Metformin. C) Caloric intake from the HFD pellet (solid line) and condensed milk (dashed line) components of the obesogenic diet, relative to week 1 gestation intake of Control dams. Boxes: results from three-way ANOVA analysis for the effect of time (T), diet type (D) and Metformin (Met), and the interactions between them. †p<0.05 using Tukey's multiple comparison test. D) Composition of the calories ingested in the three experimental groups. Numbers are n=23 Control, n=24 Obese, n=21 Obese Metformin.*

Dams ate more during lactation than pregnancy, as seen in Figure 3.7. Obese dams ate significantly more than controls during lactation (p<0.05 for time-course, Figure 3.7C).

Obese Metformin-treated dams ate significantly more than Control (p<0.0001) and Obese dams (p<0.05 for week 2, week 3 and for total caloric intake in lactation, Figure 3.7A-B). Three-way ANOVA analysis indicated that caloric intake of both the HFD pellet and condensed milk increased with progressing lactation, the latter of which was significantly higher in the metformin-treated group by the end of lactation (p<0.05 for week 3, Figure 3.7C).

3.3.6 Neonatal data

Gestation length was increased by one day in metformin-treated obese pregnancies, compared to both Control and Obese (Table 3.4). There was equal distribution of offspring sex among litters and no difference between groups. Although birth weight was not recorded, litter size and pup weight at PN2 were significantly reduced in Obese compared to Control dams, which was not rescued by metformin treatment (Table 3.4). Despite being growth restricted at PN2, offspring of obese (Ob) and obese metformin-treated dams (Ob-Met) displayed accelerated postnatal growth, catching up with offspring of control dams (Con) by PN7 and becoming heavier by PN14 (Figure 3.8). Fractional growth rate was also significantly higher in Ob and Ob-Met compared to Con offspring (Table 3.4). Gestation length, litter size or weight at PN2 did not correlate to the amount of metformin received (not shown).

	Con	Ob	Ob-Met	*p*-value
Gestation length (d)	20 [19-20] n=24	20 [20-21] n=30	21 [20.3-21] [c,e] n=20	***<0.0001****
Litter size	7.0 [6.0-9.0] n=31	6.0 [5.0-7.0] [b] n=33	6.0 [5.0-7.0] [a] n=26	***0.0015****
% males in litter	50 [33-67] n=31	50 [33-67] n=33	55 [38-75] n=26	*0.5288**
PN2 weight (gram/pup)	1.9 ± 0.04 n=29	1.6 ± 0.04 [d] n=33	1.7 ± 0.04 [c] n=25	***<0.0001**[#]
FGR (gram/d)	0.2 ± 0.00 n=27	0.3 ± 0.01 [d] n=31	0.3 ± 0.01 [d] n=26	***<0.0001***

Table 3.4: Pregnancy outcomes and neonatal body weights.
*[a]p<0.05, [b]p<0.01, [c]p<0.001, [d]p<0.0001 vs Con, [e]p<0.01 vs Ob. *Kruskal-Wallis test for non-parametric data, [#]ANOVA performed on log-transformed data. FGR = fractional growth rate. PN2 = postnatal day 2.*

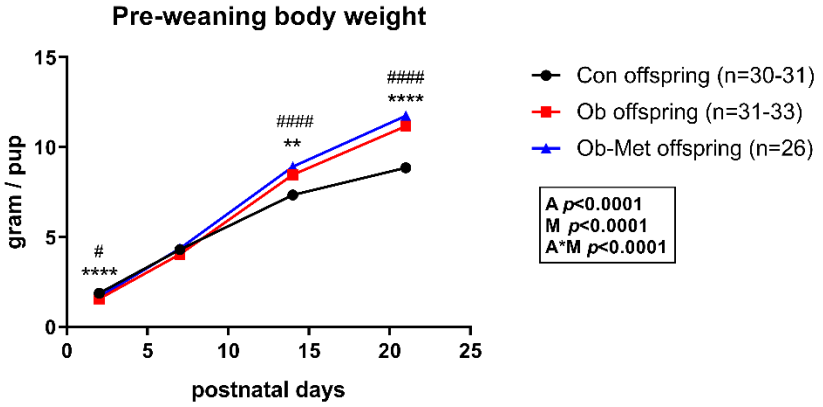

Figure 3.8: Body weight trajectory of pups in the early postnatal period.
*Box: results from mixed-effects model analysis for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). **p<0.01, ****p<0.0001 Con vs Ob, [#]p<0.05, [####]p<0.0001 Con vs Ob-Met offspring using Tukey's multiple comparison test. Con = offspring of control-fed dams, Ob = offspring of obese dams, Ob-Met = offspring of obese metformin-treated dams.*

3.4 Discussion

In our mouse model of maternal DIO, pre-conception exposure to an obesogenic diet, as expected, increased body weight and adiposity at conception and caused hyperphagia and obesity in pregnancy and lactation. Maternal obesity also decreased litter survival, litter size and introduced IUGR with excessive early postnatal catch-up growth, a hallmark sign of adverse development *in utero*. Maternal metformin treatment in obese pregnancy improved maternal body weight and fat mass at conception and at E16 but did not affect neonatal body weight trajectories or litter size despite a longer gestation time. Metformin-exposed dams were hyperphagic during lactation despite lower body weight at weaning compared to obese untreated dams.

3.4.1 Metformin dosing protocol

Metformin dosing was aimed at 200-300mg/kg/d at the start of pregnancy. This was achieved in the pre-mating period and in the first two weeks of gestation, with highest intake occurring in the pre-mating week. Only in the third week of gestation did the average amount of metformin per kg body weight drop below this threshold. Nonetheless, our mean week 3 intake of 119mg/kg/d is comparable to the 130mg/kg reported by Garbarino *et al.* during peak pregnancy weight (367). In human studies, the metformin dose is based on what is required to treat the mother (not the fetus), does not consider GWG and is not adjusted with progressing gestation (unless side effects occur or women with GDM fail to achieve glycaemic targets later in gestation)(240,260,276). Therefore, the amount of metformin received before or in early pregnancy is more reflective of treatment success in human trials. Exposure in the pre-mating and first gestational week was above the threshold for all or all but six (out of 27) dams, respectively.

Due to increasing maternal fat mass and fetal weight, calculation of the dose in mg/kg/d is no longer appropriate with advancing gestation (especially in week 3 which coincides with the most rapid fetal growth). When plotting the unadjusted amount of metformin ingested in mg/d, fewer dams ingested less than 7.2g metformin (equivalent of 200mg/kg/d based on mean body weight at E1). The decreased metformin intake per kg body weight in weeks 2 and 3 gestation thus at least partly resulted from increased maternal fat mass and fetal weight. However, the mean amount of metformin ingested remained below the 7.2g threshold in week 3. This was likely related to the drastic drop in milk intake in week 3 of gestation, which was observed in both Obese and Obese Metformin dams and therefore unlikely the result of metformin treatment. From week 1 to 2 of gestation, a slight decrease in milk intake was also observed, however we were able to counteract this by increasing the metformin concentration in the milk pot. Because metformin is hydrophilic, it was dissolved in water before mixing into the milk, and titration experiments (not shown) indicated that 420mg metformin was the

maximum that could be properly dissolved in 1ml water. At week 3 of gestation, the combination of increased body weight and decreased milk intake (see section 3.4.3) meant that the dose of metformin required to achieve 300mg/kg day often exceeded 420mg.

As metformin readily passes the placenta with reported high fetal exposure (estimated 50-200% of maternal blood concentration), a potential direct effect on the developing fetus in late gestation remains likely. One limitation of this study is that metformin levels were not measured in maternal or fetal samples. This cohort was destined for offspring (see Chapters 4-6), and therefore no maternal or offspring samples were taken to avoid additional stress to the mother. In future, maternal and fetal metformin levels will be measured in a separate fetal cohort. Preliminary analysis from this cohort shows that even at E19 metformin can be detected in both maternal and fetal serum at levels similar to those achieved in the human clinic (personal correspondence with A. Hufnagel, manuscript in preparation). Thus, metformin exposure in week 3 of gestation was probably sufficiently high to have effects on both dam and developing fetuses. Moreover, we found that neonatal outcomes including our proxy for birth weight (PN2 weight) did not correlate to maternal amount of metformin ingested. Similarly, in a RCT comparing metformin to insulin for GDM, Tertti *et al.* reported that the degree of metformin exposure did not affect fetal outcome in their study (281). Therefore, the lower metformin ingestion in week 3 of gestation is not of concern. Moreover, preliminary data from the laboratory shows that the metformin intervention improves maternal glycaemia during an ipGTT in late gestation, indicating that the dosing method is effective at a clinically relevant dose (personal communication with Dr H. Blackmore, unpublished).

3.4.2 Maternal body weight

Metformin reduces body weight in non-pregnant T2DM patients (368), and lower GWG is a common result in human RCTs in GDM, obese or PCOS women (233,278,313). In accordance with these human data, late pregnancy body weight and fat mass were decreased in obese metformin-treated compared to untreated dams in this study, consistent with a protective effect of metformin on DIO. Indeed, a decrease in late gestational body weight is reported in some (344,356) but not all (369) animal studies of metformin intervention in obese pregnancy. Interestingly, maternal obesity seems to be required for this effect as it was not observed in studies where dams were chow-fed or where body weight was not different from controls (247,301,303,366). In contrast, we did not observe an effect of the intervention on gestational or lactational weight gain using routinely collected data. Unfortunately, the TD-NMR machine became defective in the middle of the study period therefore changes in dam body composition could not be determined. Further studies are required to conclusively determine GWG and gestational or lactational fat gain in our model.

The decreased adiposity in Obese-Metformin dams in late gestation likely results from a loss of fat mass during the week of preconception metformin exposure that is maintained throughout pregnancy. It is noteworthy that Álvarez *et al.* also initiated metformin treatment one week pre-pregnancy, but in their study metformin-treated dams continued to gain rather than lose weight on the HFD during the pre-mating period (359). However, their study was performed in rats rather than mice and a different experimental diet was used (60% HFD for 4 weeks pre-mating versus longer HFHS-feeding in our model), thus complicating comparison between the studies. In humans, weight reduction with metformin is often related to decreased appetite, decreased intestinal glucose absorption or (to a lesser extent) intestinal side effects (231,368,370). Energy expenditure might also be affected by the intervention. Since pre-gestation food intake data was not collected for the current cohort, we cannot exclude that in the pre-pregnancy week a food intake effect of metformin was responsible for weight loss. Taken together, the metformin intervention seems to be effective in attenuating obesity and diabetes in obese dams.

3.4.3 Maternal food intake

Dams fed the obesogenic diet ingested more calories than dams fed the control diet, as was reported previously (87,112). As shown before in our model (87), control-fed dams increased their caloric intake throughout gestation, presumably to meet energy demands of growing fetuses since the highest caloric intake coincided with the period of rapid fetal growth. The same pattern was seen for HFD pellet intake (the main component of the diet) in dams fed the obesogenic diet, and dams decreased their weekly intake of the HFD pellet in grams (not shown) to roughly match the caloric intake of the control pellet. This tight control of caloric intake on HFD pellets is consistent with literature in rats (90) and suggests dams control their intake in pregnancy based on the fat content of the diet. In contrast to the HFD, dams drank less milk with advancing gestation, bringing back their total caloric intake to control level in late gestation. The discrepancy in feeding patterns between diet types and the lower contribution of the milk to overall intake is consistent with the sugar component being the reason dams overeat on the obesogenic diet. The increasing preference for the HFD pellet over the milk with progressing gestation (independent of metformin treatment) may be related to changes in appetite, food preference or altered palatability of the milk in late pregnancy. These changes in intake pattern are likely to result in a relative decrease in sugar intake in obese dams in the third week of pregnancy compared to earlier in gestation, with unknown consequences for the fetuses.

Caloric intake was higher during lactation compared to pregnancy in all groups. As shown previously (112), dams fed the obesogenic diet ingested more calories than controls in lactation although the hyperphagic effect of the obesogenic diet was less pronounced than in pregnancy.

An appetite suppressing effect is seen with metformin in non-pregnant humans (231,368), therefore we expected to observe lower caloric intake in metformin-treated pregnancy. Unfortunately, certain limitations of the diet used and experimental design in this study prevented comparison of food intake between Obese and Obese Metformin-treated dams in pregnancy. More recent experiments in the lab in which diet was replaced daily failed to show an effect of the metformin intervention on total caloric intake during gestation (personal correspondence with Dr L. Dearden and A. Hufnagel). This is in accordance with rodent studies that found no difference in caloric intake with metformin in chow-fed (247,301,343,371) or hypercaloric pregnancy (247,303). In lactation, metformin intervention was associated with a significant increase in caloric intake without preference for one diet component. One group previously found no effect of gestational metformin on subsequent maternal caloric intake in lactation in chow-fed animals (343,371), but this is the first animal study to assess lactational intake after metformin treatment in obese pregnancy. Human trials rarely collect food intake data and data on postpartum effects of prior metformin intervention are sparse. Although the PregMet trial (PCOS) showed less weight gain in gestation with metformin, the authors noted lower postpartum weight loss leading to increased BMI at one year postpartum compared to placebo-treated mothers (300). Dietary information was not collected but this could be related to increased appetite post-delivery. In contrast, the MiG trial (GDM) showed both decreased GWG and increased postpartum weight loss in women treated with metformin compared to insulin (260). These contrasting results may reflect the different indications for metformin use or comparison to different control groups, thus complicating extrapolation of the results. Despite increased caloric intake in lactation, Obese Metformin dams had lower body weights at weaning compared to Obese untreated dams. The lack of excessive weight gain following lactational hyperphagia suggests that metformin treatment during pregnancy may have an impact postnatally even after cessation of the drug (and consequent rapid clearance from the circulation) that may protect against maternal diet-induced weight gain in the early postpartum period. Further studies are required to elucidate the mechanisms behind gestational metformin-induced lactational hyperphagia.

3.4.4 IUGR and postnatal catch-up growth

In this study, IUGR with catch-up growth was observed in offspring exposed to maternal DIO, consistent with previous reports by ours and other groups (87,110,129,181). As illustrated by the famine studies mentioned in Chapter 1.3.2, the combination of LBW and accelerated postnatal growth is associated with long-term adverse effects on offspring cardiometabolic health (51–54). Similarly, offspring body composition and metabolic health is adversely impacted in studies from our laboratory using the 'recuperated' animal model (low protein-feeding in pregnancy followed by cross-fostering of small litters to control-fed dams)(372,373). In fact, early postnatal overnutrition in itself can have

programming effects: postnatal litter reduction increases nutrition available for offspring in smaller litters leading to hyperphagia, adiposity, metabolic and cardiovascular abnormalities in adulthood (374). The finding of growth restriction with catch-up growth in our study thus suggests predisposition to adverse cardiometabolic programming in offspring of obese dams. In humans, obese pregnancy increases the risk of both LGA and SGA babies (35). Similarly, rodent models of maternal pre-conception obesity have reported increased (359,366) or decreased (110,174) fetal or birth weight.

The effect of prenatal metformin during overnourished pregnancy on fetal or neonatal weight varies in the literature (Table 3.5). Wang *et al.* reported an increase in fetal weight with metformin at E18.5 in HFD-fed mice, leading to correction of IUGR to the level of chow-fed controls (366). An increase in birth weight was also reported in a rat model, but in this case the metformin intervention actually exaggerated the macrosomia observed in offspring of HFD-fed dams (359). Other studies found decreased fetal weight in offspring of metformin-treated overfed dams: in one study this led to induction of IUGR compared to control and high fructose-fed pregnancy (247), while in another this reflected attenuation of macrosomia in offspring of HFD-fed mice (375). Other studies, including ours, fail to report an intervention effect on fetal or neonatal weight. Tong *et al.* found no effect of a metformin intervention on maternal obesity-induced macrosomia (304), while others find no difference in fetal weight between any of their experimental groups including controls (238,376).

Study	Species	Dam diet	Offspring age	vs obese	vs chow	Effect
Alzamendi *et al.* 2012 (247)	Sprague-Dawley rats	FRD	fetal E20	decreased	decreased	IUGR induced
This study	C57Bl/6J mice	HFHS	PN2	no change	decreased	IUGR not corrected
Wang *et al.* 2013 (366)	CD-1 mice	HFD	fetal E18.5	increased	no change	IUGR rescued
Desai *et al.* 2013 (238)	Wistar rats	HFHS	fetal E19	no change	no change	No effect
Nüsken *et al.* 2019 (376)	C57Bl/6N mice	HFD	fetal E18.5	no change	no change	No effect
Vora *et al.* 2019 (375)	FVB mice	HFD	fetal E17.5	decreased	increased	Macrosomia attenuated
Tong *et al.* 2011 (304)	C56Bl/6J mice	HFD	birth weight	no change	Increased	Macrosomia not corrected
Alvarez *et al.* 2018 (359)	Sprague-Dawley rats	HFD	birth weight	increased	increased	Macrosomia exaggerated

***Table 3.5: Effect of metformin intervention on fetal or neonatal weight in rodent models of maternal overnutrition**. FRD: fructose-rich diet. HFD: high fat diet. HFHS: high fat high sugar diet. IUGR: intrauterine growth restriction.*

Ob-Met had similar body weight as Ob offspring throughout the whole lactation period, indicating that the metformin intervention did not positively or negatively affect the catchup growth phenotype. The only other study investigating neonatal growth trajectory following obese metformin-treated pregnancy found increased body weight at PN1, PN7 and PN14 in female offspring of obese compared

to control dams, which was exaggerated by metformin treatment (359). In this study, the metformin intervention also led to exaggerated adiposity and hyperleptinaemia in adulthood. Taken together with the lactational hyperphagia in our study and the known programming effects of early postnatal overnutrition (374), this stresses the importance of follow-up of metformin-exposed offspring.

In addition to early postnatal growth, the intrauterine growth trajectory is important in determining offspring outcome. Although some studies from our lab find no difference in neonatal weight at PN3 (133,192), fetal weight was decreased in obese pregnancy at various embryonic timepoints (181,201,377) indicating IUGR may have occurred even if early postnatal weights are no longer different. Indeed, intrauterine catch-up growth has been reported in obese pregnancy (378). We do not know if there were differences in intrauterine growth between the three groups and further studies are required to determine the fetal trajectory in our mouse model of metformin-treated obese pregnancy. This is especially important given the lack of data on fetal growth in human metformin-treated pregnancies (294).

In humans, the metformin intervention is associated with decreased birth weight compared to insulin in diabetic pregnancies (278). Although this is usually interpreted as beneficial (prevention of macrosomia) it may also reflect relative growth restriction that is masked by the obesogenic glucose intolerant environment. Supportive of this hypothesis, a RCT in Pakistan of pre-existing and newly diagnosed T2DM found increased rates of SGA in neonates from mothers randomised to metformin compared to insulin (336). Follow-up from the RCT by Ijäs *et al.* investigating metformin versus insulin in GDM showed decreased height at birth, normalisation of height by 6 months, and ultimately increased height and weight at 12 and 18 months of age, thus also showing an IUGR and catch-up growth phenotype (271,297). Moreover, metformin use in pregnancy significantly predicted offspring body weight at 18 months. In accordance with this observation, a recent meta-analysis from our lab reported that metformin in pregnancy leads to decreased birth weight followed by excessive postnatal growth leading to increased infant weight compared to insulin-treated GDM (294). Results from a cohort study in PCOS women show similar findings: metformin-exposed offspring were shorter and thinner between birth and 6 months of age but caught up in height and weight by 12 (females) or 18 (males) months of age (257). These observational data were strengthened by the PregMet RCT which showed excessive postnatal growth in metformin-exposed offspring with body weight and BMI (but not height) diverging from placebo-exposed offspring from 6 months of age (335).

The metformin intervention might therefore lead to a potential double hit from both metformin-induced IUGR and early postnatal maternal hyperphagia leading to offspring overgrowth (similar to the recuperated model). Although metformin did not worsen the neonatal phenotype in this study,

data from the human and animal literature combined warrant investigation into the long-term effects of the intervention.

3.4.5 Pregnancy outcomes and reproductive success

In humans, obesity is associated with an increased risk of ovulatory problems, delay to conception and early pregnancy loss (17–20). Since metformin is used to improve anovulatory fertility in PCOS women (379), the intervention may improve reproductive outcomes in obese pregnancy as well. In our mouse model, DIO decreased conception and livebirth rates (personal correspondence with Dr C. Aiken, unpublished), decreased litter size and increased litter losses post-delivery (this study).

No difference was observed in litter size or survival between Obese and Obese Metformin groups. This is consistent with data by Álvarez *et al.* who found fewer offspring in litters from both HFD-fed and HFD metformin-treated pregnancy (359), although others fail to report changes in litter size following maternal overnutrition with or without metformin (238,247,366,376). In uncomplicated pregnancy models, metformin does not adversely affect mating efficiency, implantation success, fetal survival, litter viability or size (247,315,357,371,380–384). In a PCOS-model of prenatal hyperandrogenisation, resorption rate was decreased (248). Moreover, improved reproductive hormone levels, implantation, fetal viability and/or survival have been found in experimental models of preeclampsia, PTB, PCOS and severe (genetic) diabetes (248,344,352,357,384,385), suggesting metformin may exert beneficial effects in case of pre-existing reproductive deficits. Moreover, in overnourished pregnancy metformin was found to reduce placental inflammation and promote angiogenesis leading to increased vasculature on the fetal side (238,247,366). Metformin may also restore aberrant spiral artery remodelling and uterine and umbilical artery blood flow in diabetic pregnancy (344). In the current study, no reproductive information was collected during the pre-conception period and placental function was not assessed. The current data do not suggest fertility inducing effects, but more detailed studies are required to investigate effect of metformin on reproductive outcome as well as characterisation of intervention effects on the placenta. Nevertheless, the metformin intervention seems safe for use in pregnancy as it did not adversely affect pregnancy outcomes.

Obese Metformin dams showed increased gestation time compared to Control and Obese dams (21 versus 20 days). To our knowledge this is the first study to describe such an effect. These data contrast with the MiG trial, where decreased gestational age at delivery and increased rate of PTB were found in the metformin arm (260). However, this relationship was not replicated in other trials, and recent meta-analyses find no significant risk of PTB with metformin use in GDM-complicated pregnancy (278). Contrastingly, a protective effect on preterm delivery was found with metformin treatment in PCOS women when compared to placebo (386). The relationship between metformin and PTB in humans

thus remains unclear. It must be mentioned that human trials are confounded by deliveries via non-emergency C-section in obese or GDM-complicated pregnancies to prevent macrosomia and therefore data on gestational age at delivery in humans cannot easily be compared to the increased gestation time in this mouse study. In rodents, metformin may actually prevent PTB. Indeed, metformin pre-treatment in mid-gestation decreased the incidence of spontaneous and/or LPS-induced PTB in two different mouse models of premature PTB, presumably by correcting the observed decrease in decidual AMPK activation leading to inhibition of mTORC1 signalling, a known factor in parturition timing (364,384). Moreover, metformin treatment improved decidual health and decreased markers of decidual senescence even in control animals (384). If the intervention is able to prevent PTB in at-risk pregnancies it may therefore act to delay parturition in obese pregnancies as well (as seen in the current study).

An alternative explanation for the increased gestation time is a putative developmental delay in metformin-exposed fetuses. As mentioned in section 3.4.4, no IUGR was observed in Ob-Met compared to Ob offspring at PN2 (a proxy for birth weight), but offspring are significantly lighter than Con offspring. Considering that their gestation is one day longer, it can be argued that at PN2 the metformin-exposed offspring are in fact a day older than their unexposed counterparts. Therefore, it may be that the metformin-exposed offspring are in fact growth restricted *in utero* and parturition is delayed by one day to ensure maximum growth before birth. It would be informative to measure the expression of factors involved in parturition in metformin-treated dams, including but not limited to circulating glucocorticoids and their receptors.

3.4.6 Conclusion

Maternal metformin treatment before and during obese pregnancy decreased maternal fat mass at conception, thus providing a less obese intrauterine environment for developing fetuses. The metformin intervention is safe with respect to offspring survival and early development since no differences were observed in litter size, offspring survival and neonatal growth trajectories between obese metformin-treated and untreated pregnancies. However, the increased gestation time, uncorrected IUGR and catchup phenotype of offspring, and exaggerated maternal hyperphagia during lactation warrant investigation into potential longer-term effects on offspring.

3.5 Key findings

- Obese dams were obese and hyperphagic in gestation and lactation, and gave birth to smaller litters that displayed IUGR and catch-up growth in offspring
- The metformin intervention decreased maternal body weight and fat mass pre-conception. Therefore, dams entered gestation in a healthier state, which is maintained until at least E16.
- Metformin intake did not affect litter size or survival rate and is therefore safe.
- Metformin intervention did not correct offspring IUGR despite increasing gestation time by one day. Dams that were metformin-treated during pregnancy ate significantly more during lactation, increasing risk of early postnatal overgrowth phenotype in offspring
- Based on these results, and since metformin crosses the placenta, longer-term studies into offspring phenotypes are required.

4 Short-term effects on adiposity and metabolic health

4.1 Introduction

Metformin treatment during pregnancy is characterised by improved glycaemic control, decreased GWG and reduction in preeclampsia risk in pregnant women (see Chapters 1 and 3). In our model, the metformin intervention decreased maternal weight and fat mass at conception and in late pregnancy, consistent with beneficial effects on the mother. However, the intervention did not correct IUGR with catch-up growth observed in offspring of obese and obese metformin-treated dams. It is therefore important to follow up offspring exposed to metformin *in utero* to determine how the intervention affects offspring health later in life.

4.1.1 Human offspring follow-up

Human offspring follow-up remains sparse, with only a few studies reporting childhood outcomes (Table 4.1). A single-centre RCT in Finland of metformin versus insulin in GDM pregnancy found that although babies exposed to metformin were shorter at birth, they caught up in both height and weight leading to increased body weight at 1 year of age (297). Another Finnish study reported an increased BMI z-score at 5 years of age (272,387). The longest follow-up of metformin-treated GDM pregnancies to date is from the MiG trial in Australasia. Their New Zealand subgroup showed that although there was no difference in anthropometrics between metformin and insulin arms at birth, children in the metformin arm displayed increased adiposity at the 2 and 9 year follow-up (388). Moreover, at 9 years of age abdominal fat mass (both internal and subcutaneous) was increased in the metformin arm although this did not reach statistical significance (388). This is an especially important finding since expansion of visceral adipose tissue, particularly in the abdominal region, is associated with IR and the metabolic syndrome (170). In contrast, the Australian MiG subgroup showed increased adiposity at birth despite being born at a lower gestation age, but no significant differences in body composition were found at the 2 and 7 year follow-up (388). Collectively, data from GDM trials suggest that compared to insulin, metformin-exposed babies have lower birth weight but display accelerated catch-up growth leading to childhood adiposity, as shown in a recent meta-analysis from our group (294). This meta-analysis also highlights the sparsity of follow-up data and hence the importance of follow up studies in all species looking at offspring phenotypes across the life-course.

Follow-up from the PregMet trials in PCOS women (274,276,300,334,335,389,390) showed increased BMI and incidence of overweight/obesity in metformin-exposed children as young as 4 years of age (390). This was confirmed in a larger follow-up study at 5-10 years of age that also showed an increased proportion of metabolically abnormal obese children in the metformin arm (335), suggesting that this early adiposity phenotype may influence cardiometabolic health as well. This

RCT	Neonatal	Infancy (0-2y)	Childhood
MiG Multicentre, GDM, metformin vs insulin, n=99, New Zealand (388)	No difference in birth weight, birth length, SFT or PI	**2 years**: Increased chest, mid-upper-arm, hip and waist circumference, WHR, subscapular and biceps SFT and trend for increased arm fat	**9 years**: Increased body weight, waist and mid-upper arm circumference, WHR and arm fat on DXA scan. Trend for increased BMI, triceps SFT, total and abdominal fat mass on MRI
MiG Multicentre, GDM, metformin vs insulin, n=109, Australia (388)	Increased birth weight >90th centile, mid-upper arm circumference and triceps SFT. Trend for increased birth weight centile and PI	**2 years**: No significant differences in anthropometry or fat mass on DXA scan or bio-impedance	**7 years**: No significant differences in anthropometry or fat mass on DXA, via bio-impedance or on abdominal MRI
Single centre, GDM, metformin vs insulin, n=103, Finland (297)	No difference in birth weight, macrosomia or LGA. Decreased height.	**6 months**: Less likely to be short (height <5th centile), trend for increased body weight. **12 months**: More likely to be tall (height >95th centile), increased body weight; trend for increased height **18 months**: Increased height and body weight. Trend for more likely to be heavy (body weight >95th centile)	TBD
Single centre, GDM, metformin vs insulin, n=52, Finland (272,387)	No difference in birth weight	-	**5 years**: No significant difference in height, weight, BMI and WHR, trend for increased BMI z-score
pilPregMet Single centre, PCOS, metformin vs placebo, n=40, Norway (295,299)	No difference in birth weight or length. Trend for increased head circumference and maybe increased birth weight	-	**8 years**: increased BMI without differences in height or weight
PregMet Multicentre, PCOS, metformin vs placebo, n=270, Norway (276,300,334,335,390)	No difference in birth weight or length, LGA, SGA or PI, but increased head circumference	**1 year**: increased body weight	**4 years:** increased body weight, BMI, %overweight/obese **5-10 years**: increased BMI, waist circumference, WHtR, weight z-score, and % obese and metabolically abnormal obese. Trend for increased fat mass
EMPOWaR Multicentre, obesity, metformin vs placebo, n=449, UK (274,389)	No difference in birth weight, LGA, SGA, SFT or baby fat%. Trend for increased PI	**3 months**: No difference in PI, SFT or baby fat%	TBD
MOP Multicentre, obesity, metformin vs placebo, n=151 UK (233,391)	No difference in birth weight, length, LGA, macrosomia, birth length, arm or thigh circumference, triceps or subscapular SFT	-	**4 years**: no difference in weight, height or BMI. Decreased gluteal and triceps circumference

Table 4.1: Offspring body composition outcomes in human RCTs investigating metformin use in pregnancy.
PI = ponderal index. SFT = skinfold thickness. TBD: to be determined. WHR: waist-hip-ratio. WHtR: waist-height ratio.

study also reported that maternal obesity status influenced the effect of metformin on offspring adiposity and lipid profile, with more prominent effects seen in pregnancies complicated by both PCOS and maternal pre-pregnancy obesity (335). In contrast, trials in obese glucose tolerant women failed to show a relationship between metformin exposure and offspring adiposity (233,391). However, these offspring remain relatively young (3 months in EMPOWaR and 4 years in MOP) so further follow-up studies are required.

Although these RCTs are large and well-designed, offspring phenotype beyond birth is seldom the primary outcome. Hence, they are often underpowered to detect differences in outcomes of interest for long-term offspring health. This is exacerbated by loss to follow-up after the initial study. Furthermore, offspring from RCTs remain young to date. Therefore, animal studies are critical in determining effects on offspring across the life-course.

4.1.2 Effects on offspring in animal models

Animal models are required to determine longer-term effects of maternal metformin intervention on offspring body composition and metabolic health. To date, only seven studies have been published with regards to these outcomes, with conflicting results. A set of seminal studies were published by Salomäki *et al.* in the 2010s. In their first model, they provided metformin during chow-fed pregnancy and found that metformin introduced IUGR by E18.5. When offspring were fed regular diets, no difference in body weight or glucose tolerance was found, but when given a HFD challenge later in adulthood metformin-exposed offspring developed adiposity and sex-specific metabolic dysfunction, with hypercholesterolaemia in females compared to hyperglycaemia, IGT and epididymal white adipose tissue (eWAT) IR in male offspring (301). This suggested an increased vulnerability to the metabolic syndrome if exposed to a second hit (e.g. HFD) later in life. Their follow-up study, however, found contrasting results: when metformin was given to pregnant obese HFD-fed dams, their offspring were protected against DIO and IGT when provided with HFD later in life (303). This illustrated the importance of the maternal metabolic state with regards to offspring outcome. Although extremely informative, this study provided HFD during the pre-conception and pregnancy period but switched dams to chow diets in lactation. While this demonstrates the effects of maternal HFD during pregnancy alone, it complicates extrapolation of these findings to the human clinical setting. Lastly, their study in a genetic model of maternal obesity (selective NPY overexpression in the brain and sympathetic nervous system, OE-NPY) showed that maternal metformin protected against HFD-induced obesity and hyperinsulinaemia in males but exacerbated obesity and metabolic dysfunction in female offspring, when compared to offspring of untreated OE-NPY dams (302). In addition to confirming the influence of the maternal environment in determining offspring response to metformin, this study highlighted the importance of studying both sexes in a programming context.

Gregg *et al.* used a model similar to the first Salomäki paper, with the notable exception that offspring were not exposed to HFD challenge in adulthood. Contrastingly, they found improved glucose tolerance and insulin secretion in adult male offspring while female offspring showed no obvious benefit during early adulthood (392). The discrepancy between those studies is not fully explained, although metformin dose, dosing method (gavage versus drinking water), mouse sub-strain differences and the postnatal environment may be involved. Interestingly, a follow-up study in which chow-fed dams were given metformin during lactation rather than gestation showed similar effects with improved insulin secretion and glucose tolerance as well as decreased adiposity in chow-fed male offspring. Females in this study also showed improved insulin sensitivity in absence of effects on glucose tolerance or fasted serology (307).

In clinical practice metformin is not given to pregnant women without metabolic abnormalities. Therefore, chow-fed models are not the most clinically relevant models. Instead, Tong *et al.* studied metformin intervention during HFD-induced obese pregnancy. This attenuated adiposity and glucose intolerance and prevented muscle metabolic dysfunction in 8-week-old male offspring weaned onto HFD, suggesting protective effects on offspring metabolic health (304). In contrast, Álvarez *et al.* found increased adiposity and insulin in female offspring using a similar rat model (359). However, these studies provided metformin treatment during both pregnancy and lactation, which differs from the human clinical settings. The heterogeneity of evidence highlights that post-weaning diet, timing of metformin exposure and the interaction between metformin and the maternal environment are critical, stressing the importance of designing animal models of clinical relevance. However, no studies have been published to date in which metformin is given solely during pregnancy in a maternal obesity model that provides the obesogenic diet throughout the maternal life-course (pre-conception, during pregnancy and lactation). Our well-established mouse model is therefore a clinically relevant model to study metformin intervention.

4.1.3 Wider implication of adiposity

The finding of increased adiposity in prenatally metformin-exposed children has long-term implications. Childhood obesity is associated with increased risk of obesity in later life: in an American longitudinal study, only a small proportion (7%) of obese adults had been of normal weight in childhood, whereas the vast majority (77%) of obese children went on to become obese adults (6). As outlined in Chapter 1, obesity is associated with comorbidities including the metabolic syndrome involving both cardiovascular and metabolic abnormalities (5). Indeed, childhood obesity is also associated with increased risk of dyslipidaemia, IR, T2DM, hypertension and even cardiovascular mortality (7,8,393). Thus, early adiposity phenotypes may predispose to the metabolic syndrome.

Obesity is associated with positive energy balance leading to expansion of WAT depots (170). Adult-onset obesity is predominantly characterised by adipocyte hypertrophy (increase in cell size) rather than hyperplasia (increase in cell number)(111). However, studies have shown that obese children (394) and adults with early onset obesity (111) show an increase in adipocyte number as well as size compared to lean individuals. Adipocyte hyperplasia largely occurs during childhood and plateaus in early adulthood (395). Thus, hypercellularity established in early life provides propensity for excessive WAT expansion and obesity in adulthood.

4.1.4 Adipose tissue dysfunction

It is increasingly acknowledged that adipose tissue is not simply an energy storage depot, but an active endocrine organ that secretes a variety of circulating factors including hormones, lipids, cytokines and chemokines that impact on whole body energy balance and metabolic health (396). Altered WAT function may thus underlie some of the adverse health implications in obesity. Indeed, pathological WAT expansion is characterised by excessive production of inflammatory adipokines, oxidative stress and IR which together with elevated circulating free fatty acids (FFAs) leads to whole body IR and cardiovascular dysfunction (88).

Adipocyte size significantly correlates with indices of insulin sensitivity in men and women (308,397), and this relationship is maintained after adjustment for adiposity (397). In non-obese individuals with T2DM, adipocyte size is increased compared to normoglycaemic controls and correlates to measures of IR across the cohort, indicating that adipocyte hypertrophy is associated with impaired insulin sensitivity and glucose tolerance even in absence of obesity (398). Local WAT IR (characterised physiologically by decreased glucose uptake and failure to suppress lipolysis, and molecularly by downregulation of insulin signalling pathways) often precedes systemic IR and IGT and may thus affect whole-body insulin sensitivity and glucose control (399). The metabolic consequences of adiposity depend on which depot is expanding: expansion of subcutaneous depots may be beneficial and associated with improved insulin sensitivity whereas hyperplasia of the visceral, more insulin-resistance prone depots leads to the opposite (400).

Abnormal FFA liberation from WAT and consequent storage of lipid in other organs is one mechanism by which WAT dysfunction can cause whole-body IR (399,401). Increased FFA flux may occur when energy intake exceeds the lipid storage capacity of WAT (399,402). According to this theory, not adiposity itself but rather the limit of WAT expandability predisposes to the metabolic comorbidities of obesity. This is supported by lipodystrophy patients who show a metabolic syndrome-like phenotype (hyperlipidaemia, fatty liver and IR) despite having low fat mass (402). Early life exposures leading to low adipocyte number at birth, impairments in remodelling required for WAT expansion or

a failure to elicit compensatory hyperplastic responses could therefore all contribute to insufficient WAT expandability (398,399,402).

Obesity is associated with excessive production of pro-inflammatory factors by WAT, including cytokines TNFα, IL-6, IL-1β, chemokine CCL2/MCP-1 and the chemokine receptor CCR2 (170). Some of these are produced at low levels by adipocytes but the majority instead originate from macrophages (403–405). Accordingly, increased presence of macrophages in WAT from obese compared to lean individuals is commonly reported in both rodents and humans (403,404,406–409). Bone marrow transplantation studies in HFD-fed mice showed that these macrophages are blood-derived (403), suggesting external macrophage infiltration. This is supported by the observation of increased chemokine expression in WAT of obese rodents (403,404). Using several genetic and/or dietary models of obesity, Xu *et al.* found increased expression of macrophage infiltration and inflammation markers in WAT, but not in other metabolic and/or macrophage-rich tissues such as muscle, liver, lung or spleen indicating a WAT-specific inflammatory phenotype in obesity (404).

While adipose tissue-resident macrophages are often of the protective M2-type, the macrophages upregulated in obese WAT express markers reflective of classical M1-type polarisation, such as CD11c, TNFα, IL-6, CCL3/MIP-1α and iNOS (403,407,408). Levels of activated M1-type macrophages and the M1:M2 ratio were increased in obese compared to non-obese women, with highest levels reported in obese women with the metabolic syndrome (407). These parameters also significantly correlated with HOMA-IR. Obesity thus promotes selective expansion of activated M1 macrophages causing a pro-inflammatory state that induces IR. This relationship may be independent of obesity as M1 density and M1:M2 ratio were also increased in non-obese T2DM patients compared to normoglycaemic controls (398). Importantly, upregulation of inflammatory genes in obese rodents occurred after development of adiposity but preceded the increase in glucose or insulin in the study by Xu *et al.*, indicating a causal role for macrophage-associated WAT inflammation in the development of systemic metabolic disturbance (404). Culturing the SGBS human adipocyte cell line in M1-conditioned media decreased insulin-stimulated glucose uptake (407), suggesting factors produced by infiltrated macrophages may contribute to development of (local) IR. Accordingly, treatment of Zucker rats with neutralising antibodies to the TNFα receptor drastically improves insulin sensitivity during a hyperinsulinaemic euglycaemic clamp indicating a direct role for this cytokine (410). Other cytokines and chemokines including IL-6 and MCP-1 were also shown to affect insulin sensitivity (411–417). Notably, adipocyte size in non-obese individuals correlates with HOMA-IR as well as TNFα and M1:M2 ratio (398). WAT inflammation may thus at least partly underlie the relationship between hypertrophy and IR.

Macrophages in adipose tissue of obese individuals are not only increased in number but also histologically distinct. While in lean mice tissue-resident macrophages are small and scattered across the tissue, in obese mice macrophages aggregate around certain adipocytes (403,404). Macrophages in these aggregates or 'crown-like structures' (CLS) are recruited to facilitate phagocytosis of dead adipocytes, the necrotic adipocyte being a persistent M1 activator stimulating continued pro-inflammatory cytokine production during phagocytosis (406). Accordingly, CLS preferentially contain classically activated $CD11c^{+}$ macrophages (407). Presence of CLS in subcutaneous WAT of obese humans is associated with increased levels of pro-inflammatory cytokines and CRP, IR and hyperinsulinaemia compared to those without CLS (418). Moreover, macrophage content directly correlated with insulin and HOMA-IR across the cohort suggesting presence of these structures as a strong predictor of local and (future) systemic IR.

Adipocyte hypertrophy is thought to initiate macrophage recruitment into WAT (Figure 4.1). This can occur independent of obesity as mice lacking the hormone-sensitive lipase gene (*Hsl*) have excessively large adipocytes, macrophage infiltration and WAT inflammation but are not obese, indicating hypertrophy as the underlying signal (406). As WAT hypertrophies, blood supply becomes insufficient to sustain metabolic demands, resulting in tissue hypoxia, upregulation of HIF1α, pro-inflammatory cytokine production and CLS recruitment to hypoxic areas (408,419–421). This may initially facilitate physiological WAT expansion since inflammatory mediators and monocytes can promote angiogenesis and extracellular matrix remodelling (422). Physical pressure on the extracellular matrix may also play a role (423). Hypertrophic adipocytes also produce high amounts of leptin, which promotes chemo-attraction, monocyte activation and upregulation of adhesion molecules in WAT endothelial cells facilitating capture of circulatory monocytes (424–428). Conversely, production of anti-inflammatory adiponectin, which promotes macrophage polarisation to M2-type and prevents transformation into phagocytic foam cells (429,430), is decreased in hypertrophic adipocytes (431). Lastly, adipocyte hypertrophy is associated with increased adipocyte death in obese humans and mice (406,409). Following subsequent phagocytosis of dead adipocytes, the tissue undergoes remodelling and hyperplasia to replace the lost adipocytes leading to an increase in newly formed small adipocytes (Figure 4.1)(409). This 'obesity-induced adipocyte death' has been suggested as the limiting factor to adipocyte hypertrophy and may underlie the switch from hypertrophic to hyperplastic adiposity (406). Although initially protective, in obesity these processes are insufficient and/or persistent resulting in WAT dysfunction (422). Adipocytes and macrophages can activate one another in a paracrine manner (405), causing a feed-forward loop of pro-inflammatory cytokine/chemokine production by M1 and dysfunctional adipocytes, leading to M1 polarisation of newly recruited monocytes. A similar cascade may also exist for FFAs and adipokines (405). Lastly, adipocyte necrosis stimulates release of cytotoxic

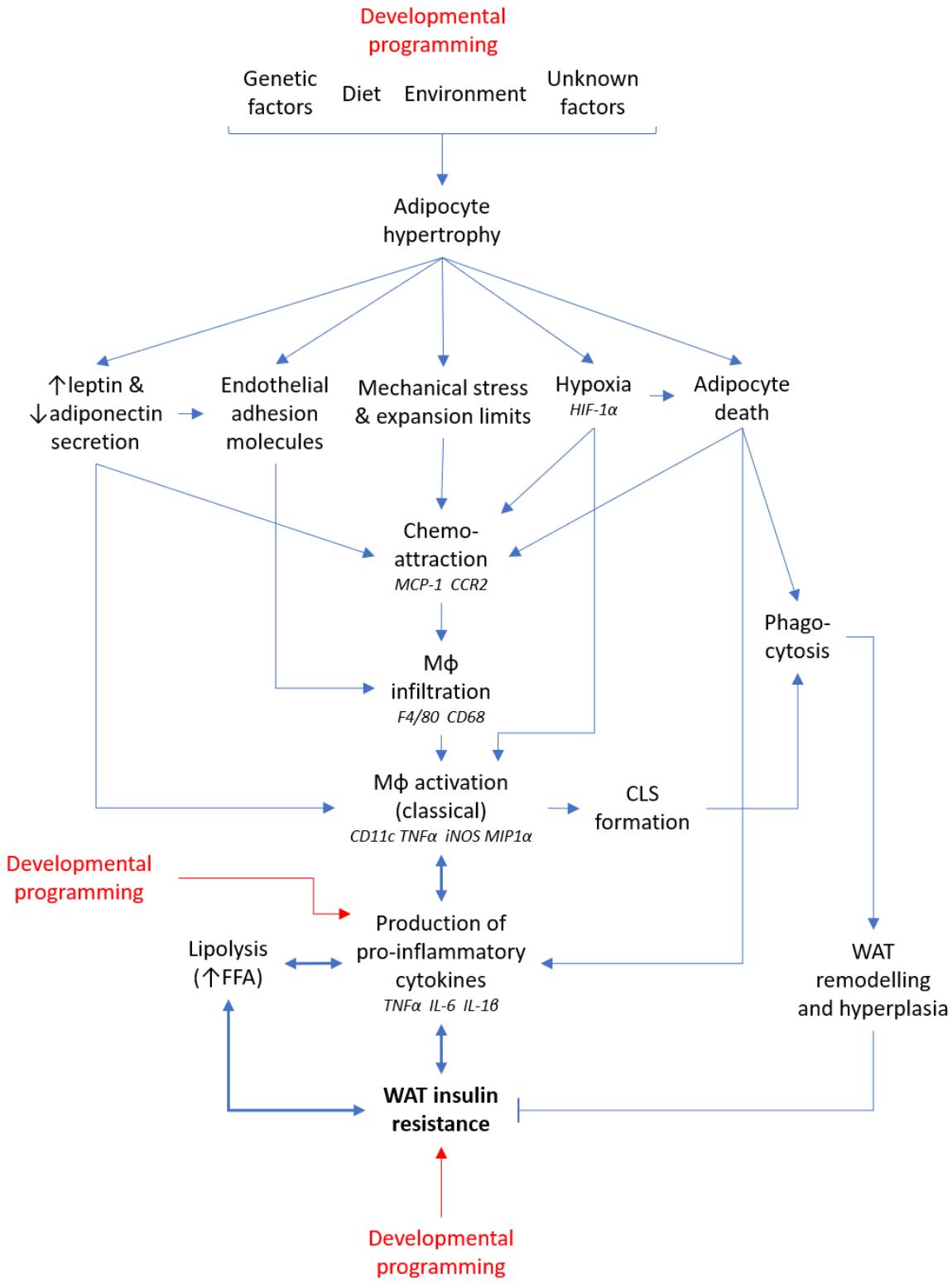

Figure 4.1: WAT inflammation in hypertrophic obesity.
WAT expansion in obesity is associated with increased leptin and decreased adiponectin production, enhanced FFA release, hypoxia, upregulation of adhesion molecules and adipocyte death. This stimulates chemoattractant production by WAT, recruiting blood-derived monocytes to the hypertrophied WAT proportionally to the degree of adiposity. These macrophages (MΦ) activate to form pro-inflammatory M1 macrophages, leading to excessive cytokine production which promotes local and systemic insulin resistance. This inflammatory cascade triggers a feed-forward loop that stimulates further macrophage infiltration and activation. Infiltrated macrophages aggregate around dead adipocytes, phagocytose them and ultimately stimulate their replacement through WAT remodelling and adipocyte hyperplasia. Thick arrows represent bidirectional relationships, red arrows the involvement of developmental programming by maternal obesity.

and inflammatory mediators from surrounding macrophages (406), thus further exacerbating the inflammatory cascade (Figure 4.1). Interestingly, certain pro-inflammatory genes such as MCP-1 maintain the capacity to respond to insulin even in IR adipocytes (432), aggravating the inflammatory WAT phenotype when exposed to hyperinsulinaemia. Accordingly, the development of hyperinsulinaemia coincided with significantly worsening inflammation in obese mice (404). Therefore, although macrophage infiltration is initially proportional to the degree of adiposity, this relationship is lost when WAT dysfunction becomes self-propagating (433).

4.1.5 Adiposity and metabolic health in our mouse model

WAT dysfunction and adiposity can be also programmed in the absence of overnutrition or high fat feeding, as evidenced by the commonly observed obesity phenotypes in chow-fed offspring exposed to a variety of insults *in utero* (summarised in 1.4.3.1). In our model of diet-induced maternal obesity no differences in body weight or adiposity exist in 8-week-old offspring of obese dams of either sex (112,132,133,138,192), but male offspring show epididymal adipocyte hypertrophy (138). Although glucose tolerance is not affected in male offspring in young adult life, they display adipose tissue IR and systemic hyperinsulinaemia (112,126,133). Importantly, these changes in WAT function and metabolic health precede the development of obesity, indicating programming effects not confounded by postnatal obesity. There were signs of WAT inflammation in 8-week-old male offspring of obese dams, evidenced by microarray analysis showing an enrichment of pro-inflammatory pathways (138). The increased mRNA expression of *TNFα*, *Mcp1* and *Mcp3* was validated in eWAT, although macrophage marker expression and histological assessment of $F4/80^{+}$ cells in eWAT was not significantly different (138). Although female offspring, like males, showed hyperinsulinaemia at 8 weeks (192), evidence of improved pancreatic function was recently reported in females, suggesting the capacity to compensate for programmed metabolic dysfunction (126). The effect of maternal obesity on adipose tissue biology in female offspring has not yet been investigated in our model.

A maternal exercise intervention corrected fasting insulin and attenuated eWAT IR in 8-week-old male offspring (136). This shows that programmed adipose tissue and metabolic changes are susceptible to gestational interventions. Whether metformin treatment during obese pregnancy has similar beneficial effects or detrimental effects due to its ability to cross the placenta and act directly on fetal tissues remains unknown. Therefore, the aim of this chapter was to investigate the effect of maternal metformin intervention during obese pregnancy on adiposity, WAT function and metabolic health in male and female offspring during early adulthood (8 weeks of age).

4.2 Methods

4.2.1 Animal model

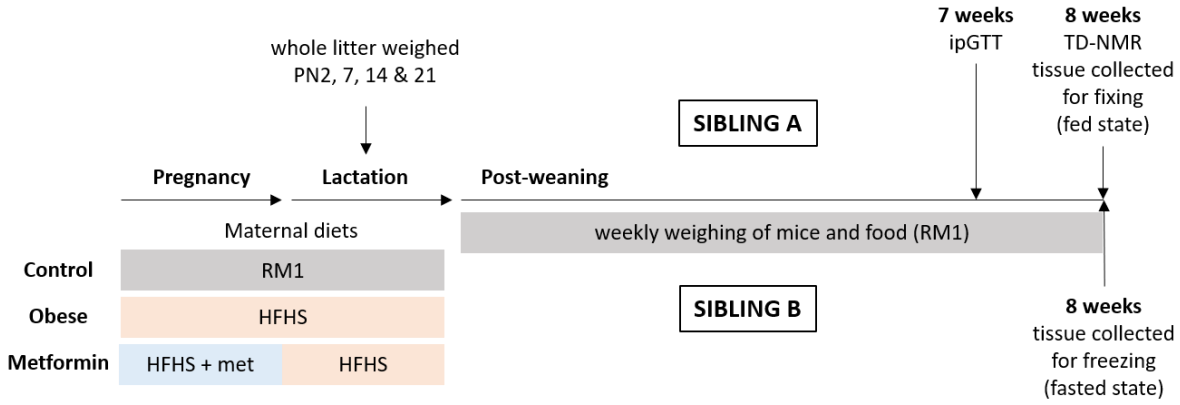

Figure 4.2: Animal model for 8-week-old offspring. *Total n-numbers are n=61 males from 36 independent litters (Con n=11 fed, n=8 fasted; Ob n=12 fed, n=9 fasted; Ob-Met n=12 fed, n=9 fasted siblings) and n=63 females from 37 independent litters (Con n=10 fed, n=8 fasted; Ob n=11 fed, n=9 fasted, Ob-Met n=15 fed, n=10 fasted siblings).*

Offspring were generated as described in Chapter 2.1. For each sibling pair, one animal was designated for *in vivo* phenotyping and collection of fixed tissues (sibling A), whereas the other was left untouched until tissue collection following an overnight fast (sibling B, Figure 4.2). Breeding was continued until an ipGTT (4.2.3) was performed in *n*=10 animals and fasted tissue was available for at least *n*=8 offspring in each group.

4.2.2 Body composition

Body weight and food intake were recorded weekly[13] and body composition was measured by TD-NMR as described in Chapter 2.2.1.

4.2.3 Intraperitoneal glucose tolerance test

An ipGTT was performed in 7-week-old offspring according to methods described in Chapter 2.2.2.

4.2.4 Tissue collection

After culling via Schedule 1 (rising CO_2 concentration), white and brown adipose tissue depots (epididymal/gonadal, intraperitoneal, retroperitoneal and subcutaneous WAT and subscapular BAT) were excised and either snap-frozen (fasted) or fixed in formalin (fed tissues).

4.2.5 Serum analysis

Serum analysis was performed according to methods described in Chapter 2.4.1.

[13] Weights recorded by C. Custance or T. Ashmore, analysis by JMS

4.2.6 Adipose tissue histology

H&E-stained epididymal and gonadal WAT sections[14] were analysed for cell size (Figure 4.3), presence of CLS (Figure 4.4) and WAT browning (Figure 4.5) using the Indica Labs HALO image analysis software as outlined in section 2.5.

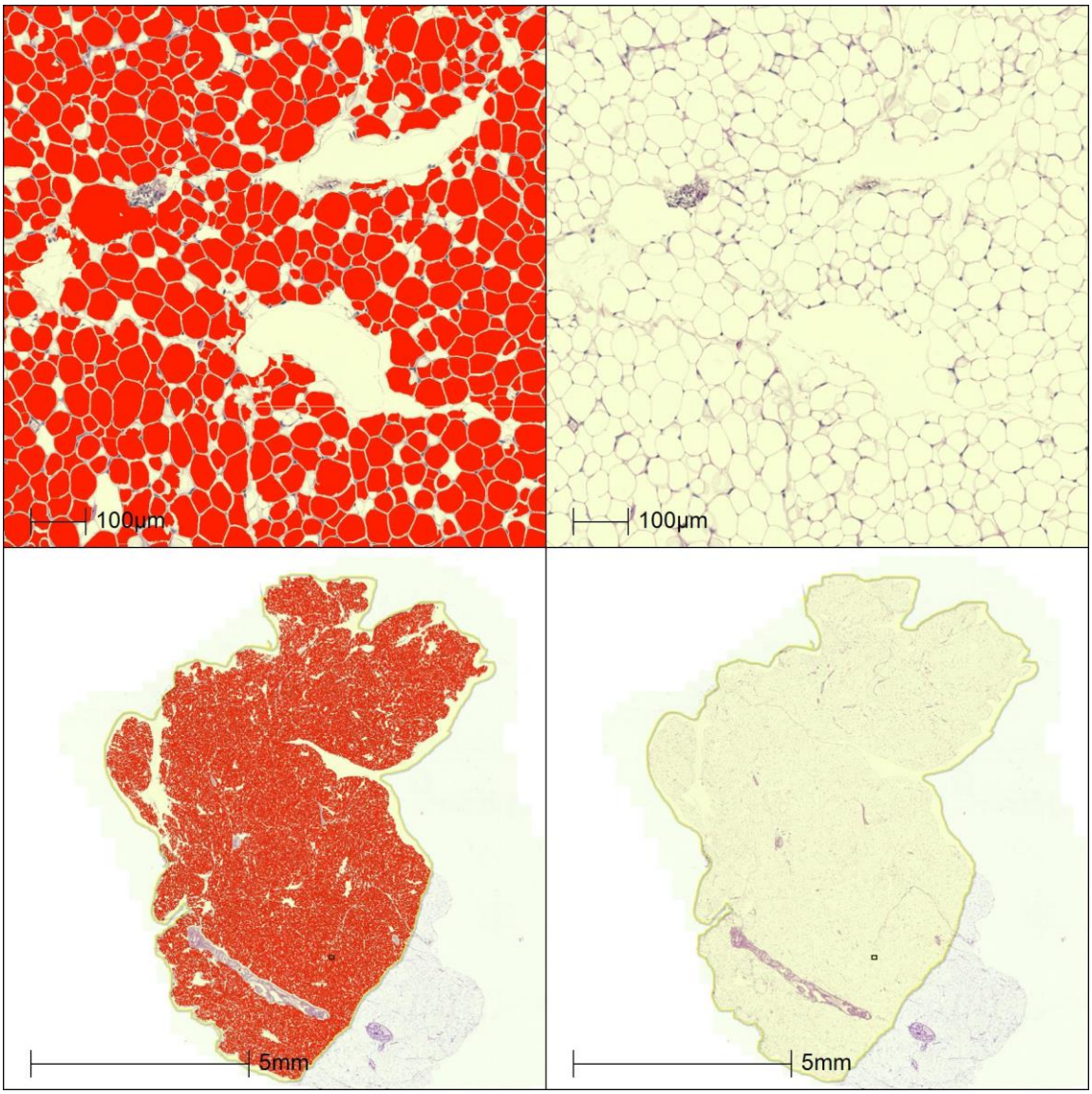

Figure 4.3: Example of cell size analysis in an 8-week-old animal.

4.2.7 Gene expression

The methods for RNA extraction, cDNA synthesis and qPCR are detailed in section 2.4.2. RNA extraction was performed using 50mg adipose tissue. For cDNA synthesis, 1000ng RNA was used. cDNA was initially diluted 1:20 and primer efficiencies were tested using standard curves generated from this dilution. A 1:80 working dilution was ultimately used for qPCR analysis. Data was normalised

[14] Histological processing expertise provided by T. Ashmore

using the comparative CT method against the geometric mean of *Sdha, Ppia* and *Hprt* as this was unaffected by experimental group or sex. Primer sequences are shown in Chapter 2.4.2 (Table 2.3).

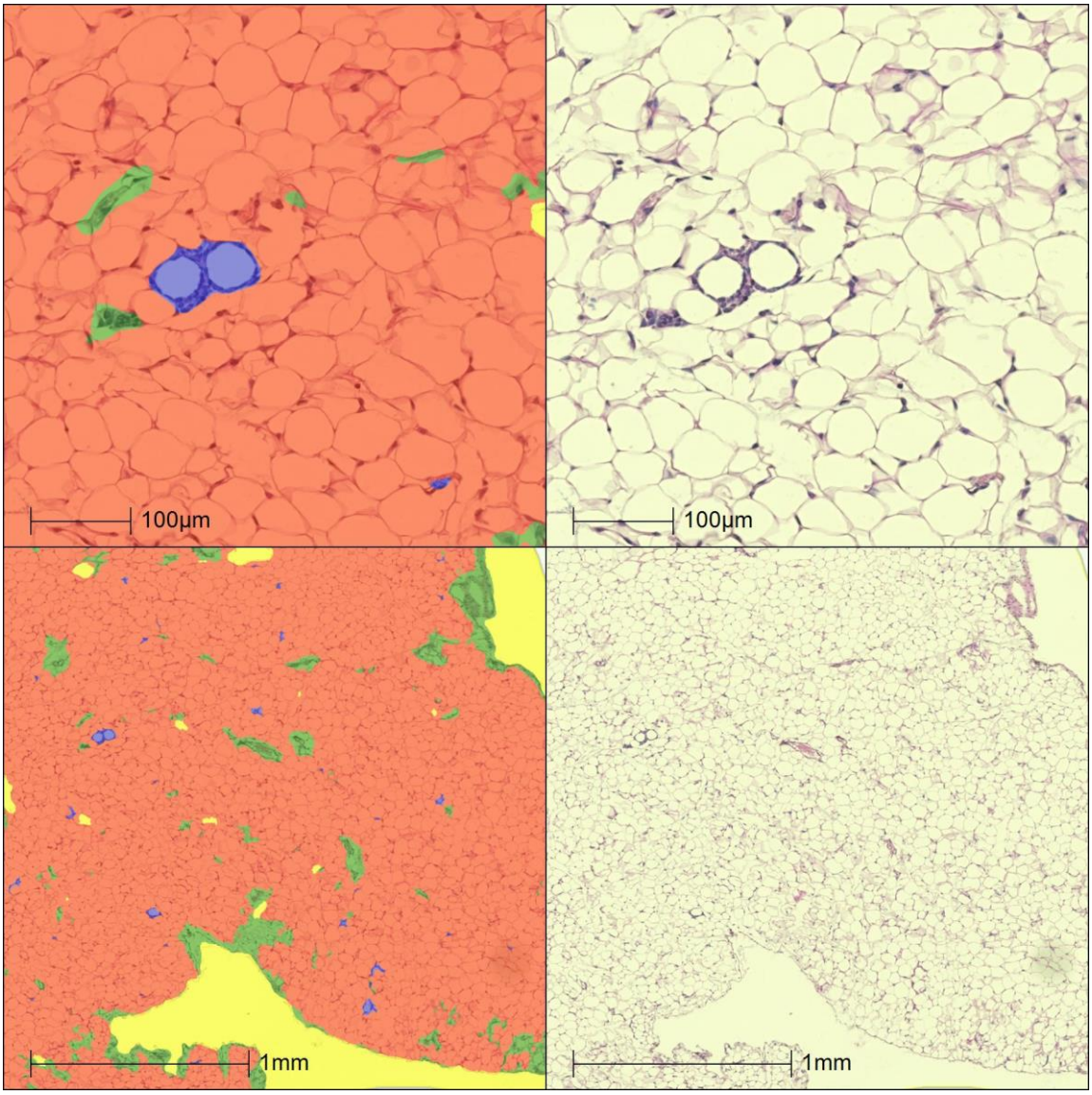

Figure 4.4: Example of crown-like structure analysis in an 8-week-old animal.
Yellow = background, red = adipose tissue, blue = CLS, green = 'other'.

4.2.8 Statistical analysis

Data were analysed using Prism 8.0 (GraphPad). Data are presented as mean ± SEM or median [interquartile range] where appropriate. A p-value <0.05 is considered statistically significant. For linear regression used to compute correlations, p<0.01 is considered statistically significant.

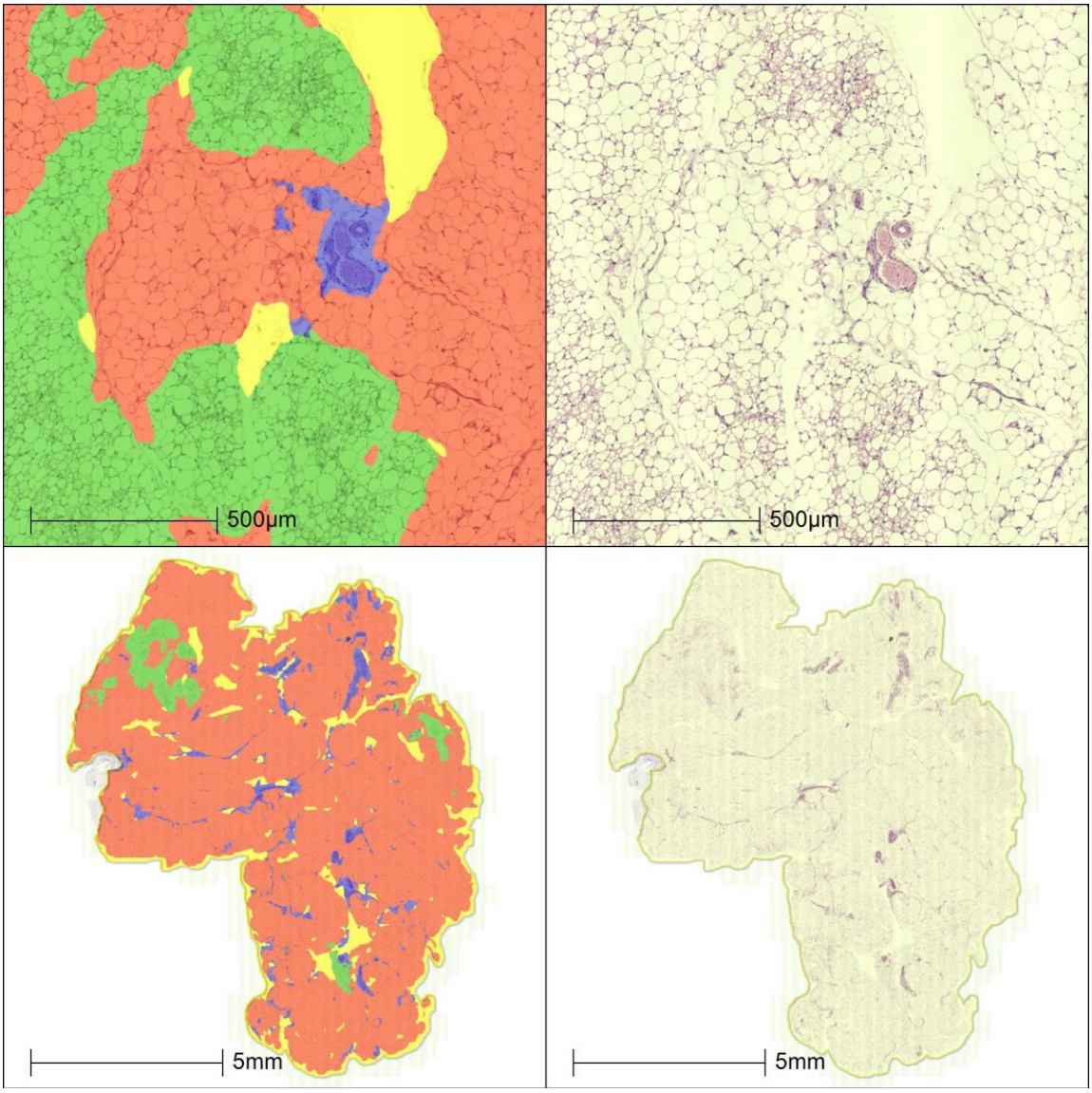

Figure 4.5: Example of white adipose tissue browning analysis in an 8-week-old animal.
Yellow = background, red = adipose tissue, green = BAT-like areas, blue = 'other'.

4.3 Results

4.3.1 Body weight and food intake

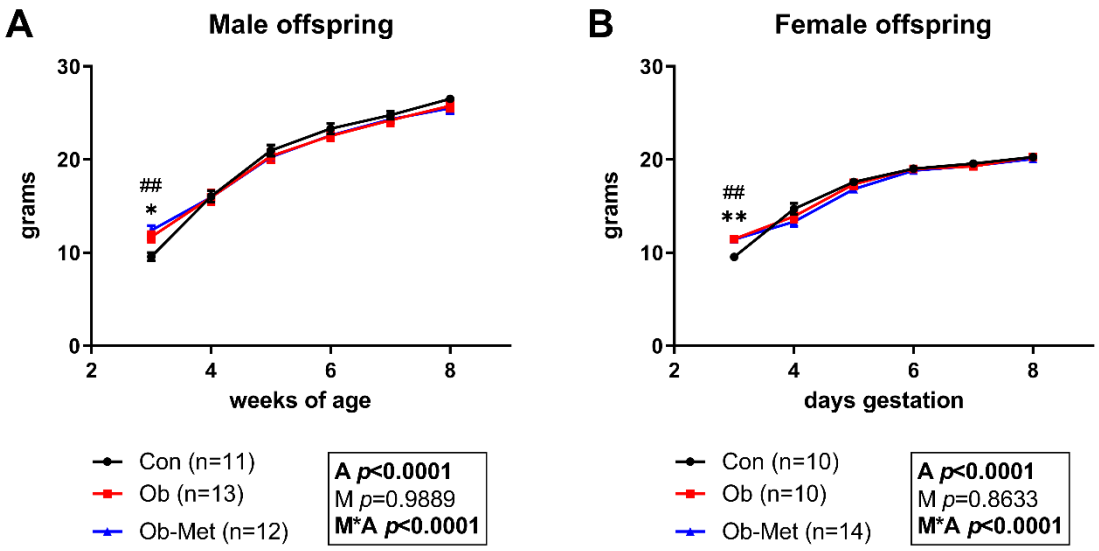

Figure 4.6: Body weight trajectories of male and female offspring up to 8 weeks of age.
*Box: results from repeated measures two-way ANOVA for the effect of age (A), the maternal environment (M) and the interaction between them (M*A). *p<0.05, **p<0.01 Con vs Ob offspring, ##p<0.01 Con vs Ob-Met offspring.*

As demonstrated in Chapter 3.3.6, at weaning both male and female Ob and Ob-Met offspring were significantly heavier than Con offspring (Figure 4.6). However, by 4 weeks of age there was no difference in body weight. Body weight and absolute caloric intake remained unchanged between 4 and 8 weeks of age (Figure 4.6, Table 4.2).

Male offspring	**Con**	**Ob**	**Ob-Met**	***p*-value**
Body weight (g)	26.5 ± 0.41 *(n=11)*	25.8 ± 0.6 *(n=13)*	25.5 ± 0.6 *(n=12)*	*0.4599*
Caloric intake	367 ± 16 *(n=7)*	357 ± 11 *(n=9)*	351 ± 7 *(n=8)*	*0.6442*
Female offspring	**Con**	**Ob**	**Ob-Met**	***p*-value**
Body weight (g)	20.3 [19.7-20.8] *(n=10)*	20.5 [19.4-21.1] *(n=11)*	20.3 [19.0-20.9] *(n=16)*	*0.7791**
Caloric intake	320 ± 5 *(n=8)*	322 ± 12 *(n=6)*	320 ± 3 *(n=11)*	*0.9592*

Table 4.2: Body weight and caloric intake in 8-week-old male and female offspring.
*The p-values reflect outcomes of one-way ANOVA or*Kruskal-Wallis test for non-parametric data.*

4.3.2 Body composition of male offspring

4.3.2.1 TD-NMR

There was no significant effect on body composition in 8-week-old male offspring as assessed by TD-NMR (Table 4.3).

Male offspring	Con (n=19)	Ob (n=9)	Ob-Met (n=12)	*p*-value
Body weight (g)	25.4 ± 0.4	24.4 ± 0.7	25.9 ± 0.7	*0.2496*
Lean mass (g)	16.2 ± 0.5	15.7 ± 0.6	16.0 ± 0.6	*0.8397*
Fat mass (g)	2.1 ± 0.1	2.3 ± 0.2	2.5 ± 0.2	*0.1225*
Fat mass (%BW)	8.3 ± 0.6	9.4 ± 0.6	9.8 ± 0.6	*0.1995*

Table 4.3: Body composition in 8-week-old male offspring using TD-NMR.
Numbers reflect mice in the cohort outlined in 4.2.1 as well as non-littermate mice at 8 weeks of age from the cohort used to generate data in Chapters 5 and 6. The p-values reflect outcomes of one-way ANOVA testing. BW = body weight.

4.3.2.2 Male fat depots

Fed sibling								
	Absolute weights (mg)				Relative weights (% of BW)			
Depot	Con (n=11)	Ob (n=11-12)	Ob-Met (n=12)	*p*-value	Con (n=11)	Ob (n=10-12)	Ob-Met (n=12)	*p*-value
BW	26.6 ± 0.4	26.4 ± 0.6	26.2 ± 0.5	*0.8336*	-	-	-	-
Epididymal	280 ± 16	427 ± 37 [b]	445 ± 28 [c]	***0.0004***	1.05% ± 0.05	1.63% ± 0.13 [b]	1.71% ± 0.11 [c]	***0.0001***
Intra-peritoneal	154 ± 16	222 ± 22 [a]	241 ± 21 [b]	***0.0043#***	0.57% ± 0.05	0.83% ± 0.07 [a]	0.93% ± 0.08 [b]	***0.0014#***
Retro-peritoneal	69 ± 5	109 ± 14 [a]	96 ± 11	***0.0248#***	0.26% ± 0.02	0.40% ± 0.04 [a]	0.37% ± 0.04	***0.0282***
Sub-cutaneous	212 ± 8	297 ± 19 [b]	304 ± 15 [c]	***0.0002***	0.80% ± 0.02	1.12% ± 0.05 [c]	1.17% ± 0.06 [d]	***<0.0001***
Fasted sibling (16 hours)								
	Absolute weights (mg)				Relative weights (% of BW)			
Depot	Con (n=8)	Ob (n=8-9)	Ob-Met (n=9)	*p*-value	Con (n=8)	Ob (n=8-9)	Ob-Met (n=9)	*p*-value
BW	22.6 ± 0.6	21.2 ± 0.7	21.4 ± 0.8	*0.3681*	-	-	-	-
Epididymal	187 ± 20	248 ± 31	314 ± 9 [a]	*0.0616*	0.82 ± 0.09	1.15 ± 0.11	1.45 ± 0.18 [a]	***0.0160***
Intra-peritoneal	82 ± 11	90 ± 21	144 ± 23	*0.0720*	0.36 ± 0.04	0.41 ± 0.09	0.67 ± 0.10 [a]	***0.0350***
Retro-peritoneal	31 ± 4	53 ± 12	60 ± 13	*0.1644*	0.14 ± 0.02	0.24 ± 0.05	0.27 ± 0.05	*0.1157*
Sub-cutaneous	107 ± 10	133 ± 17	173 ± 23 [a]	***0.0490***	0.46 [0.36-0.60]	0.51 [0.46-0.84]	1.02 [0.63-1.06] [a]	***0.0110****
BAT	41 ± 2	42 ± 3	50 ± 5	*0.3013#*	0.18 ± 0.01	0.20 ± 0.0	0.24 ± 0.02	*0.063*

Table 4.4: Absolute and relative weight of excised adipose tissue depots from 8-week-old male offspring.
*BAT = brown adipose tissue. BW = body weight. [a]p<0.05, [b]p<0.01, [c]p<0.001, [d]p<0.0001 vs Con, one-way ANOVA with Tukey's multiple comparison test, #ANOVA performed on log-transformed data or *Kruskal-Wallis test for non-parametric data.*

In utero exposure to an obesogenic diet, irrespective of maternal metformin treatment, increased weight of all WAT depots collected from fed 8-week-old male offspring when expressed in absolute terms and relative to body weight (Table 4.4). The same was observed when considering the sum of WAT depots collected (a proxy for total body fat mass, Figure 4.7A).

In the fasted state, no difference in adiposity was found between male Con and Ob offspring (Table 4.4). However, fasted Ob-Met offspring show increased adiposity compared to Con for all WAT depots except the retroperitoneal depot when expressed relative to body weight (Figure 4.7B). BAT weight was not significantly different between groups.

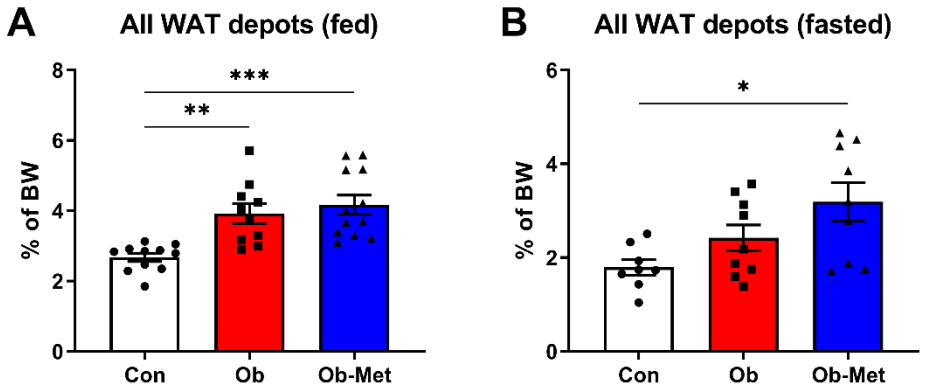

Figure 4.7: Adiposity in fed and fasted 8-week-old male offspring.
*Combined weights of all WAT depots collected in A) the fed state (n=11 Con, n=10 Ob, n=12 Ob-Met) and B) following a 16 hour fast (n=8 Con, n=9 Ob, n=9 Ob-Met),relative to body weight (BW) at the study endpoint. *p<0.05, **p<0.01, ***p<0.001 one-way ANOVA with Tukey's multiple comparison test.*

4.3.2.3 Epididymal adipocyte size

Both Ob and Ob-Met offspring showed epididymal adipocyte hypertrophy compared to Con offspring, evidenced by a rightward shift in cell size distribution favouring larger adipocytes (Figure 4.8A). Consistent with the lack of difference in adiposity between male Ob and Ob-Met offspring in the fed state, no effect of the metformin intervention on fed adipocyte size was observed. However, the increased estimated cell number in the dissected eWAT suggests excessive adipocyte hyperplasia in Ob-Met offspring (Figure 4.8D).

Because, unlike Ob animals, Ob-Met also showed adiposity compared to Con offspring in the fasted state, the adiposity phenotype was explored further using fixed fasted eWAT from a different cohort (the pilot cohort used to generate data in Figure 1.4), since all fasted eWAT from the current cohort was frozen for molecular analysis (see Figure 4.2). As for fed animals, there was a significant rightward shift in adipocyte size distribution in 8-week-old offspring exposed to maternal obesity (Figure 4.9A). Ob-Met offspring tended to have more cells larger than the 90th percentile but this did not reach statistical significance (p=0.06), likely due to lack of power (Figure 4.9C).

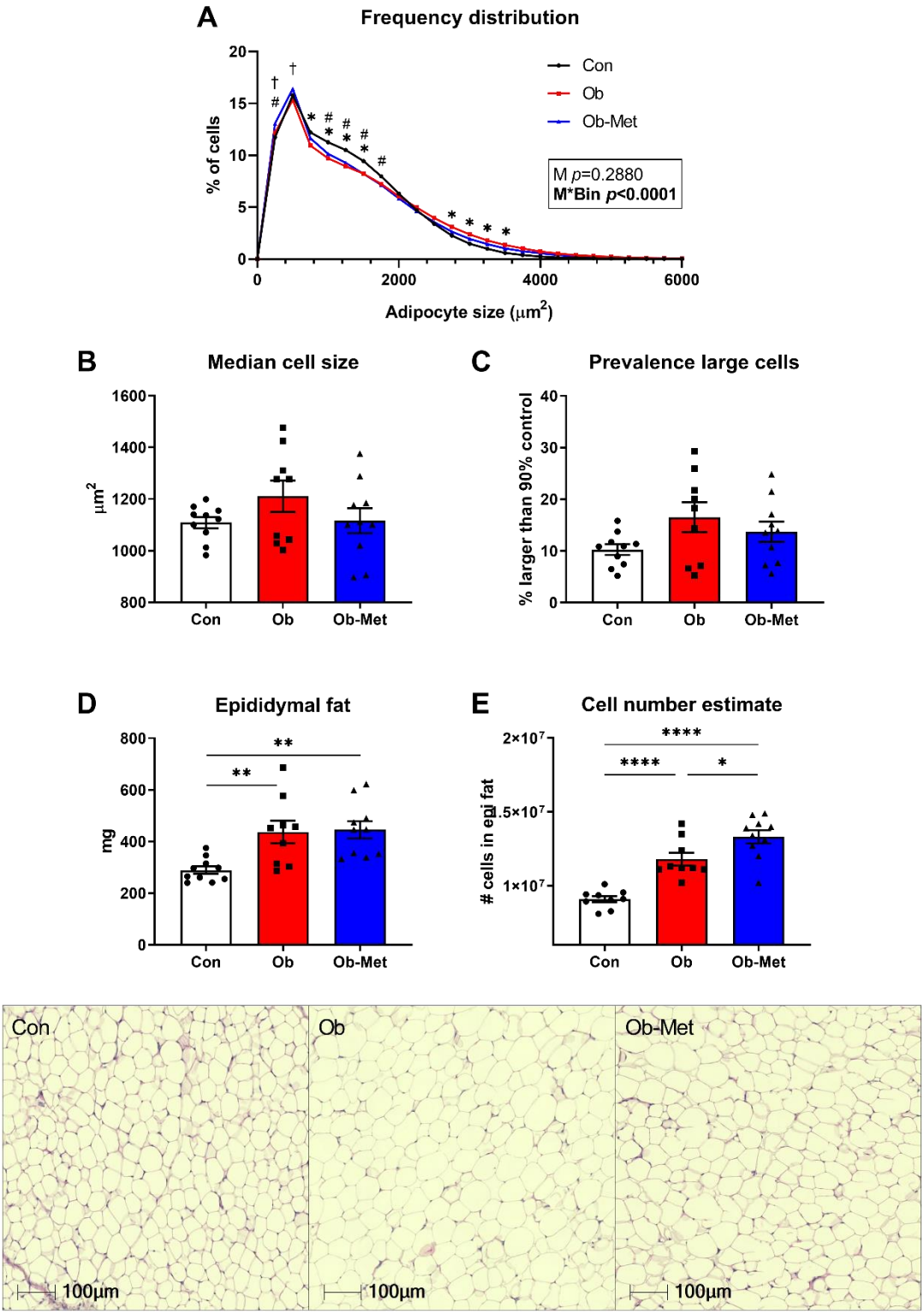

Figure 4.8: Epididymal adipocyte size in 8-week-old male fed offspring.

*A) Frequency distribution of cell size. Box: results from repeated measures two-way ANOVA for the effect of the maternal environment (M) and its interaction with adipocyte size (M*Bin). *p<0.05 (or less) Con vs Ob, #p<0.05 (or less) vs Ob-Met, †p<0.05 (or less) Ob vs Ob-Met offspring. B) Median cell size, C) the percentage of cells larger than the 90th centile of Con offspring, D) epididymal fat depot weight of animals used for cell size analysis, E) estimated adipocyte number based on the weight of the dissected fat depot and mean adipocyte area. *p<0.05, **p<0.01, ****p<0.0001 one-way ANOVA with Tukey's multiple comparison test. Bottom: representative images of male Con (n=10), Ob (n=9) and Ob-Met (n=10) offspring.*

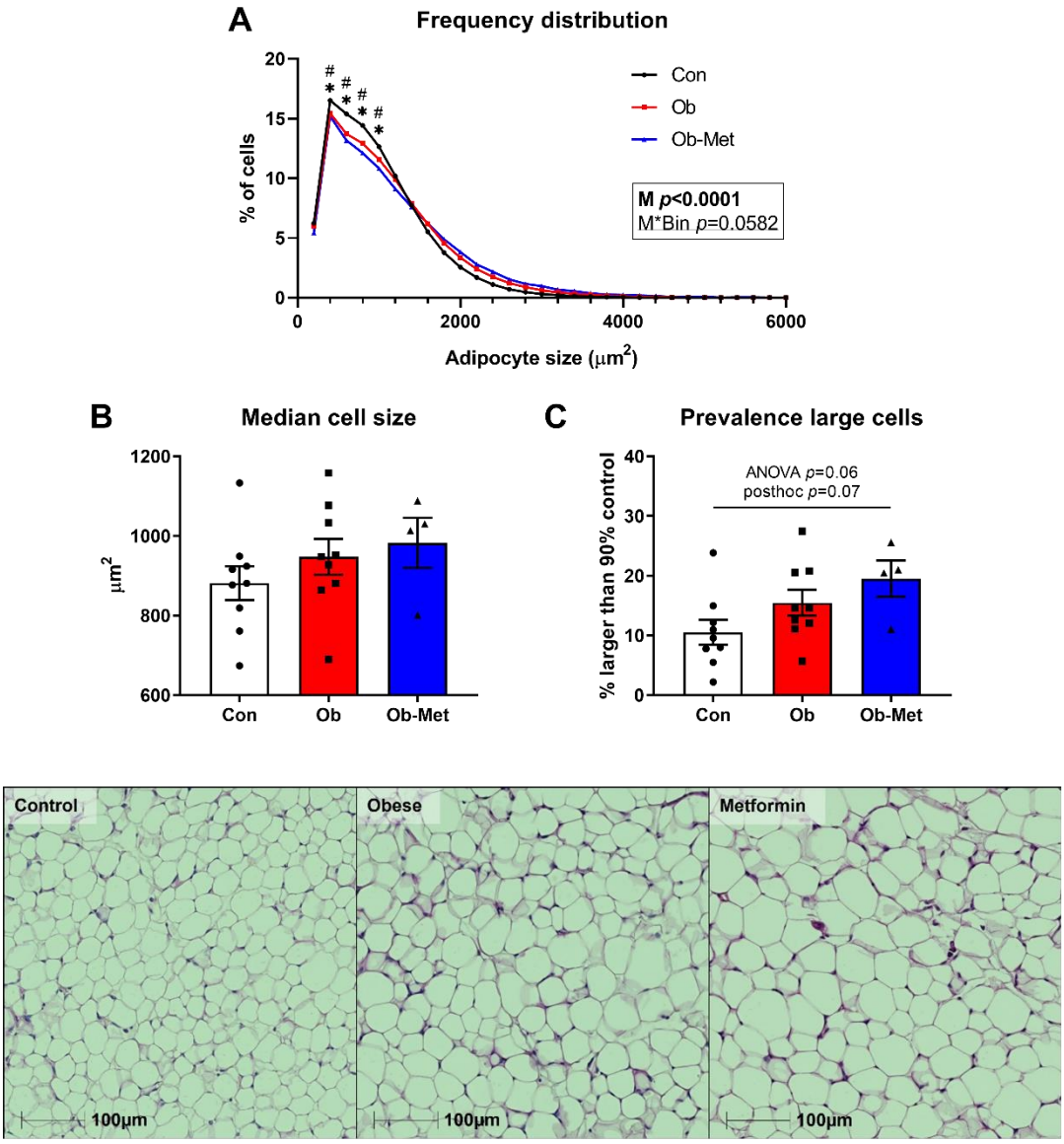

Figure 4.9: Epididymal adipocyte size in 8-week-old fasted male offspring from a different cohort.
*A) Frequency distribution of cell size. Box: results from repeated measures two-way ANOVA for the effect of the maternal environment (M) and its interaction with adipocyte size (M*Bin). *p<0.05 (or less) Con vs Ob, #p<0.05 (or less) Con vs Ob-Met offspring. B) Median cell size, C) the percentage of cells larger than the 90th centile of Con offspring. Bottom: representative images of male Con (n=9), Ob (n=9) and Ob-Met (n=4) offspring.*

4.3.3 Body composition of female offspring

4.3.3.1 TD-NMR

There was no significant effect on whole body composition in 8-week-old female offspring assessed by TD-NMR (Table 4.5).

Female offspring	Con (n=16)	Ob (n=13)	Ob-Met (n=15)	*p*-value
Body weight (g)	19.9 ± 0.2	20.1 ± 0.4	20.8 ± 0.4	*0.2325*
Lean mass (g)	12.1 ± 0.5	12.0 ± 0.5	12.6 ± 0.3	*0.5989*
Fat mass (g)	2.3 ± 0.1	2.4 ± 0.2	2.7 ± 0.2	*0.1670*
Fat mass (%BW)	11.5 ± 0.5	11.7± 0.7	13.1 ± 0.9	*0.3130#*

Table 4.5: Body composition in 8-week-old female offspring using TD-NMR.
Numbers reflect mice in the cohort outlined in 4.2.1 as well as non-littermate mice at 8 weeks of age from the cohort used to generate data in Chapters 5 and 6. The p-values reflect outcomes of one-way ANOVA or #ANOVA performed on log-transformed data. BW = body weight.

4.3.3.2 Female fat depots

Fed sibling								
	Absolute weights (mg)				Relative weights (% of BW)			
Depot	Con (n=9-10)	Ob (n=10-11)	Ob-Met (n=13-15)	*p*-value	Con (n=9-10)	Ob (n=11)	Ob-Met (n=13-15)	*p*-value
BW	20.2 ± 0.2	20.4 ± 0.4	20.5 ± 0.5	*0.8044*	-	-	-	-
Gonadal	252 ± 17	309 ± 27	326 ± 23	*0.0894*	1.25 ± 0.08	1.50 ± 0.10	1.57 ± 0.09 [a]	*0.0556*
Intra-peritoneal	118 ± 11	171 ± 16 [a]	175 ± 10 [b]	***0.0070***	0.58 ± 0.05	0.83 ± 0.06 [a]	0.86 ± 0.05 [b]	***0.0029***
Retro-peritoneal	44 ± 3	47 ± 3	52 ± 2	*0.0928*	0.22 ± 0.01	0.25 ± 0.02	0.26 ± 0.01	*0.1175*
Sub-cutaneous	216 [194-249]	279 [234-347]	296 [237-313]	***0.0314****	1.10 ± 0.05	1.36 ± 0.12	1.32 ± 0.11	*0.2240*
Fasted sibling (16 hours)								
	Absolute weights (mg)				Relative weights (% of BW)			
Depot	Con (n=8)	Ob (n=9)	Ob-Met (n=10)	*p*-value	Con (n=8)	Ob (n=8-9)	Ob-Met (n=10)	*p*-value
BW	17.8 ± 0.1	17.9 ± 0.6	17.2 ± 0.4	*0.3961*	-	-	-	-
Gonadal	169 ± 21	246 ± 36	183 ± 21	*0.1205#*	0.94 ± 0.11	1.34 ± 0.15	1.05 ± 0.10	*0.0854*
Intra-peritoneal	74 ± 12	102 ± 17	83 ± 13	*0.3376#*	0.41 ± 0.07	0.49 ± 0.04	0.48 ± 0.07	*0.6661*
Retro-peritoneal	23 ± 4	36 ± 7	25 ± 3	*0.1287*	0.13 ± 0.02	0.19 ± 0.03	0.14 ± 0.01	*0.0967*
Sub-cutaneous	128 [87-170]	155 [153-210]	154 [127-174]	*0.1599**	0.72 ± 0.07	0.95 ± 0.04[a]	0.92 ± 0.06	***0.0259***
BAT	38 ± 2	45 ± 3	40 ± 2	*0.1217*	0.21 ± 0.01	0.25 ± 0.01	0.23 ± 0.01	*0.2015*

Table 4.6: Absolute and relative weight of excised adipose tissue depots from 8-week-old female offspring.
*BAT = brown adipose tissue. BW = body weight. [a]p<0.05, [b]p<0.01 vs Con offspring, one-way ANOVA with Tukey's multiple comparison test, *Kruskal-Wallis test for non-parametric data or #ANOVA performed on normally distributed log-transformed data.*

In the fed state, WAT weight in female Ob-Met offspring was consistently increased compared to Con offspring, but statistical significance was only reached for the intraperitoneal (absolute and relative to body weight) and subcutaneous depot (absolute weight). Ob offspring only had significantly increased intraperitoneal WAT weight (Table 4.6).

In the fasted state, Ob females showed increased adiposity compared to Con offspring: the relative weight of the subcutaneous depot was significantly increased with a trend for gonadal and retroperitoneal weights (Table 4.6). WAT weights were comparable between Con and Ob-Met offspring. The same patterns were observed when looking at the combined weight of WAT depots: adiposity was observed in fed Ob-Met offspring with a trend for fasted Ob offspring (Figure 4.10).

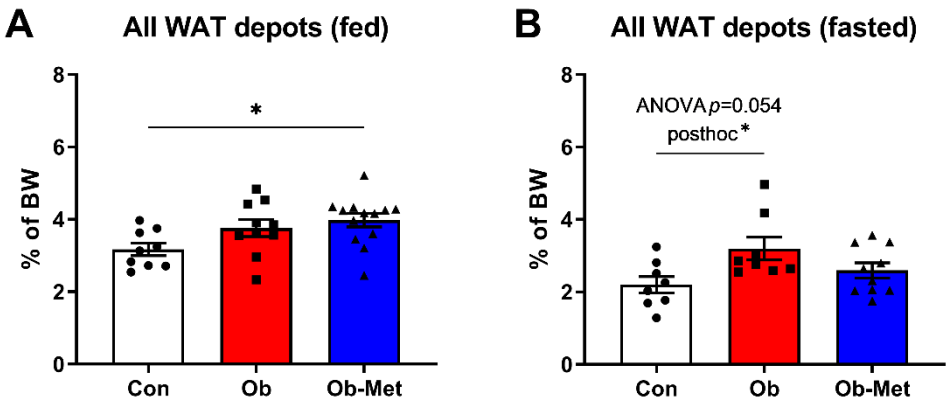

Figure 4.10: Adiposity phenotype in fed and fasted 8-week-old female offspring.
*Combined weights of all WAT depots collected in A) the fed state (n=9 Con, n=10 Ob, n=13 Ob-Met) and B) following a 16 hour fast (n=8 Con, n=8 Ob, n=10 Ob-Met), relative to body weight (BW) at the study endpoint. *p<0.05 one-way ANOVA with Tukey's multiple comparison test.*

4.3.3.3 Gonadal adipocyte size

There was no significant difference in gWAT weight, median adipocyte size or estimated adipocyte number. However, the distribution of adipocyte size was significantly altered by exposure to maternal obesity, with a rightward shift seen for Ob offspring which was not corrected by the metformin intervention. This may be driven by a specific increase in exceptionally large cells (Figure 4.11).

No fasted gWAT was available for histological analysis.

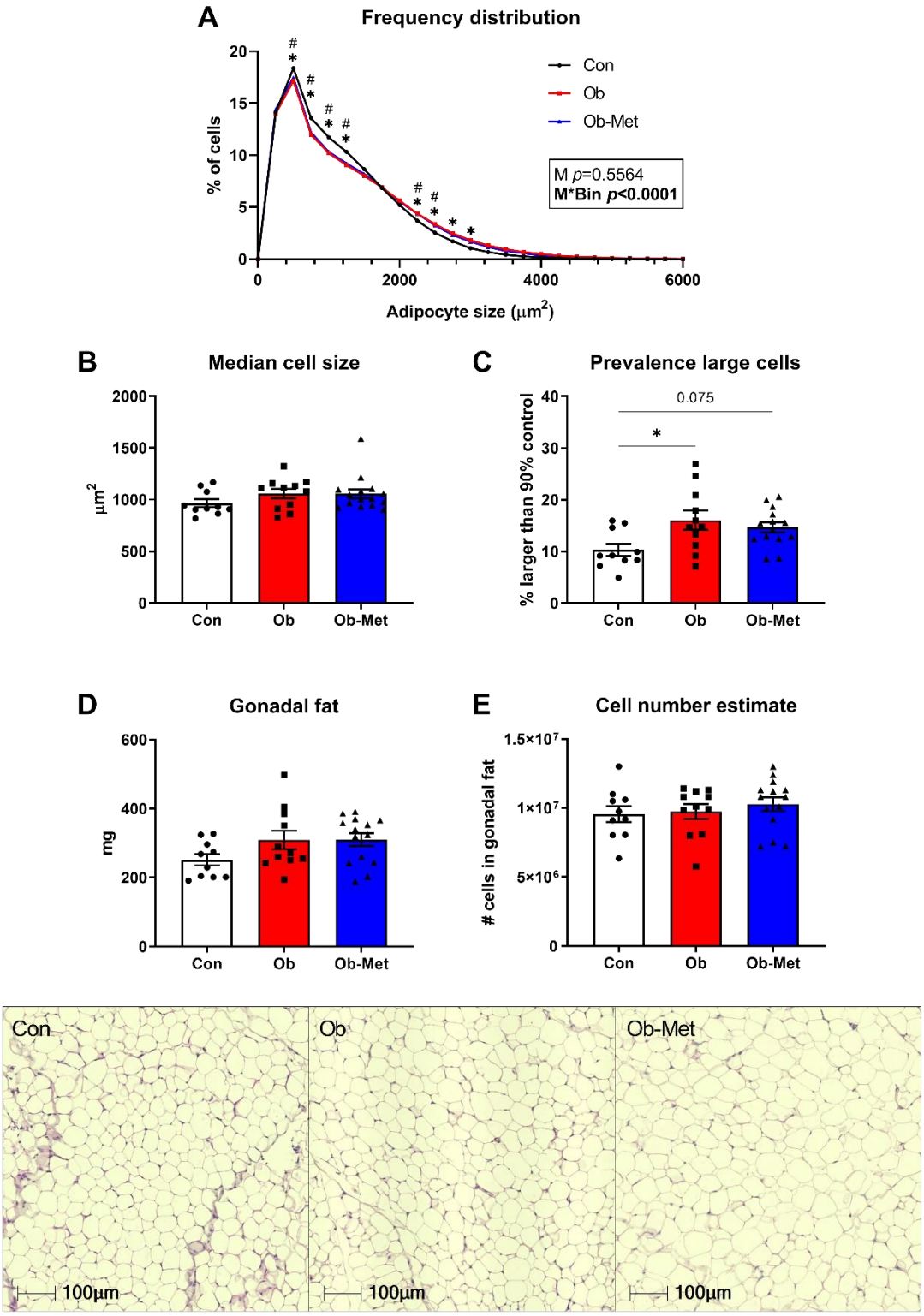

Figure 4.11: Gonadal adipocyte size in 8-week-old fed female offspring.

*A) Frequency distribution of adipocyte size. Box: results from repeated measures two-way ANOVA for the maternal environment (M) and the interaction between the maternal environment and adipocyte size (M*Bin). *p<0.05 (or less), #p<0.05 (or less) Con vs Ob-Met offspring. B) Median cell size, C) the percentage of cells larger than the 90th centile of Con offspring, D) gonadal fat depot weight of animals used for cell size analysis, E) estimated adipocyte number based on the weight of the dissected fat depot and mean adipocyte area. *p<0.05 one-way ANOVA with Tukey's multiple comparison test. Bottom: representative images of female Con (n=10), Ob (n=11) and Ob-Met (n=14) offspring.*

4.3.4 Response to fasting

	Male offspring					*Female offspring*				
	Ob (fed)	**Ob (fasted)**	**Ob-Met (fed)**	**Ob-Met (fasted)**	***p***	**Ob (fed)**	**Ob (fasted)**	**Ob-Met (fed)**	**Ob-Met (fasted)**	***p***
Glucose (mmol/l)	13.5 ± 0.8	5.8 ± 0.3	12.1 ± 0.9	5.6 ± 0.3	*ns*	11.7 ± 0.5	6.1 ± 0.4	11.5 ± 0.6	5.7 ± 0.2	*ns*
BW (gram)	25.7 ± 0.6	21.3 ± 0.8	26.1 ± 0.4	21.4 ± 0.8	*ns*	20.3 ± 0.5	17.9 ± 0.6	21.4 ± 0.4	17.3 ± 0.4	***0.036***
Epi/Gon fat (gram)	407 ± 46	241 ± 34	466 ± 32	314 ± 47	*ns*	303 ± 31	246 ± 36	365 ± 25	189 ± 22	***0.008***
Epi/Gon fat %/BW)	1.57 ± 0.16	1.11 ± 0.12	1.78 ± 0.12	1.44 ± 0.18	*ns*	1.47 ± 0.12	1.34 ± 0.15	1.70 ± 0.09	1.08 ± 0.11	***0.010***
SC fat (gram)	267 ± 18	131 ± 19	309 ± 17	173 ± 23	*ns*	277 ± 35	182 ± 17	306 ± 12	165 ± 13	*ns*
SC fat (%/BW)	1.04 ± 0.06	0.61 ± 0.07	1.19 ± 0.07	0.88 ± 0.09	*ns*	1.31 ± 0.16	0.95 ± 0.04	1.30 ± 0.13	0.83 ± 0.08	*ns*
All depots (%/BW)	3.76 ± 0.33	2.33 ± 0.30	4.28 ± 0.31	3.19 ± 0.41	*ns*	4.04 ± 0.36	3.20 ± 0.31	4.34 ± 2.69	2.69 ± 0.21	***0.048***

Table 4.7: Response to fasting in Ob and Ob-Met offspring.
P-values for the interaction between metformin treatment and fasting using two-way ANOVA in siblings. Epi/Gon: epididymal/gonadal. BW: body weight. SC: subcutaneous. Males: n=8 Ob, n=9 Ob-Met. Females: n=8-9 Ob, n=9 Ob-Met.

The difference in adiposity phenotype between the fed and fasted sibling suggests prenatal metformin may alter the response to fasting in offspring of obese dams. To address this, the interaction between metformin exposure and fasting was tested by two-way ANOVA using adiposity and metabolic data from fed and fasted Ob and Ob-Met sibling pairs. No difference in response to fasting was found for male offspring (p>0.05, Table 4.7). In contrast, female Ob-Met responded significantly differently than Ob offspring to an overnight fast with respect to gWAT weight, total fat mass and body weight, losing more weight and fat mass after an overnight fast (Table 4.7). No interaction was found for glucose or subcutaneous WAT. Examples of how female animals respond to a fast are shown (Figure 4.12).

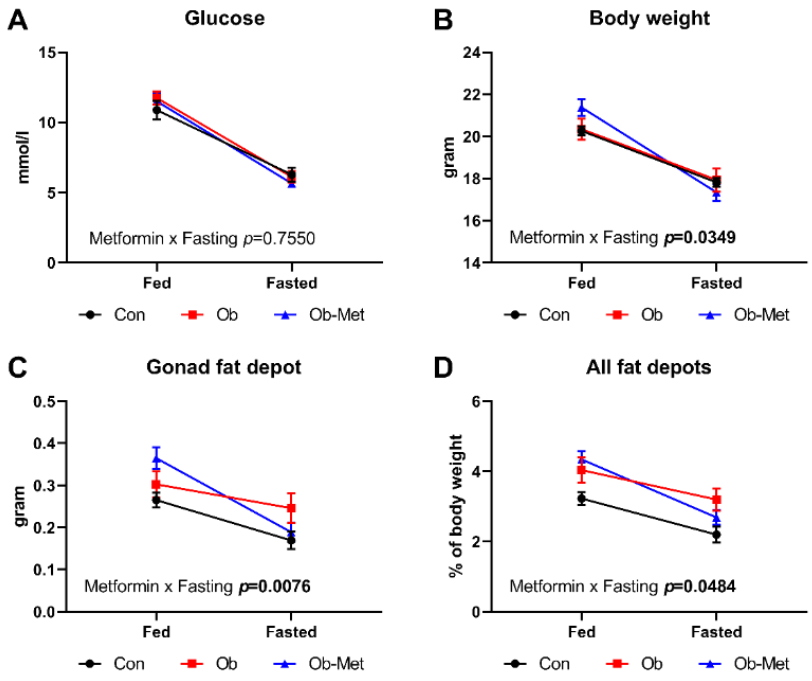

Figure 4.12: Response of female offspring to fasting.
Analysis was performed on Obese and Ob-Met only, but the Control trajectory is included on graphs for completeness. Numbers are n=8 for Con, n=8-9 for Ob, n=9 for Ob-Met females.

4.3.5 Metabolic health

4.3.5.1 Glucose tolerance

To assess whether exposure to maternal obesity and/or metformin affected metabolic health, an ipGTT was performed at 7 weeks of age. There was no difference in baseline parameters (age, body weight, baseline glucose) between groups in male or female offspring (not shown). There was no effect of the maternal environment on absolute glucose levels during the ipGTT or area under the curve (Figure 4.13).

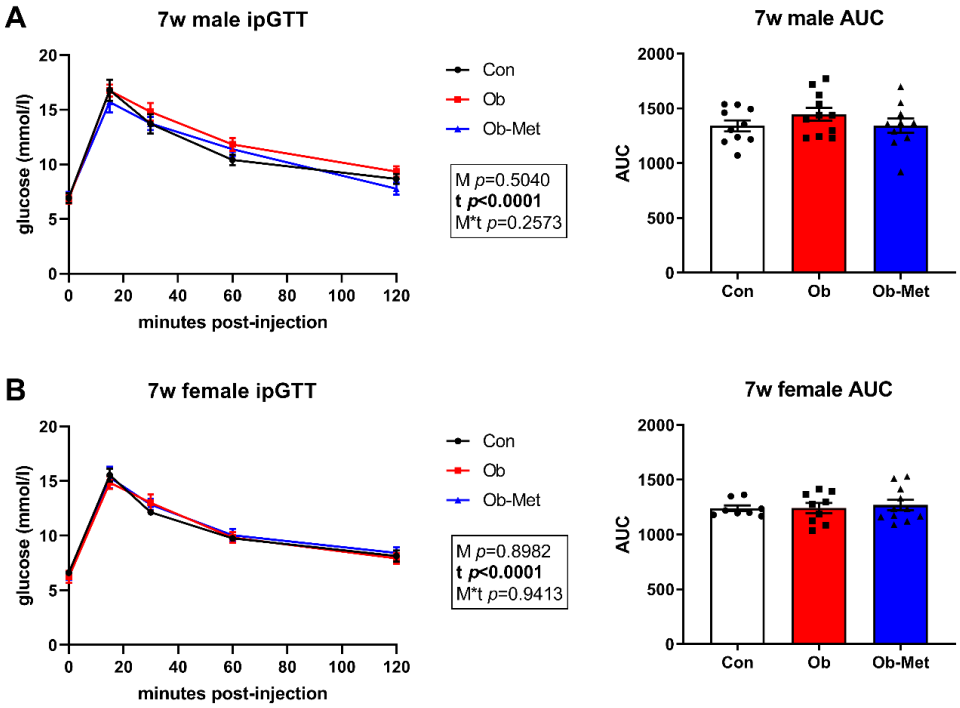

Figure 4.13 Glucose tolerance in 7-week-old male and female offspring.
*A) Male (n=10 Con, n=11 Ob, n=10 Ob-Met) and B) female offspring (n=8 Con, n=9 Ob, n=11 Ob-Met) ipGTT curve and area under the curve for 10% glucose injection. Box: results from repeated measures two-way ANOVA for the effect of time (t), the maternal environment (M) and the interaction between them (M*t).*

4.3.5.2 Serum analysis in male offspring

Male offspring	Con (n=7-8)	Ob (n=8-9)	Ob-Met (n=9)	*p*-value
Glucose (fed, mmol/l) *from different animals*	13.6 ± 0.5 (n=11)	12.9 ± 0.8 (n=12)	11.8 ± 0.7 (n=12)	*0.1474*
Glucose (fasted, mmol/l)	7.2 ± 0.5	5.9 ± 0.3 [a]	5.6 ± 0.3 [a]	***0.0109***
Insulin (pmol/l)	50 ± 6	67 ± 6	73 ± 10	*0.1697*
HOMA-IR	2.4 ± 0.4	2.5 ± 0.2	2.6 ± 0.4	*0.8830*
HOMA-%B	41 ± 4	74 ± 9	113 ± 21 [b]	***0.0111***
Cholesterol (mmol/l)	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.2	*0.9263*
Triglycerides (mmol/l)	1.36 ± 0.10	1.29 ± 0.14	1.31 ± 0.07	*0.8978*
FFAs (mmol/l)	1.87 ± 0.15	1.50 ± 0.11	1.89 ± 0.14	*0.0657*

Table 4.8 Serological analysis in 8-week-old male offspring.
All analysis was performed on serum from 16-hour fasted animals, except fed glucose which was taken from fed siblings at tissue collection. [a]p<0.05, [b]p<0.01 vs Con offspring, one-way ANOVA with Tukey's multiple comparison test. FFAs = free fatty acids.

There was no difference in fed glucose before tissue collection. In contrast, fasting glucose was decreased in Ob and Ob-Met males compared to Con offspring following a 16-hour fast (Table 4.8). Fasting insulin and HOMA-IR index were not significantly different, but Ob-Met males showed significantly higher HOMA-%B index indicative of increased β-cell activity (Table 4.8). Fasted serum cholesterol, triglycerides or FFAs were not significantly different.

4.3.5.3 Serum analysis in female offspring

Female offspring	**Con (n=6-8)**	**Ob (n=8-9)**	**Ob-Met (n=9-10)**	***p*-value**
Glucose (fed, mmol/l) *from different animals*	11.4 ± 0.6 (n=10)	11.6 ± 0.4 (n=11)	12.2 ± 0.5 (n=15)	*0.5360*
Glucose (fasted, mmol/l)	6.3 ± 0.5	5.5 ± 0.1	5.6 ± 0.2	*0.2363*
Insulin (pmol/l)	77 ± 15	67 ± 7	52 ± 4	*0.1417*
HOMA-IR	3.1 ± 0.5	2.5 ± 0.3	1.8 ± 0.1 [a]	***0.0221***
HOMA-%B	64 ± 15	90 ± 12	82 ± 14	*0.3467*
Cholesterol (mmol/l)	2.3 ± 0.2	2.4 ± 0.1	2.2 ± 0.1	*0.6568*
Triglycerides (mmol/l)	0.95 ± 0.05	1.07 ± 0.09	0.93 ± 0.05	*0.3019*
FFAs (mmol/l)	1.59 ± 0.17	1.82 ± 0.22	1.46 ± 0.09	*0.2851*

Table 4.9 Serological analysis in 8-week-old female offspring.
All analysis was performed on serum from 16-hour fasted animals, except fed glucose which was taken from fed siblings at tissue collection. [a]p<0.05 vs Con offspring, one-way ANOVA with Tukey's multiple comparison test. FFAs = free fatty acids.

There was no difference in glucose, insulin, HOMA-%B index, cholesterol, triglyceride or FFA levels of 8-week-old female offspring. However, the HOMA-IR index was significantly lower in Ob-Met offspring indicative of improved insulin sensitivity (Table 4.9).

4.3.6 Adipose tissue biology

4.3.6.1 WAT browning

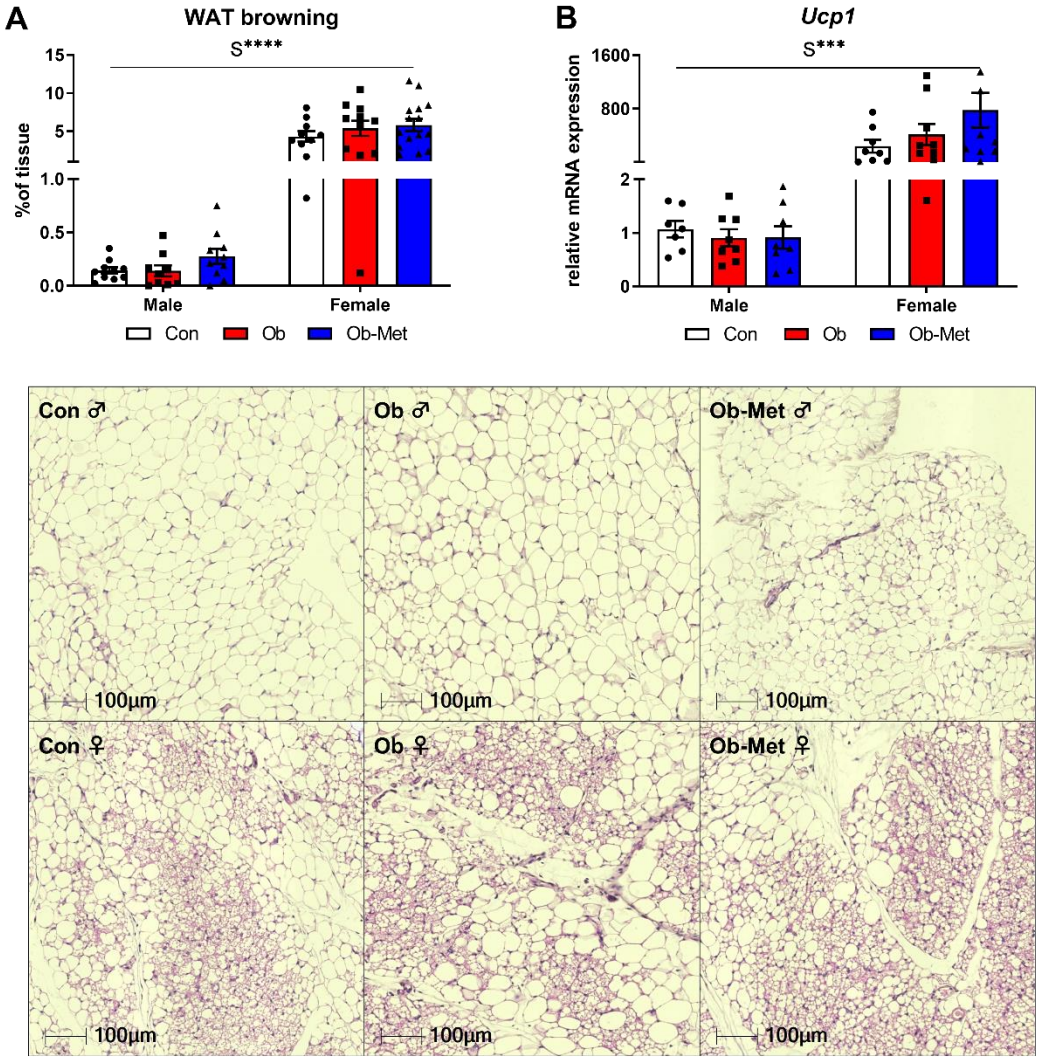

Figure 4.14: Foci of adipose tissue browning in epididymal or gonadal fat depots.
*A) Percentage BAT-like tissue in H&E-stained adipose tissue sections from 8-week-old fed animals (examples below; n=10 Con, n=9 Ob, n=10 Ob-Met males; n=10 Con, n=11 Ob, n=15 Ob-Met females). B) Expression of Ucp1 mRNA in fasted 8-week-old adipose tissue relative to geomean of housekeeper genes Ppia, Sdha and Hprt (n=7 Con, n=8 Ob, n=8 Ob-Met males; n=8 Con, n=9 Ob , n=10 Ob-Met females). ***p<0.001, ****p<0.0001 for the effect of sex (S), two-way ANOVA.*

To assess whether the differences in adiposity or response to fasting could be explained by differences in WAT browning, prevalence of BAT-like areas (Figure 4.14A) and *Ucp1* mRNA expression (Figure 4.14B) was measured in in eWAT/gWAT from fed and fasted siblings, respectively. There were clear sex differences for both parameters with females showing increased WAT browning and *Ucp1* expression, but no difference between the groups.

4.3.6.2 WAT inflammation

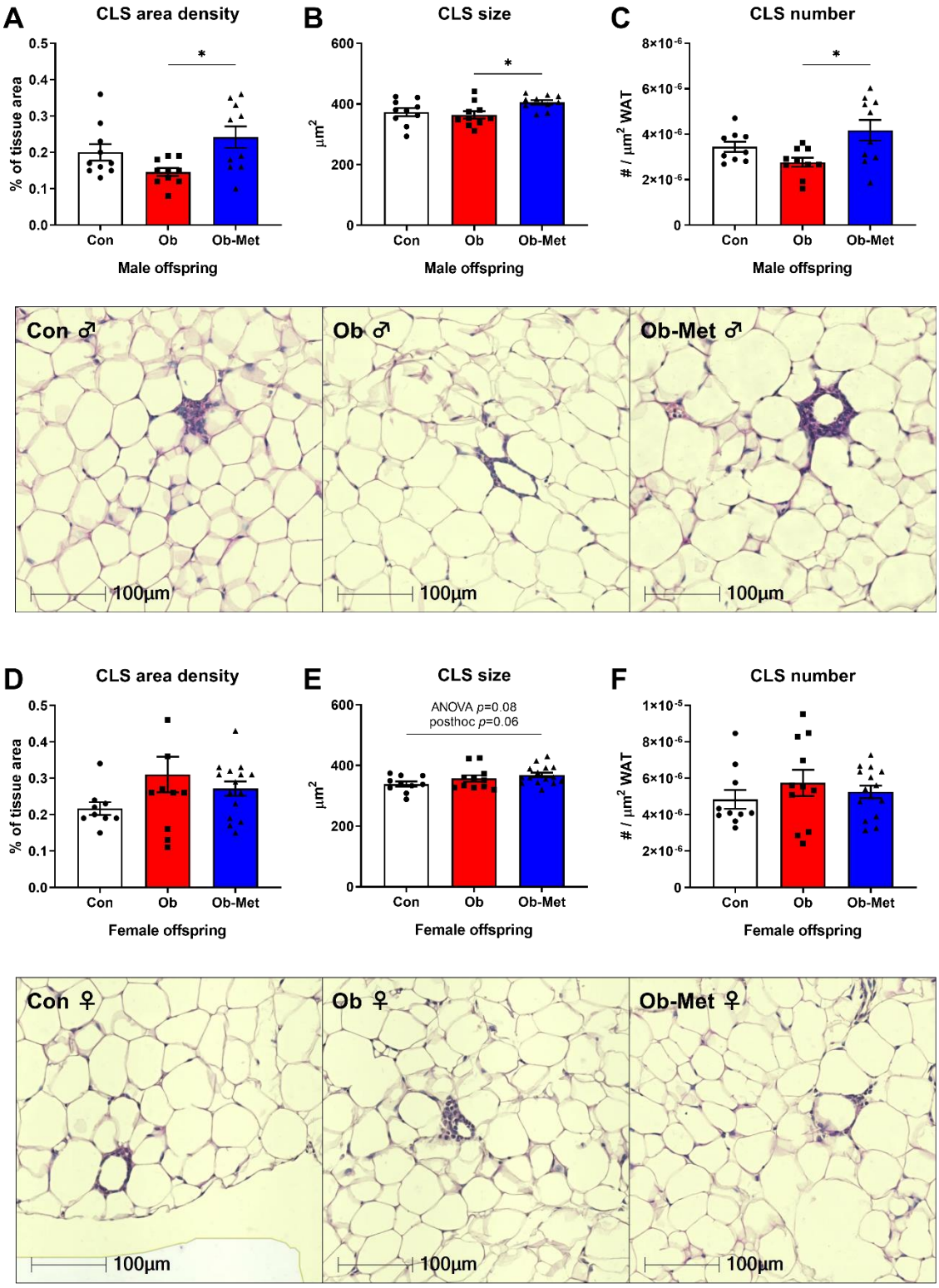

Figure 4.15: Crown-like structures (CLS) in 8-week-old epididymal/gonadal adipose tissue.
A/D) Percentage of WAT tissue area consisting of CLS. B/E) Median size of CLS in the tissue. C/F) Number of CLS per µ² WAT tissue. A-C) male offspring (n=10 in all groups), D-F) female offspring (n=10 Con, n=11 Ob, n=15 Ob-Met offspring).

Male Ob-Met offspring showed significantly higher CLS density in eWAT compared to Ob offspring, associated with an increase in both CLS number and size (Figure 4.15A-C). In female offspring, there were no significant differences in CLS density, size or number (Figure 4.15D-F).

4.3.6.3 Gene expression

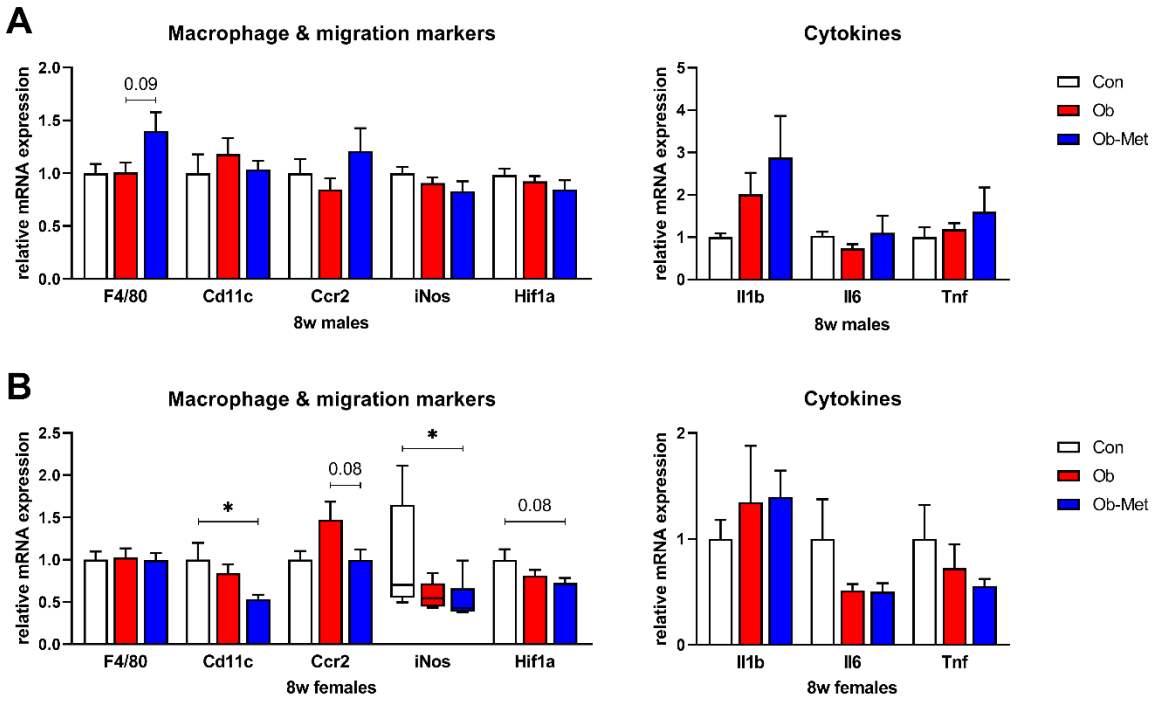

Figure 4.16: Expression of genes related to macrophage infiltration and inflammation in epididymal/gonadal WAT
*Gene expression in A) male and B) female offspring relative to expression of housekeeper genes Sdha, Ppia and Hprt. Data reflect mean ± SEM except for iNos expression in female offspring, where the median [range] is shown. *p<0.05, one-way ANOVA (or Kruskal-Wallis test for iNos) with Tukey's multiple comparison test. Numbers are n=7-8 for Con, n=8-9 for Ob, n=8-9 for Ob-Met males; n=8 for Con (n=6 for Il1b), n=8-9 for Ob, n=9-10 for Ob-Met females.*

Expression of macrophage marker *F4/80* was increased in eWAT of fasted male Ob-Met offspring, although this did not reach statistical significance (p=0.06 one-way ANOVA, Figure 4.16A). Expression of markers for activated M1-type macrophages, immune cell migration or inflammatory cytokines were not different in male offspring (Figure 4.16A). In females, expression of M1-type macrophage markers *Cd11c* and *iNos* were decreased in Ob-Met offspring (Figure 4.16B).

In male offspring, exposure to maternal obesity irrespective of metformin intervention was associated with lower *Adiponectin* expression in eWAT, indicative of local IR (Figure 4.17A). Markers of lipolysis were decreased in male Ob and Ob-Met compared to Con offspring (Figure 4.17A). Female Ob-Met had significantly decreased *Adiponectin* expression compared to Ob offspring, but expression of other WAT-associated genes was not significantly different (Figure 4.17B).

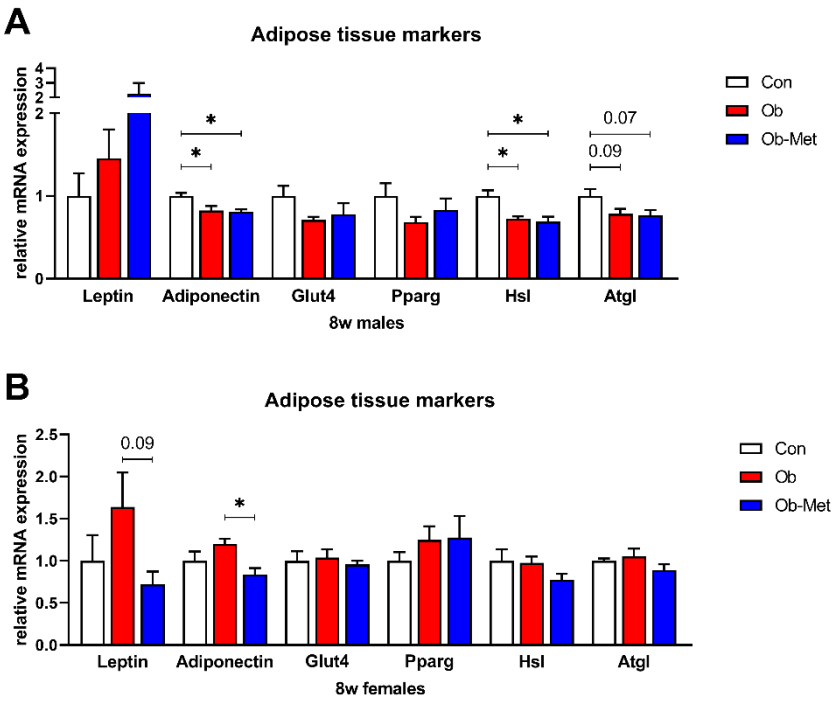

Figure 4.17: Gene expression of WAT markers in epididymal/gonadal adipose tissue of 8-week-old offspring
*Gene expression in A) male and B) female offspring relative to expression of housekeeper genes Sdha, Ppia and Hprt. *p<0.05, one-way ANOVA with Tukey's multiple comparison test. Numbers are n=8 for Con, n=8-9 for Ob, n=9 for Ob-Met males (n=9 for adiponectin); n=8 for Con (n=6 for Atgl), n=9 for Ob, n=10 for Ob-Met females (n=9 for Pparg).*

4.3.7 Correlations

4.3.7.1 Male offspring

Correlations between WAT inflammation and adiposity were assessed to investigate whether macrophage infiltration was related to the degree of adiposity. There was no correlation between CLS data and adiposity or adipocyte size in fed male offspring (not shown). In fasted male offspring, *F4/80* expression significantly correlated with eWAT mass and our proxy of total body WAT (Figure 4.18A-B) but not to body weight (not shown). Next, the relationship between WAT inflammation and metabolic function was assessed. In fasted offspring, *F4/80* mRNA did not correlate to serum insulin or markers of insulin sensitivity (HOMA-IR, *Glut4* and *Adiponectin* mRNA, not shown). Histological measures of CLS density (% area) and adipocyte death (CLS number per WAT area) negatively correlated to fed glucose (Figure 4.18C-D).

4.3.7.2 Female offspring

Macrophage density, CLS number and *F4/80* expression were not related to measures of adiposity in female offspring (not shown). However, there was a significant correlation between CLS size and gWAT weight or adipocyte hypertrophy (Figure 4.19). Inflammatory parameters did not correlate to metabolic health in female offspring (not shown).

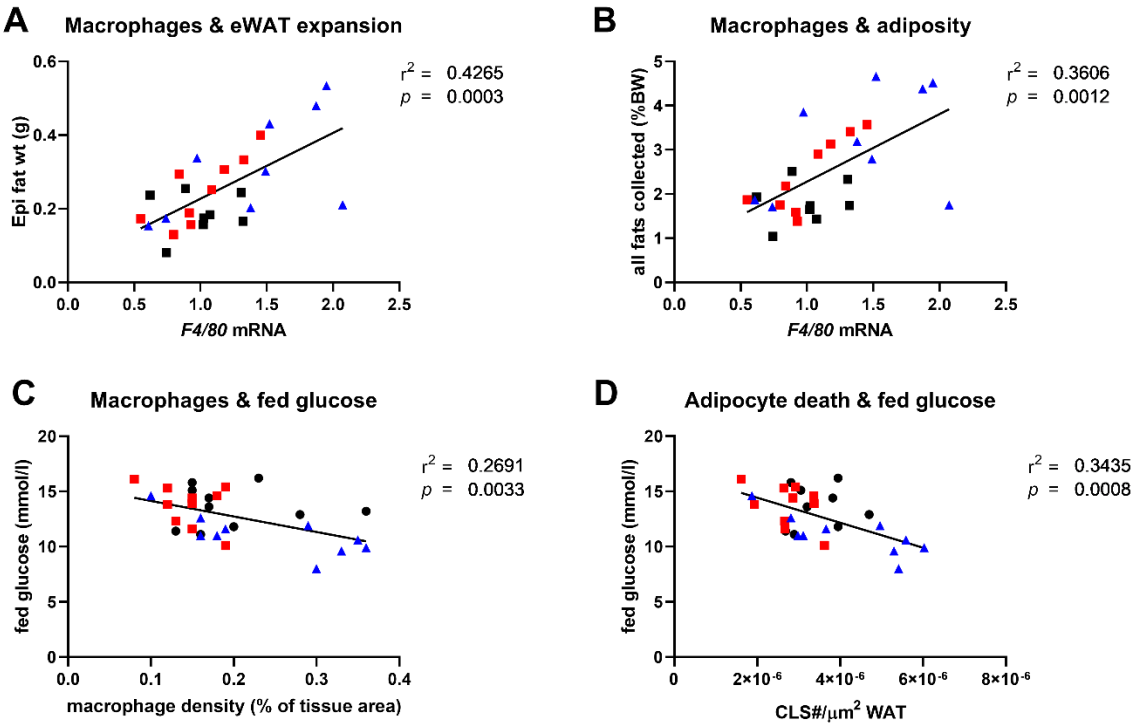

Figure 4.18: Correlations of macrophage invasion with adiposity/glucose homeostasis in 8-week-old male offspring. *Black circles = Con, red squares = Ob, blue triangles = Ob-Met offspring. Numbers are n=36 for panels A-B (n=8 Con, n=9 Ob, n=9 Ob-Met offspring) and n=30 for panels C-D (n=10 in all groups).The r^2 and p-values reflect outcomes of linear regression analysis.*

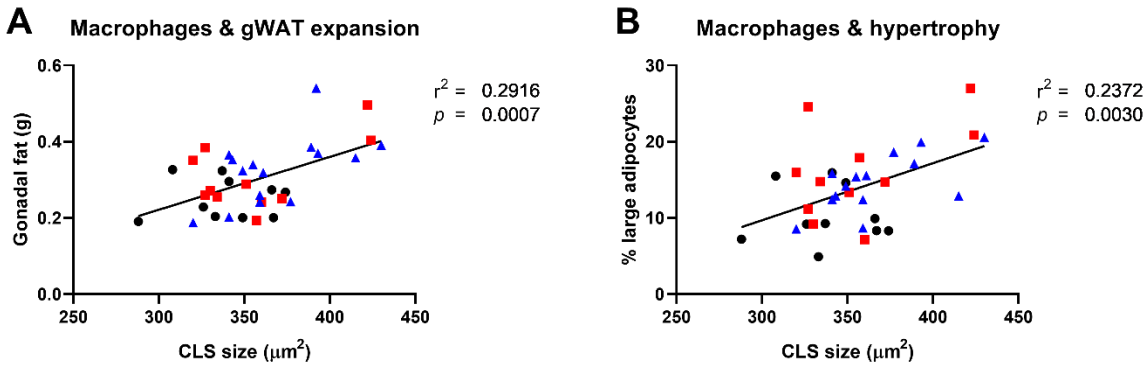

Figure 4.19: Correlations of macrophage invasion with adiposity in 8-week-old female offspring.
Black circles = Con, red squares = Ob, blue triangles = Ob-Met offspring. Numbers are n=35-36 (n=10 Con, n=11 Ob, n=14-15 Ob-Met offspring). The r^2 and p-values reflect outcomes of linear regression analysis.

4.3.7.3 Macrophages are M1 and inflammatory

F4/80 expression in fasted male and female offspring combined significantly correlated to *Cd11c* and *Ccr2* expression, suggesting the WAT macrophages are of migratory (*Ccr2*) and M1 (*Cd11c*) phenotype (Figure 4.20). *F4/80* expression was unrelated to expression of other genes (not shown).

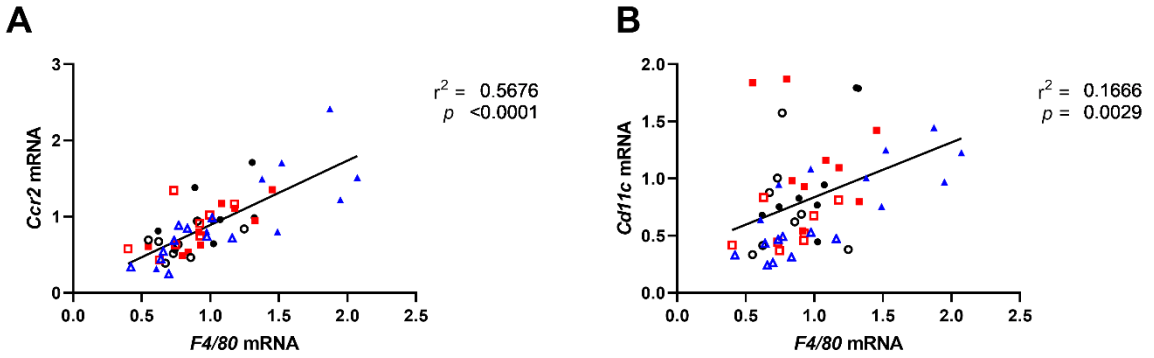

Figure 4.20: Correlations of F4/80 with M1 markers in 8-week-old offspring.
Correlation with A) Cd11c (n=52) and B) Ccr2 mRNA (n=51) in epididymal or gonadal WAT from 8-week-old male (solid symbols, n=8 Con, n=8 Ob, n=9 Ob-Met) and female offspring (open symbols, n=8 Con, n=8 Ob, n=9-10 Ob-Met). Black circles = Con, red squares = Ob, blue triangles = Ob-Met. The r^2 and p-values reflect outcomes of linear regression analysis.

4.3.7.4 Correlations with dam parameters

Correlations with dam parameters were performed to explore what maternal factors could potentially mediate offspring phenotypes. When the entire cohort was included, a relationship existed between maternal E1 body weight and measures of adiposity in fed male offspring (Figure 4.21). However, when only dams on the obesogenic diet were included (Obese and Obese Metformin-treated) there was no correlation. Therefore, offspring adiposity at 8 weeks seems influenced by presence but not necessarily degree of maternal obesity. In female offspring, prevalence of large adipocytes significantly correlated with maternal body weight at all time-points in lactation but not pregnancy, suggesting lactation as a critical period for programming adipocyte hypertrophy (Table 4.10). Similar trends were observed for male offspring but did not reach statistical significance (not shown).

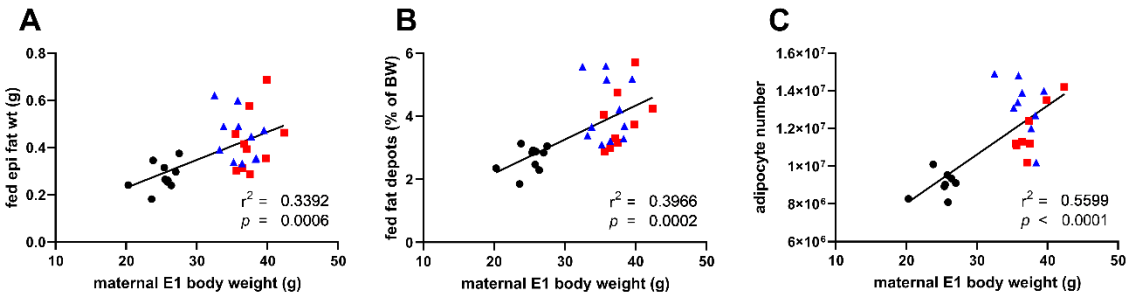

Figure 4.21: Correlation of maternal early pregnancy body weight with male offspring adiposity.
Numbers are n=30-31 for panels A-B (n=10 Con, n=9-10 Ob, n=11 Ob-Met) and n=25 for panel C (n=8 Con, n=8 Ob, n=9 Ob-Met). Black circles = Con, red squares = Ob, blue triangles = Ob-Met offspring. The r^2 and p-values reflect outcomes of linear regression analysis.

Day gestation	slope	r^2	*p*-value	*n*	Day lactation	slope	r^2	*p*-value	*n*
1	0.329	0.091	*0.143*	*27*	**2**	0.357	0.240	***0.006***	*14*
7	0.372	0.089	*0.156*	*26*	**7**	0.402	0.252	***0.003***	*14*
14	0.272	0.055	*0.271*	*26*	**14**	0.462	0.240	***0.004***	*13*
19	0.052	0.002	*0.898*	*13*	**21**	0.579	0.214	***0.007***	*15*

Table 4.10: Correlations of body weight in dams on the obesogenic diet with female offspring adipocyte hypertrophy.
Correlations between dam weight and levels of large adipocytes in gonadal adipose tissue of 8-week-old female offspring from Obese and Obese Metformin dams. The r^2 and p-values reflect outcomes of linear regression analysis.

There was no significant relationship between maternal body weight and male offspring metabolic health (not shown). In female offspring, maternal body weight at E1 correlated positively with HOMA-%B index across the cohort, a relationship that was weakened but maintained when only dams on the obesogenic diet were included (Figure 4.22A-B). Maternal parameters did not relate to measures of WAT inflammation in male offspring, but in female offspring dam body weight at PN2 significantly correlated to CLS size (Figure 4.22C).

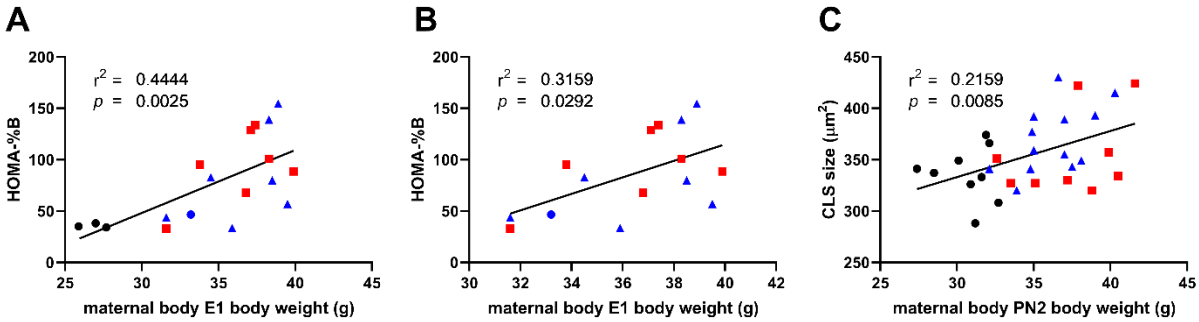

Figure 4.22: Correlation of maternal body weight with female offspring parameters.
Black circles = Con, red squares = Ob, blue triangles = Ob-Met offspring. Numbers are n=23 and n=17 for panels A-B (n=6 Con, n=8 Ob, n-9 Ob-Met) and n=36 for panel C (n=10 Con, n-11 Ob, n=15 Ob-Met). The r^2 and p-values reflect outcomes of linear regression analysis.

Using the daily dose of metformin in mg/kg/d across the entire dosing period as a marker of maternal metformin effect, and daily metformin entering the maternal system during pregnancy in mg/d as proxy of fetal exposure, the influence of the metformin intervention on offspring parameters was assessed. After exclusion of one visual outlier, there was a strong negative correlation between fetal metformin exposure and adipocyte number in male (Figure 4.23A) but not female offspring (not shown). In female offspring there was no relationship between metformin dose and adiposity or metabolic parameters, but maternal metformin dose correlated significantly with *F4/80* and *Ccr2* expression in fasted offspring (Figure 4.23B-C).

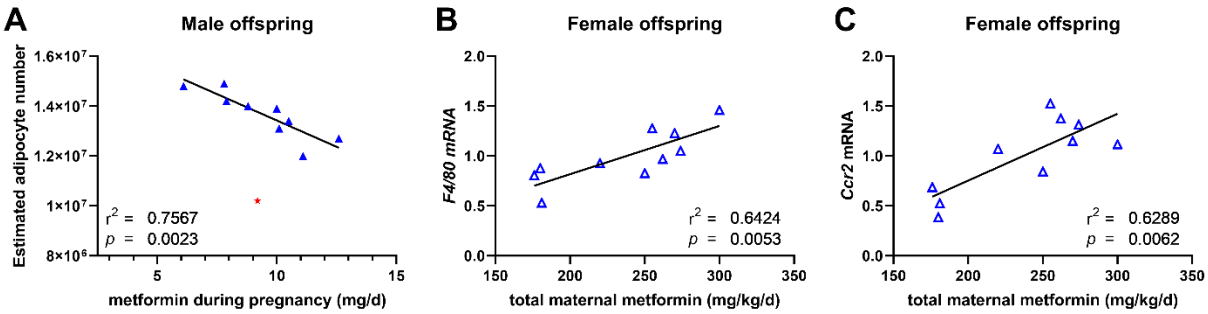

Figure 4.23: Correlations of offspring parameters with metformin dose.
Closed and open triangles refer to male (n=11) and female offspring (n=10), respectively. The red star in panel A refers to a visual outlier. The r^2 and p-values reflect outcomes of linear regression analysis.

4.4 Discussion

4.4.1 Body weight in young animals is unaffected

Despite being larger at 3 weeks, offspring of Obese and Obese Metformin-treated dams quickly normalise their body weight after weaning and no difference between groups was found up to 8 weeks of age. This growth trajectory is consistent with previous observations in male offspring from our maternal obesity model (138). Between 4 and 8 weeks of age, basal intake of the chow diet was unaltered in this study. This is in accordance with previous reports in offspring of obese dams from our lab (116) as well as in offspring exposed to metformin *in utero* (303).

4.4.2 Metformin (and maternal obesity) induce adiposity in offspring

Despite having similar body weights, fed 8-week-old male Ob and Ob-Met offspring had epididymal adipocyte hypertrophy and a consistent increase in absolute and relative weight of all WAT depots compared to controls. Maternal obesity therefore increased offspring adiposity that was not corrected by the metformin intervention. In previous reports from our lab, visceral WAT (epididymal, intra- and retroperitoneal) weight was not different between fasted Con and Ob offspring (138). Accordingly, in the fasted state increased adiposity was not observed in Ob compared to Con offspring, although there was evidence of adipocyte hypertrophy consistent with previous reports (138). In contrast to Ob offspring, the adiposity phenotype in Ob-Met offspring was also observed in fasted offspring with almost all depots (except the retroperitoneal depot) increased in mass compared to controls. BAT weight was not significantly increased, suggesting specific expansion of WAT rather than all adipose tissue types. In female offspring a milder adiposity phenotype was found in fed Ob-Met offspring only, characterised by significantly increased weight of the combined WAT depots, increased intraperitoneal and gWAT weight and hypertrophy of gonadal adipocytes. Together, these data indicate an exaggerated adiposity phenotype in Ob-Met offspring, especially in males.

No increase in whole-body fat mass measured by TD-NMR was found in this study. Although consistent with previous studies finding unaltered total body fat in male or female offspring of obese dams using DEXA (128,133,192), the lack of metformin effect was surprising considering the increased weight of individual depots. However, TD-NMR may not be sensitive enough to detect subtle differences in total body fat mass. Isolated depot weights at post-mortem may therefore be more informative.

This study reports an increased adiposity phenotype induced by the maternal metformin intervention. The mechanisms underlying this phenotype are unclear. No detectable difference in food intake was observed in the current study where animals were fed a chow diet postweaning. However, recent work from our group has shown that offspring from obese dams show hyperphagia when challenged with HFD or following an overnight fast (116). Hence, although basal intake may be unaffected,

regulation of energy balance can still be disturbed in the face of a challenge, likely due to programmed changes in the hypothalamus. Changes in this system could thus underlie the development of adiposity in metformin-exposed offspring. Alternatively, decreased energy expenditure may play a role. Our previous studies showed this was unchanged in offspring of obese dams (112). Although energy expenditure was not assessed in this study, the molecular data did not imply a role for decreased uncoupling as no differences in in WAT browning or *Ucp1* expression were found between groups. This contrasts with a study by Liang *et al.* who found improved thermogenic capacity in BAT with early life metformin intervention (345). However, they provided metformin directly to the pups during lactation, suggesting the developmental window for WAT browning may not be targeted with gestational intervention.

It is possible that adiposity is induced by direct intrauterine metformin exposure. Although timing of differentiation depends on the WAT depot (subcutaneous adipocytes differentiate earlier than visceral adipocytes) and maturation continues into postnatal life, adipocyte lineage commitment in both humans and rodents largely occurs during gestation and could thus be influenced by metformin exposure (434). Moreover, the description of cell-autonomous programming of (pre-)adipocyte dysfunction in our maternal obesity and low-protein models show that programmed effects on early adipocytes can persist into adulthood and consequently have long-lasting effects on offspring health (112,435). Moreover, metformin was shown to introduce epigenetic alterations in fetal tissues as shown by upregulation of the *H19* long non-coding RNA in fetal livers from metformin-treated chow-fed dams, leading to fetal hepatic *Hnf4a* promotor DNA hypo-methylation, increased *Hnf4a* expression and ultimately alterations in hepatocyte differentiation and gene transcription (381). This suggests the intervention may influence the epigenetic signature of developing adipocytes as well.

Metformin has been shown to decrease lipid uptake, maturation and differentiation of mouse and human pre-adipocytes *in vitro* (436). Others report that metformin drives differentiation of human mesenchymal stem cells from the adipogenic to the osteogenic lineage, suggesting direct inhibitory effects on adipocyte lineage commitment (437). It is therefore possible that prenatal metformin disturbs adipogenesis in developing offspring. Accordingly, maternal metformin treatment during pregnancy and lactation corrected the increase in ectopic adipocytes of mice exposed to prenatal HFHS by preventing adipogenesis in skeletal muscle (304). Metformin exposure *in utero* could thus decrease adipocyte number at birth. Exposure to *ad libitum* nutrients in postnatal life may consequently 'overflow' the lipid-storing capacity of WAT leading to elevated circulating lipids, IR, ectopic lipid deposition and lipotoxicity (402). Consistent with this theory, a significant inverse relationship between fetal metformin exposure and eWAT cellularity was observed, indicating that offspring exposed to higher levels of metformin have fewer epididymal adipocytes at 8 weeks of age.

Paradoxically, the exaggerated adiposity in male Ob-Met offspring seemed to result from hyperplasia as evidenced by the significantly higher estimated adipocyte number (but not cell size) compared to Ob offspring at 8 weeks. On first thought, this observation seems to reject the hypothesis that metformin prevents pre-adipocyte formation. However, as mentioned in 4.1.3, early onset obesity is often hypercellular as well as hyperplastic (111,394). The observed increase in epididymal adipocyte number could thus be explained by early postnatal hyperplasia following cessation of metformin treatment to the dam. Moreover, the excessive hyperphagia in lactation displayed by Obese Metformin-treated dams (which was increased compared to both Control and Obese dams) might have stimulated adipogenesis in early postnatal life, when development of most (especially visceral) depots is particularly vulnerable (91,434). The relationship between cellularity and metformin exposure might thus reflect a different starting point from which hyperplasia occurred rather than decreased postnatal hyperplastic capacity, leading to elevated adipocyte number at 8 weeks. Accordingly, postnatal overfeeding induced by maternal HFD in lactation resulted in both hypertrophy and hyperplasia in eWAT (but not subcutaneous WAT) in 6-month-old male rat offspring (91). This was associated with altered epigenetic signature (DNA methylation changes) in epididymal adipocytes that was present in both pre-weaning and adult offspring indicating a programming role for lactational hyperphagia. In female offspring dam weight in lactation (but not pregnancy) significantly correlated to the presence of excessively hypertrophic adipocytes, further suggesting a role for this time-period in programming of obesity in early adulthood.

It is commonly accepted that programming by maternal undernutrition is worsened by postnatal nutrient excess (153). Although the precise molecular mechanism mediating metformin signalling is unclear, the consensus is that it leads to AMPK activation (225). AMPK acts as the major cellular nutrient sensor, and activation by metformin has been suggested to reflect cellular starvation (438). Indeed, metformin induces a starvation-type response in adult mice following 8 weeks metformin treatment that was worse than the caloric restriction model used as comparison (439). We may thus have created a model of IUGR (due to metformin) with catch-up growth (following metformin removal at birth) similar to studies using recuperated offspring following low-protein exposure *in utero* (372). This was also proposed by Salomäki *et al.* who drew comparison between their model of metformin treatment to chow-fed dams and developmental programming by maternal undernutrition (301). Indeed, when looking at studies investigating male offspring adiposity in non-transgenic models, metformin was only detrimental to offspring body composition in situations where this cellular 'starvation' was followed by restored or increased energy balance in lactation, whereas continued metformin treatment or switching to a healthy diet postnatally was associated with protective effects

(Table 4.11). Whether this relates to direct effects of metformin signalling in fetal or maternal cells or indirectly through effects on maternal metabolism is unclear.

Study	Prenatal	Postnatal	Offspring outcome
This report	HFHS + metformin	HFHS	Adiposity on chow
Salomäki et al. 2013 (301)	Chow + metformin	Chow	Adiposity on HFD
Salomäki et al. 2014 (303)	HFD (non-obese) + metformin	Chow	Protects against DIO
Tong et al. 2011 (304)	HFHS + metformin	HFHS + metformin	Protects against DIO

Table 4.11: Overview of studies investigating adiposity in mouse offspring following metformin treatment.
Only studies in wildtype dams included. DIO = diet-induced obesity. HFD = high fat diet. HFHS = high fat high sugar diet.

It is critical that these offspring are followed up into adulthood as IUGR with catch-up growth is associated with poor cardiometabolic outcomes in adulthood in both humans and animal models (reviewed in Chapter 1). However, increased adiposity could also reflect physiological WAT expansion to protect against ectopic lipid deposition (399). Accordingly, Rowan *et al.* suggested that the increased adiposity in metformin-exposed offspring in the MiG trial resulted from expansion of the subcutaneous depot, as no change was seen in visceral fat mass at 2 years of age (298). Subcutaneous depots are less associated with IR than visceral depots and relative expansion of these depots may thus protect against metabolic disturbance. The current study does not show depot-specificity but a global adiposity phenotype in exposed offspring, and both Salomäki *et al.* and this study showed increased weight of visceral depots (301). Although both Ob and Ob-Met male offspring show hypertrophy and hyperplasia compared to controls, adipocyte number was significantly higher in the Ob-Met group. Rather than interpreting this as a sign of more extensive WAT expansion, it could be hypothesised that Ob offspring show insufficient compensatory hyperplasia which is improved by the metformin intervention. Although more research is required to elucidate the cause of adiposity in Ob-Met offspring, the functional consequences can be addressed by the characterisation of WAT function.

4.4.3 Metformin promotes WAT dysfunction in male offspring

Histological evidence of macrophage infiltration was observed in WAT from male Ob-Met offspring, evidenced by increased CLS density. Absolute CLS number was similar between Con and Ob offspring, but section area was larger in Ob offspring (Appendix B). Therefore, Ob offspring WAT has hypertrophied, but at this age this was insufficient to initiate excessive macrophage infiltration leading to a relative decrease in CLS per section. In contrast, Ob-Met offspring showed an increase in absolute CLS number as well as section area (Appendix B). Therefore, both hypertrophy and immune cell recruitment had occurred in Ob-Met offspring by 8 weeks of age, suggesting they are one step further along the WAT inflammation cascade and have progressed to adipocyte dysfunction. This increase in CLS density is driven by both increased CLS number and size. As CLS tend to aggregate around a single necrotic adipocyte, CLS number can be considered a marker of adipocyte death rate (406). Therefore,

Ob-Met offspring show increased adipocyte death compared to Ob offspring. In contrast, CLS size more likely reflects the extent of macrophage recruitment to the necrotic adipocyte, either by recruiting more macrophages or via formation of phagocytic foam cells (404,406). As both histological and molecular data are suggestive of a mild pathological phenotype it is unlikely that the increase in CLS size is attributable to foam cell formation at this age. Therefore, these data indicate that the metformin intervention is associated with initial signs of an inflammatory cascade in male offspring, which is likely to deteriorate as animals age and the phenotype progresses.

Despite increased CLS density, expression of pro-inflammatory cytokines as measured by qPCR were unchanged and mRNA levels were generally low in 8-week-old samples. However, *Cd11c* and *Ccr2* are often co-expressed in obese WAT macrophages (407) and CLS contain preferentially *Ccr2*-expressing and *Cd11c*$^{+}$ monocytes (407,424). Therefore, the correlation of expression of macrophage marker *F4/80* with *Cd11c* and *Ccr2* suggests the macrophages are migratory and of the classically activated M1-type. Perhaps at 8 weeks we are seeing the start of immune cell infiltration where macrophages are recruited but not yet abundant or fully activated. Consequently, the qPCR data may reflect cytokine expression by adipocytes, which is relatively low compared to WAT macrophages that are responsible for the majority of pro-inflammatory cytokine production in obese WAT (403–405). It would thus be interesting to measure TNFα, IL-6 and IL-1β protein in the collected tissue and to perform *ex vivo* cytokine production studies with fresh WAT to investigate the production of pro-inflammatory signals at the protein level. Hotamisligil *et al.* highlighted the role of adipose tissue as a whole regarding cytokine production. The authors proposed that since mRNA expression is measured per unit tissue it is only reflective of the expression in that number of cells, and therefore any increase in overall adipocyte number may increase the total amount of *Tnf* mRNA and protein produced by the tissue (410). Since Ob-Met males show increased adipocyte number, net production of *Tnf* and other pro-inflammatory factors may still be increased despite relative mRNA expression being unchanged.

Hypertrophic WAT expansion is considered a major initiator of macrophage infiltration. It is unclear whether this was the main driver in this cohort, as CLS content did not correlate with eWAT weight. This is not unexpected since hypertrophy is a better determinant of inflammation than adiposity per se. However, hypertrophy was not different between fed Ob and Ob-Met offspring suggesting inflammation may also be driven by something else in Ob-Met offspring. In contrast to CLS, *F4/80* offspring in fasted animals correlated with WAT weight and our proxy of total body adiposity, suggesting a role for adiposity in the fasted state. Additionally, excessive liberation of FFAs from obese adipocytes is thought to contribute to pro-inflammatory signature, directly via activating Toll-like receptors and indirectly by upregulating cytokine production from WAT (405). Contrastingly, expression of the lipolytic enzyme *Hsl* was downregulated in Ob and Ob-Met offspring suggesting

decreased rather than increased lipolysis. Although excessive FFA liberation is a feature of more pronounced obesity, initial stages of adiposity depend on adipogenesis and can be associated with downregulated lipolysis (170). Despite decreased lipolytic capacity of individual adipocytes reported in obesity, net lipolysis can still be increased due to the increase in total fat mass (401).

Ob and Ob-Met offspring had decreased expression of *Adiponectin* mRNA in eWAT. Both circulating and WAT levels of adiponectin are inversely related to macrophage content and pro-inflammatory cytokines (405,418), and adiponectin has direct anti-inflammatory effects on macrophage polarisation and recruitment (429,430,440). However, since *Adiponectin* expression was not different between Ob and Ob-Met offspring despite macrophage recruitment being upregulated exclusively in Ob-Met, its involvement in inducing macrophage infiltration in this study is unclear. Adiponectin has potent insulin sensitising as well as anti-inflammatory properties (5), therefore its decreased expression in Ob and Ob-Met male offspring could lead to eWAT IR. WAT inflammation is closely associated with local IR (441), posing the question if the inflammatory phenotype of Ob-Met WAT may underlie changes in metabolic function. However, no correlation between *F4/80* and insulin or HOMA-IR was observed in male offspring. This is not surprising as WAT inflammation is known to precede systemic hyperinsulinaemia (404).

4.4.4 WAT function in female offspring

In female offspring, no pro-inflammatory phenotype was observed. Because the adiposity phenotype was also milder in female offspring, it seems female offspring are relatively protected against the WAT effects of the metformin intervention at this age. However, levels of *F4/80* and *Ccr2* correlated with the degree of metformin exposure suggesting the intervention may have influenced gWAT inflammation to some extent. Interestingly, although macrophage content measured by CLS density and *F4/*80 expression was not different, Ob-Met females show decreased expression of markers of M1-type macrophage activation (*Cd11c* and *iNos*). This suggests that in contrast to male offspring, the metformin intervention is associated with a decreased pro-inflammatory state in female Ob-Met offspring where the majority of gWAT macrophages may not be classically activated. This could reflect a higher proportion of tissue-resident macrophages in WAT as these are generally $Cd11c^{-}$ (407).

Ob-Met females showed exaggerated loss of fat mass after an overnight fast. Induction of lipolysis (whether through pharmacological stimulation or prolonged fasting) has been shown to cause expansion of $Cd11c^{-}$ M2-type macrophages in rodents (442,443). Increased lipolysis in Ob-Met female offspring could thus cause both weight loss and a rise in M2-type macrophages. However, there were no significant changes in the expression of lipolytic enzymes or serum FFAs in fasted female offspring. Nevertheless, lipase activity is partly regulated at the protein level and changes in FFA liberation from

WAT without altered mRNA expression have been shown before (443). In fasting conditions FFAs are the primary source of energy for peripheral tissues such as skeletal muscle and WAT (414), therefore it is not inconceivable that lack of difference in serum levels reflects increased peripheral FFA uptake and drainage from the circulation. Exaggerated fat mass loss was observed in gWAT and total WAT but not subcutaneous WAT, suggesting this phenotype is confined to visceral depots. Since FFAs from subcutaneous WAT are thought to contribute more to serum FFA concentration than visceral WAT (444,445), increased gWAT lipolysis need not necessarily be accompanied by elevated serum FFAs.

Regardless of the mechanism, putative expansion of the 'healthy' resident macrophage population may be beneficial, as M2-macrophages may phagocytose excess lipid in states of lipolysis and are associated with improved WAT insulin sensitivity (441,442). Indeed, whole-body insulin sensitivity was improved in female Ob-Met offspring (see 4.4.5). In contrast, *Adiponectin* expression was downregulated, indicating local reduction in gWAT insulin sensitivity. Metformin-mediated suppression of adiponectin production has been shown in mature 3T3-L1 cells and subcutaneous WAT from neonates exposed to HFD and metformin *in utero* (303,446). Further experiments, such as the measurement of M2 markers in female gWAT, are required to characterise the activation state of macrophages in female gWAT and its relation to local insulin sensitivity. Lastly, macrophage content in female offspring may not be associated with WAT dysfunction at 8 weeks of age, as inflammatory parameters failed to correlate with offspring metabolic parameters. Instead CLS size only correlated with gWAT expansion and our proxy of total fat mass. Therefore, CLS size may simply reflect the increased degree of adiposity in fed females.

4.4.5 Maternal metformin affects metabolic health in a sex-specific manner

The finding that maternal metformin intervention during obese pregnancy leads to altered WAT function and immune signature could have implications for offspring metabolic health. In this study, no significant differences were found in serum lipids. This is in accordance with previous reports from our model in male offspring (128,132,138). In contrast, glucose homeostasis was affected by the metformin intervention in both male and female offspring, but in a sex-specific manner.

There were no overt differences in glucose tolerance in this study. This is consistent with reports in male offspring from our maternal obesity model following a 4-hour or overnight fast (112,126). However, previous work from the group described increased fasting insulin in 8-week-old male offspring of obese dams, which contrasts with our findings (112,133,136). The reported insulin levels for offspring of control and obese dams were 50 and 100pmol/l, respectively. In our study, values for Con offspring were similar (50 ± 6pmol/l) but male Ob offspring showed lower fasting insulin than previously reported (67 ± 6pmol/l). The discrepancy in insulin levels between this work and previous

studies is likely related to the significantly lower fasting glucose in Ob and Ob-Met compared to Con offspring. Fasting glucose was not different in previous work using our model, presumably caused by slight cohort differences in both Con (6.5 versus 7.2mmol/l) and Ob (6.2 versus 5.9mmol/l) offspring glucose (138). Fed glucose levels in the current study were comparable to previous reports (126). There was no significant difference between Con and Ob offspring in systemic (or hepatic) insulin sensitivity as measured by HOMA-IR, consistent with previous reports (129). As local IR often precedes whole-body IR (112), the lack of significant difference in HOMA-IR is not surprising.

HOMA-modelling showed that male Ob-Met offspring had increased pancreatic β-cell activity in absence of changes in whole-body insulin sensitivity. It is unclear what mechanisms underlie the increased β-cell action in male Ob-Met offspring. Metformin could have directly affected the developing pancreas, for instance by introducing epigenetic alterations as is reported for fetal mouse livers (including *Hnf4a* promotor DNA hypo-methylation as previously described)(381). Gregg *et al.* showed that *ex vivo* metformin treatment increases proliferation of progenitor cells in pancreatic buds from murine E13.0 fetuses, leading to enhanced bud size (331). *In vivo,* the intervention increased pancreatic bud size, total cell number and number of endocrine progenitors at E14, consequently leading to increased β-cell fraction and decreased blood glucose at birth (331). Metformin could therefore have directly increased insulin secretory capacity by increasing β-cell mass and improving calcium flux. Intriguingly, the authors saw a similar phenotype (improved glucose tolerance via improved insulin secretion without changes in insulin sensitivity) in 8-week-old male offspring whose mothers were given metformin during lactation (307).

Ob-Met females showed a significant reduction in HOMA-IR indicative of improved insulin sensitivity. Improved insulin sensitivity in female metformin-exposed offspring was also found during an insulin tolerance test in the aforementioned model of lactational treatment, although interestingly HOMA-IR was not different (307). In contrast, insulin sensitivity was unaffected in the gestational metformin study by the same group (447). In obese pregnancy, treatment with metformin during both pregnancy and lactation did not affect the hyperinsulinaemia in female offspring of obese dams, suggesting no intervention effect on glucose/insulin homeostasis in that model (359). The Salomäki studies also reported no difference in glucose tolerance during chow diet-feeding in early adulthood, although insulin sensitivity was not assessed (301,303). Our finding of improved insulin sensitivity in female offspring exposed to metformin *in utero* is therefore novel. Interestingly, this phenotype occurred despite decreased *Adiponectin* expression in gWAT (marker of local IR). Perhaps other peripheral organs, such as liver or muscle, are affected by the intervention and consequently drive the systemic improvement of insulin sensitivity observed in Ob-Met females. The hypothesised alternative activation of macrophages in Ob-Met gWAT may also play a role.

4.4.6 Sex differences

The metformin intervention had sex-specific effects in this study. Although both male and female Ob-Met offspring show adiposity in the fed state, fasting exaggerated the male phenotype but prevented increased adiposity in female Ob-Met offspring. The adiposity phenotype is also stronger in males with more drastic and significant upregulation of fat depot weights in both Ob and Ob-Met offspring. Male offspring may have an inherent propensity to develop adiposity following developmental insults. Male rats reared in small litters (model of early postnatal overnutrition) show exaggerated body weight gain from weaning to 7 weeks as well as hyperleptinaemia, which was not observed in females (448). Similarly, weight gain was accelerated in male but not female 4-month-old mice exposed to gestational HFHS-feeding when given HFD postnatally (449). Notably, although body weight in 8-week-old wild-type offspring of GDM-like Lepr$^{db/+}$ dams was increased only in males, WAT depot weight and adipocyte size were increased in both sexes (84) suggesting programmed hypertrophy can occur in absence of obesity which is consistent with the current study. In the same Lepr$^{db/+}$ model, 24-week-old female offspring had developed obesity but male body weight was no longer different (85) proposing adiposity in female offspring may be delayed rather than prevented. Indeed, several studies including those using the same model as our laboratory showed that both sex and age influenced the presence of obesity (87,450). Although in these studies the body weight increase remained less drastic in females, this highlights the importance of studying developmental programming at various offspring ages (metformin-exposed females might develop overt adiposity at a later age than males). In contrast, there are also reports of females developing adiposity before male offspring, for instance in a study of maternal high fructose feeding where females and males became obese at 4 and 7 weeks of age, respectively (451).

WAT function was also different between sexes in this study. While adiposity in males was driven by both hypertrophy and hyperplasia (especially in Ob-Met offspring), in females only hypertrophy was observed. Hyperplasia might follow if females develop more severe adiposity at later ages. Female offspring also showed drastically increased WAT browning and *Ucp1* expression compared to male offspring. This phenotype is most commonly associated with white-to-brown transition of mature cells to form 'beige' adipocytes, leading to increased uncoupling of mitochondrial respiration and thermogenesis (452). Accordingly, prolonged HFD-feeding increased presence of BAT-like regions and *Ucp1* expression in visceral WAT in female mice only, which was associated with protection against DIO and IGT (453). Upregulation of *UCP1* expression was also seen in subcutaneous WAT from women compared to men and was associated with increased metabolic rate per kg fat mass independent of sex steroids, HOMA-IR or waist:hip ratio (454). The finding of increased WAT browning in female offspring in the current study may thus partly explain the milder adiposity phenotype.

The metformin intervention affected WAT inflammatory signature in a sex-specific way. Male Ob-Met offspring had increased macrophage infiltration in eWAT, associated with larger CLS surrounding more adipocytes. In contrast, female offspring showed no difference in macrophage density but instead a down-regulation of pro-inflammatory macrophage markers. Hence, male Ob-Met offspring showed a more pro-inflammatory phenotype than female offspring in this study. This is consistent with reports that basal cytokine and chemokine expression is higher in male compared to female WAT (443), which was also seen in this study. Furthermore, WAT inflammation (CLS and cytokine production) occurred readily in DIO males but female mice required a second hit such as stimulation of lipolysis before seeing drastically increased macrophage accumulation (443). Many studies also indicate increased susceptibility to (WAT) inflammation in males in a programming context. Six-month-old male offspring of LPS-treated or HFD-fed dams have increased hypothalamic inflammation and circulating leukocytes, despite females showing a more severe metabolic and adiposity phenotype at this age (455). Similarly, maternal HFD initiated at conception led to macrophage invasion in chow-fed male offspring but female offspring required postnatal HFD to show signs of inflammation (*Tnf, Cd11c* and *Mcp-1* expression). Even then, no significant upregulation of *Il-6* or CLS formation was seen and expression of *Cd11c* and *Mcp-1* remained lower than in HFD-fed males (141). Anti-inflammatory actions of oestrogen in female offspring are suggested as one explanation for this sexual dimorphism (141,306).

Most other studies investigating long-term effects of early metformin exposure only investigate one sex (304,345,359), while others find minimal sexual dimorphism in intervention effect in early adulthood, and suggest sex differences emerge when animals have reached older age (301,303). The only exception is a study in OE-NPY dams. In this study gestational metformin is associated with adiposity and glucose intolerance in females, while male offspring had lower body weight compared to controls (302).

4.4.7 Strengths and limitations

This study provided extensive adipose tissue and metabolic phenotyping in offspring of obese dams treated with or without metformin. Unlike most programming papers, both male and female offspring were included. Moreover, phenotyping was performed in both the *ad libitum* fed and overnight fasted state which facilitated investigation of differential effects with nutritional status, which has not been done before with prenatal metformin. To our knowledge this is also the first study addressing WAT inflammation in a metformin intervention model. Another strength is the HALO image analysis software used for analysis of WAT histology. The whole section approach (rather than selected images) allows for unbiased detection of rare events, such as adipocyte and CLS sizes at the edges of the spectrum. This could explain the slight difference in adipocyte size between ours and previously published studies (138), although the observation of a similar adipocyte size range (200-6000µm^2) is

reassuring and suggests ours is a more accurate approach. Lastly, a major strength of this study is the clinical relevance of the study design. Although metformin intervention in chow-fed pregnancy is common in rodent models, in clinical practice metformin is only prescribed to pregnant women with GDM. Our model of metformin intervention in obese glucose intolerant pregnancy with a clinically relevant dose of metformin is therefore more translational to the human situation.

There are a few limitations to this study. Since differences between the fed and fasted state in this study were unexpected, fixed tissues for hypertrophy and CLS measurements were not collected in the fasted state and similarly, fed tissues were not frozen for molecular analysis. This prevented the investigation of relationships between molecular and histological data, such as those between leptin expression and cell size or CLS and pro-inflammatory gene expression. Lastly, the work for this thesis was produced during the COVID-19 pandemic. Periods of government-enforced departmental closure, quarantine and self-isolation prevented laboratory access for several months. For this reason, qPCR experiments for inflammatory phenotypes were prioritised over Western Blotting for insulin signalling and other adipocyte markers, which is why this data is missing from the thesis.

4.5 Conclusion

Metformin intervention during obese pregnancy induces adiposity and WAT hypertrophy in exposed 8-week-old offspring prior to development of obesity. This is more prominent in male offspring, who also show adipocyte hyperplasia, WAT inflammation and local IR. However, male metformin-exposed offspring also show increased β-cell output which may be protective against glucose intolerance programmed by maternal obesity in young adult life. In female offspring, metformin exposure is associated with an improvement in systemic insulin sensitivity, altered WAT response to fasting and alternative macrophage activation in gWAT. As it is unsure how these changes relate to long-term health, it is imperative that these offspring are followed up into later adulthood to determine potential sex-specific long-term effects on adiposity and metabolic health.

4.6 Key findings

- Gestational metformin treatment induced adiposity in 8-week-old exposed offspring
- Metformin exposure caused eWAT dysfunction in male offspring, characterised by hypertrophy, hyperplasia, inflammation, insulin resistance and altered lipolysis
- In female offspring metformin led to improved systemic insulin sensitivity and alternative macrophage activation in adipose tissue
- Follow-up of metformin-exposed offspring is required to determine consequences of the intervention for metabolic health

5 Longitudinal adiposity and metabolic health

5.1 Introduction

5.1.1 Obesity and T2DM are age-related diseases

Prevalence of non-communicable diseases such as obesity, T2DM and CVD increase with advancing age. Furthermore, even 'normal' ageing in humans is associated with progressive increases in body weight, (predominantly visceral) fat mass and IR suggesting a causative role of the ageing process (456,457). Certain aspects of ageing, such as dysregulation of epigenetic modifications, cellular senescence, chronic inflammation and oxidative stress have been implicated in the pathogenesis of age-related diseases (458). Some of these processes are also thought to contribute to developmental programming as a consequence of maternal obesity and other *in utero* insults (reviewed Chapter 1), suggesting potential mechanistic overlap between the increased risk of cardio-metabolic disease with ageing and developmental insults.

5.1.2 Programming becomes more deleterious with age

Programmed differences in adiposity and glucose tolerance have previously been shown to become more apparent in older offspring. For instance, male rat offspring of low protein-fed dams had improved muscle insulin sensitivity at 3 months but displayed skeletal muscle IR and glucose intolerance by 15 months of age (459,460), suggesting compensation at a young age was insufficient to prevent disease later in life. By 17 months of age these offspring had progressed to T2DM (461) again showing age-related worsening of the programmed phenotype. Similarly, progressive dysregulation of lipid metabolism and increased hepatic lipid accumulation with age (3-18m) was demonstrated in a "recuperated" rat low protein model where offspring of low-protein fed dams were cross-fostered to control-fed dams during lactation (462).

Increased age also affects outcomes in models of maternal obesity or overfeeding. Male mouse offspring of DIO dams showed progressively increasing differences in body weight as well as hyperleptinaemia and hyperglycaemia at 6 months, which were not present at one month of age (463). Similarly, female offspring of non-obese HFD-fed rats showed no difference in body weight or serology at 80 and 180 days but had developed obesity, hyperglycaemia and dyslipidaemia by 360 days of age (117). A study using the same mouse model as this thesis demonstrated deterioration of insulin secretion in male offspring of obese dams between 3 and 6 months of age, indicating ageing was required for progression from glucose intolerance to T2DM in this model (87). Similarly, maternal HFD-feeding from 2 weeks pre-conception led to progressively increased glycaemia with age during an ipGTT challenge in male and female mouse offspring compared to controls (120,121). Both basal and stimulated hepatic glucose output was increased throughout life but drastically increased

between 12 and 24 weeks of age in female offspring of HFD-fed dams, clearly showing an ageing effect (120). A different murine study of maternal HFD-feeding without overt obesity found glucose intolerance and IR in female offspring at 9 but not 6 or 12 months of age (464), stressing the importance of investigating several time-points across the life-course.

If the ageing process itself adds to the incidence of obesity and metabolic disease, then insults that increase 'cellular ageing'-like processes could contribute to disease incidence as well. Accordingly, markers of accelerated ageing such as excessive telomere shortening and decreased lifespan have previously been reported in the recuperated rat low protein model (465–467). Accelerated ageing was also evident in female mouse offspring of dams fed a HFD from one week pre-mating: offspring were heavier as adults (12 weeks) but reached their maximum weight sooner and lost relatively more fat mass with further ageing when compared to offspring of control-fed dams (89). They also showed increased prevalence of senescence-associated T-cells as well as hepatic inflammation and fibrosis at 70 weeks of age (89), indicative of immunological ageing. In another study of maternal overfeeding, increased fur thinning and markers of osteoporosis were indicative of accelerated ageing in female offspring of HFD-fed mice (93).

5.1.3 Maternal obesity and aged offspring

Few studies have investigated the effects of maternal obesity (rather than gestational HFD-feeding) in offspring aged for 12 months or longer. A seminal study in rats by Rodríguez-González *et al.* showed that both male and female offspring of obese dams showed excessive age-related adiposity, IR, hyperinsulinaemia, hyperleptinaemia and fatty liver disease associated with oxidative and nitrosative stress (468). Moreover, age-related patterns of worsening phenotypes were initiated earlier in these animals indicative of accelerated ageing accompanied by shortened lifespan (468). Desai *et al.* also found obesity upon longitudinal follow-up of male offspring of HFD-fed overweight dams. In their study, male offspring were born macrosomic and showed basal hyperphagia and accelerated body weight gain throughout life, leading to increased adiposity at 12 months of age (469). Glucose tolerance and WAT function were not assessed in either study.

Data from our lab showed that although body weight, fat mass and glucose tolerance were not altered in male offspring of diet-induced obese dams at 8 weeks of age (112,128,129,132,133,138,139), male offspring developed increased adiposity and metabolic abnormalities by 6 months of age (470). These 6-month-old offspring showed eWAT expansion with adipocyte hypertrophy, altered lipogenic enzymes, ER stress, systemic and eWAT IR as well as glucose intolerance following a 4h-fasted ipGTT (470). Further longitudinal studies in male offspring from the laboratory showed that maternal obesity increased body weight after 20 weeks of age, although fat mass was increased at earlier ages. These

animals also showed increased lipid deposition in the liver and alterations in serum lipids at 12 months of age, although glucose tolerance and insulin homeostasis were no longer different (Vales Mennitti *et al.* submitted for publication). These data clearly demonstrate a role for ageing in our model of maternal DIO and stress the importance of phenotyping across the life-course. However, WAT function has not previously been assessed in male offspring beyond 6 months of age. Moreover, any long-term effects in our model of maternal obesity on female offspring beyond 8 weeks are unknown.

5.1.4 Long-term follow-up of metformin interventions is scarce

As mentioned in Chapter 4, long-term follow-up of human trials investigating metformin use in pregnancy is limited by the age of the exposed offspring, and long-term outcomes will not be reported on for many years. Most animal studies into metformin intervention effects (reviewed in Chapters 3-4) focus on maternal, fetal and neonatal outcomes, with few investigating offspring beyond weaning. Only two groups carried out longer-term follow-up of offspring exposed to metformin *in utero.*

In their studies on gestational metformin exposure in wild-type mouse models, Salomäki *et al.* followed up offspring until 4 months following a postnatal HFD challenge initiated at 10 weeks of age (301,303). Offspring exposed to metformin during chow-fed pregnancy exhibited hypercholesterolaemia (females), hyperglycaemia, glucose intolerance, WAT IR (males), adiposity and hepatomegaly (both males and females) by 20 weeks of age (301). In contrast, when the metformin intervention was given during HFD-fed pregnancy, female offspring gained less weight on HFD (associated with adipocyte hypotrophy and decreased leptin), whereas male offspring had lower serum lipid levels (303). Moreover, maternal metformin treatment prevented the increase in glucose excursion during an ipGTT in offspring of overnourished dams after 6 weeks of HFD-feeding when compared to the ipGTT performed prior to initiation of HFD at 10 weeks of age (303). In the study of gestational metformin treatment using OE-NPY dams, offspring were provided with regular diet until 5 months of age followed by HFD for a further two months (302). Female offspring already showed diverging body weight with age during the regular diet phase, but HFD exposure and/or further ageing post 5 months of age was required for this difference in body weight to become significant. In contrast, the IGT in 4-month-old metformin offspring disappeared after two months of HFD-feeding as a result of ageing/HFD-related increased glycaemia in both control and metformin groups. Ultimately, 7-month-old female metformin offspring showed increased adiposity but no metabolic abnormalities except for elevated serum cholesterol (302). Male offspring had a different phenotype: chow-fed metformin-exposed males were lighter than controls from 18 weeks of age. In the HFD phase metformin males continued to gain less weight than controls leading to decreased body weight at 7 months of age accompanied by improved insulin sensitivity (302). Although in these studies the adverse [chow-fed pregnancy (301) & female OE-NPY (302)] or beneficial [HFD in pregnancy only (303)

& male OE-NPY (302)] effects of prenatal metformin intervention on offspring adiposity and metabolic health emerged only after 10/20 weeks of age, it is unclear what changes were due to ageing or postnatal HFD, respectively. Further studies are thus required to determine the long-term consequences of metformin intervention in these models.

The longest follow-up of metformin-exposed rodent offspring to date was carried out by Gregg *et al.* When provided during chow-fed pregnancy, metformin intervention improved glucose tolerance and insulin secretion in adult male offspring by 6 and 10 weeks of age, respectively. These effects persisted but became less pronounced with age: glucose tolerance at 9 months of age was less markedly improved, and while *in vitro* insulin secretion remained enhanced in 3-month-old islet explants, the *in vivo* insulin response to ipGTT was no longer significant at 6 months (392). In contrast, while the metformin intervention did not affect young female offspring, 15- and 18-month-old animals showed improved glucose tolerance that disappeared by 2 years of age (392), indicating sex-specific interactions between ageing and prenatal metformin effects. The authors also investigated the effect of lactational maternal metformin intervention on aged offspring (307). In this study, 2-month-old male offspring had decreased body weight and improved glucose tolerance compared to controls, associated with enhanced insulin secretion but not increased insulin sensitivity. When aged on a chow diet, body weight was no longer different but offspring showed eWAT hypotrophy (5 months) in the absence of changes in eWAT insulin signalling (5 months) or insulin secretion (12 months of age)(307). Female offspring of dams treated with metformin during lactation showed improved insulin tolerance at two months of age (in absence of changes in fasted glucose, insulin or glycaemia during GTT), but no difference in gWAT insulin signalling was observed at 12 months of age (307). These data suggest dilution of metabolic effects of prenatal metformin on (especially male) offspring with advanced age.

Although interesting from a scientific perspective, these studies involve transgenic models, maternal chow-feeding or switching from HFD to chow diet at parturition, and thus do not mimic the human situation. Therefore, long-term follow-up in a clinically relevant model of maternal metformin intervention is required to address concerns of long-term safety of metformin treatment in pregnancy.

5.1.5 Aims of the chapter

Ageing is associated with cardiometabolic disease risk and has been shown to contribute to the development of the programmed phenotype. It is therefore vital that early life exposed offspring are followed up throughout life to assess relevant outcomes that may be affected by advancing age. This chapter therefore aims to elucidate the effects of maternal obesity and maternal metformin intervention on adiposity, WAT function and metabolic health of male and female offspring in a longitudinal study up to 12 months of age.

5.2 Methods

5.2.1 Animal model

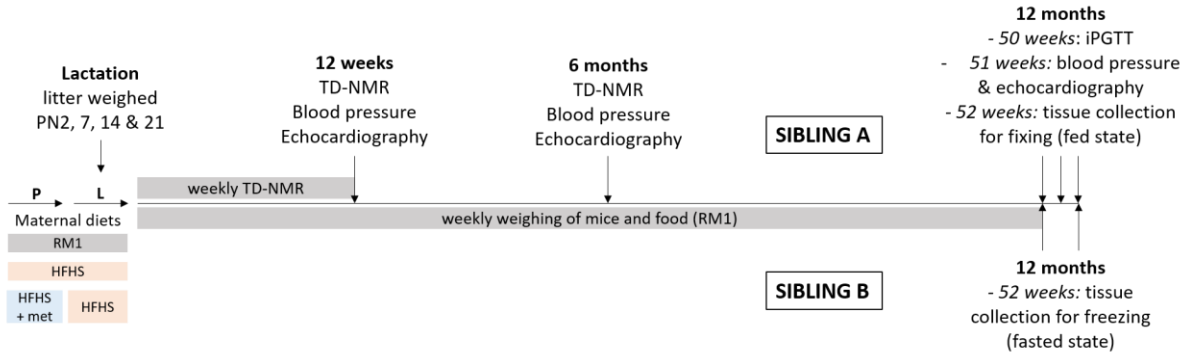

Figure 5.1: Animal model for 12-month-old offspring.
Data from body composition assessment and metabolic phenotyping is presented in this chapter. Data from cardiovascular phenotyping will be presented in Chapter 6. Total n-numbers are n=68 males from 37 independent litters (Con 12 fed, 12 fasted; Ob 13 fed, 8 fasted; Ob-Met 12 fed, 11 fasted siblings) and n=71 females from 36 independent litters (Con 12 fed, 12 fasted; Ob 12 fed, 12 fasted, Ob-Met 12 fed, 11 fasted siblings).

Offspring were generated according to previous chapters. For each sibling pair, one animal was designated for *in vivo* phenotyping and collection of fixed tissues (sibling A) and one was left untouched until tissue collection following an overnight fast (sibling B, Figure 5.1). Body weight and food intake was recorded weekly and body composition was assessed by TD-NMR as previously described. An ipGTT was performed at 11.5 months (see section 2.2.2). At 12 months of age, animals were culled via Schedule 1 (rising CO_2 concentration for fasted offspring, cervical dislocation for fed offspring[15]). At post-mortem, adipose tissue depots and other tissues were excised and either snap-frozen (fasted) or fixed in formalin (fed tissues). Breeding was continued until cardiovascular phenotyping (Chapter 6) was performed in *n*=12 animals and fasted tissue was available for at least *n*=8 offspring in each group.

5.2.2 Serum analysis

Serum analysis was performed according to methods described in section 2.4.1.

5.2.3 Adipose tissue histology

H&E-stained epididymal and gonadal WAT sections[16] were analysed for cell size (Figure 5.2) and presence of CLS (Figure 5.3) using the Indica Labs HALO image analysis software as outlined in section 2.5.

[15] Cervical dislocation by T. Ashmore or P. Wilsmore
[16] Histological processing expertise provided by T. Ashmore

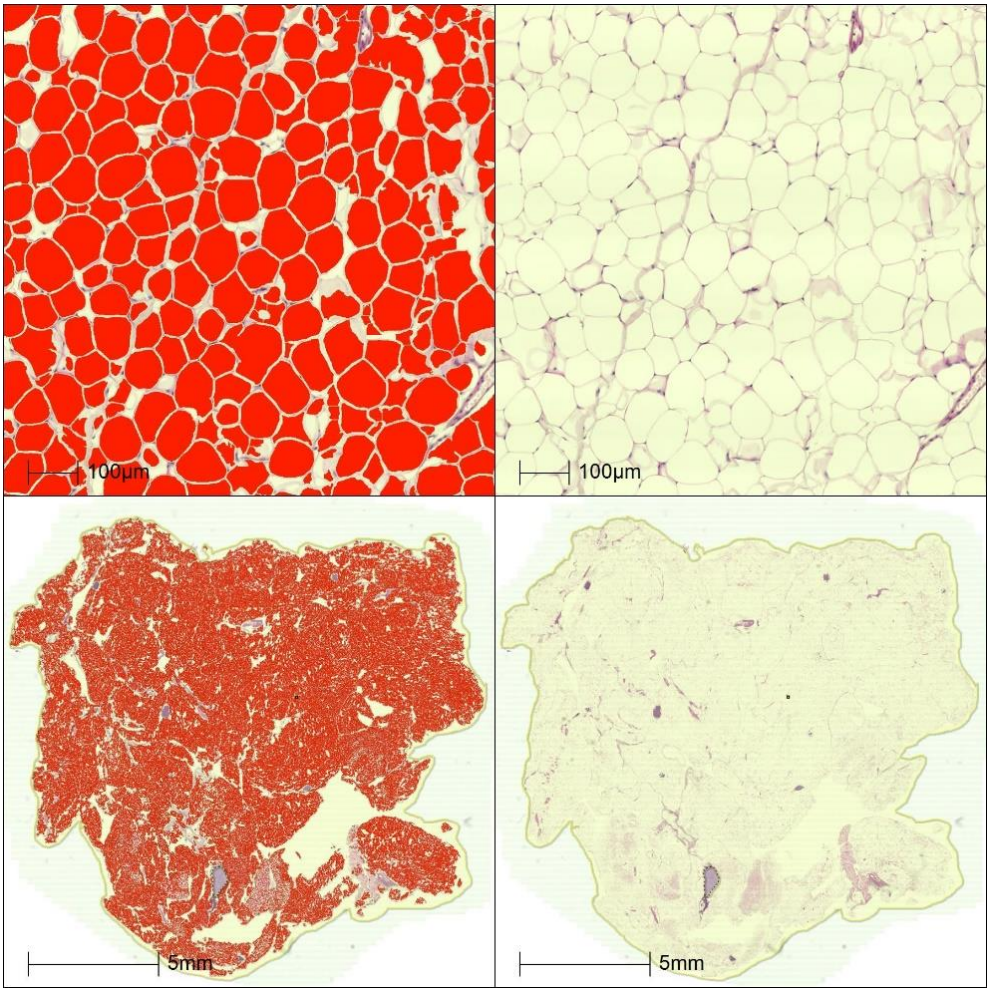

Figure 5.2: Example of cell size analysis in a 12-month-old animal.

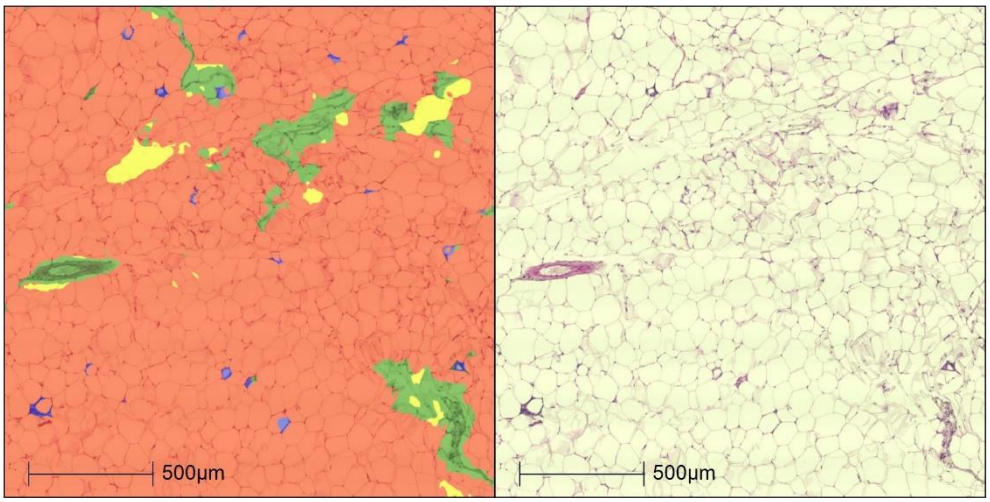

Figure 5.3: Example of crown-like structure analysis in a 12-month-old animal
Yellow = background, red = adipose tissue, blue = CLS, green = 'other'.

5.2.4 Gene expression

The methods for RNA extraction, cDNA synthesis and qPCR are detailed in section 2.4.2. RNA extraction was performed using 100mg adipose tissue. For cDNA synthesis, 450ng RNA was used. cDNA was initially diluted 1:10 and primer efficiencies were tested using standard curves generated from this dilution. A 1:40 working dilution was ultimately used for qPCR analysis. Data was normalised using the comparative CT method against the expression of housekeeping gene *Ppia* as this was unaffected by experimental group or sex. Primer sequences are shown in Chapter 2 (Table 2.3).

5.2.5 Statistical analysis

Data were analysed using Prism 8.0 (GraphPad). Data are presented as mean ± SEM or median [interquartile range] where appropriate. A p-value <0.05 is considered statistically significant. For linear regression used to compute correlations, p<0.01 is considered statistically significant.

5.3 Results

5.3.1 Longitudinal effects on male offspring body composition

5.3.1.1 Short-term effects on male body composition

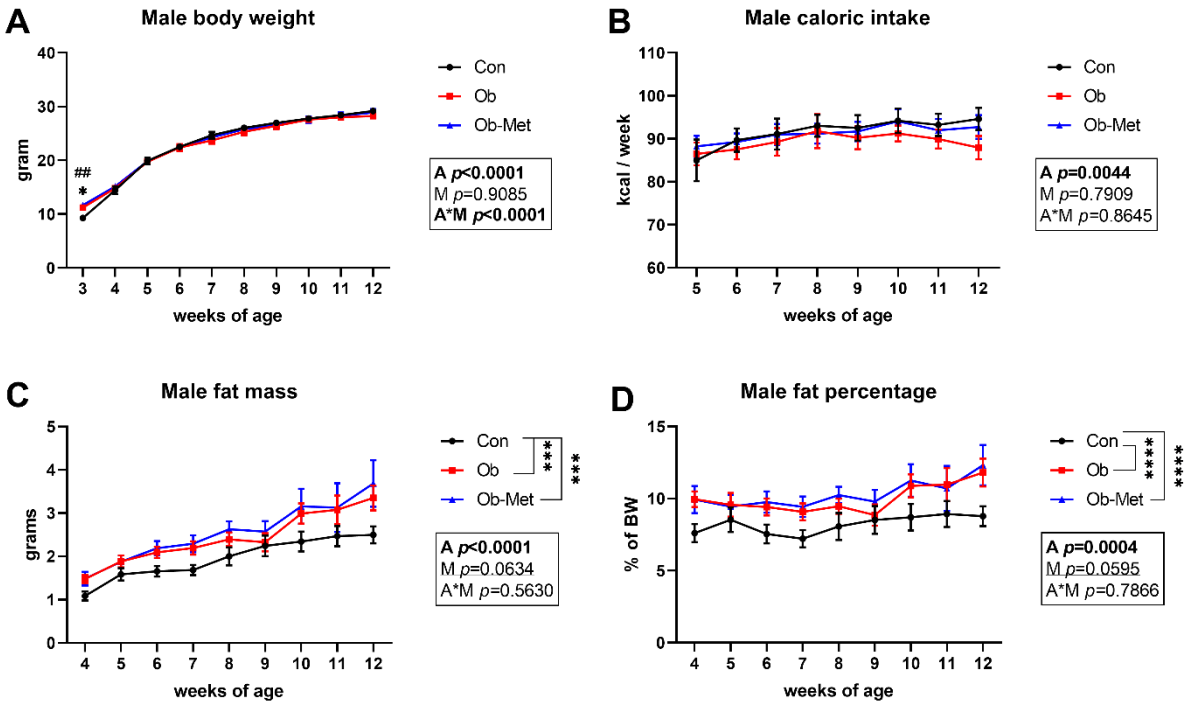

Figure 5.4: Body composition and caloric intake in male offspring until 12 weeks of age.
*A) Body weight (n=12 Con, n=13 Ob, n=12 Ob-Met) and B) caloric intake trajectory until 12 weeks of age (n=10 Con, n=9-10 Ob, n=11 Ob-Met)(data are littermate averages). C) Absolute and D) relative fat mass trajectory between 4-12 weeks of age in the littermate destined for physiological phenotyping (n=10-12 Con, n=10-11 Ob, n=11-12 Ob-Met). Box: results from repeated measures two-way ANOVA (panel A) or mixed effects model analysis (panels B-D) for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). *p<0.05, ***p<0.001, ****p<0.0001 vs Con offspring, ##p<0.01 Con vs Ob-Met offspring using Tukey's multiple comparison test.*

Despite increased body weight at 3 weeks reflective of catch-up growth, there was no difference in body weight or caloric intake between male offspring until 12 weeks of age (Figure 5.4A-B). Total caloric intake (Con 733 ± 22, Ob 705 ± 17, Ob-Met 730 ± 18 kcal, p=0.555) and body weight gain (Con 15.1 [13.5-16.4], Ob 14.1 [12.8-14.4], Ob-Met 13.7 [12.5-15.4g], p=0.231) between 4-12 weeks of age was not different between groups. However, there was a borderline significant effect of the maternal environment on fat mass and fat percentage with Ob and Ob-Met having increased adiposity compared to Con offspring (Figure 5.4C-D). Lean mass was not different throughout the study period (not shown) including at 12 weeks of age (Con 18.1 ± 0.4, Ob 17.1 ± 0.5, Ob-Met 17.4 ± 0.6g, p=0.404).

5.3.1.2 Body composition until 6 months of age

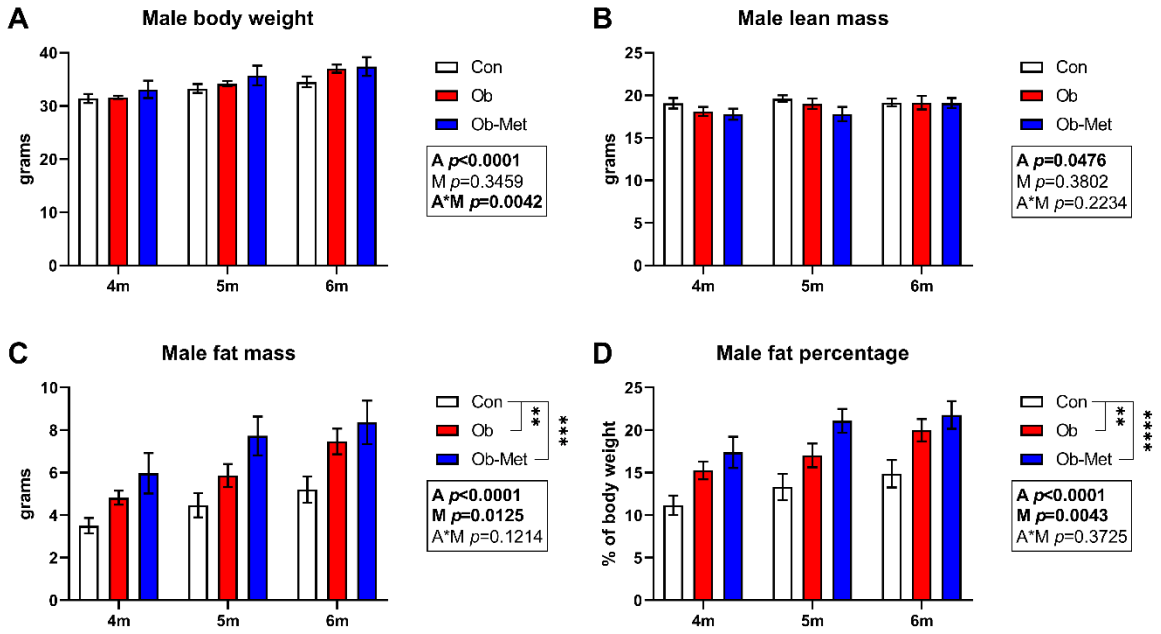

Figure 5.5: Body composition in male offspring between 4-6 months of age by TD-NMR.
*A) Body weight, B) lean mass, C) fat mass and D) fat mass relative to body weight. Box: results from repeated measures two-way ANOVA for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). **p<0.01, ***p<0.001, ****p<0.0001 using Tukey's multiple comparison test. Numbers are n=10 for Con, n=10 for Ob and n=9 for Ob-Met offspring.*

Body weight and lean mass was not affected by the maternal environment between 4 and 6 months of age. In contrast, fat mass and fat mass percentage were significantly increased in both Ob and Ob-Met compared to Con offspring with the Ob-Met having the highest adiposity at each age (Figure 5.5).

5.3.1.3 Body weight and caloric intake until 12 months of age

Both body weight and weekly caloric intake increased with age (Figure 5.6). Before 12 weeks of age this predominantly resulted from lean mass gain, but from 3 months of age body weight gain was largely explained by increased fat mass (Appendix C). Ob and Ob-Met offspring diverged in body weight from Con offspring from 6 months of age, reaching significantly increased body weight by 9 and 10 months, respectively. There was no overall effect of the maternal environment on weekly caloric intake, but the interaction between age and the maternal environment was significant suggesting subtle differences in caloric intake (perhaps driven by the slightly higher intake in Ob offspring in the last few weeks).

A drop in body weight and caloric intake was observed between 26 and 28 weeks of age, coinciding with cardiovascular phenotyping under anaesthesia (see Chapter 6). Since animals lost body weight following anaesthesia at the 6- and 12-month time-points (not shown), cumulative food intake and body weight change was assessed between 12-25 and 28-49 weeks of age (Table 5.1) to avoid confounding effects associated with metabolic or cardiovascular phenotyping.

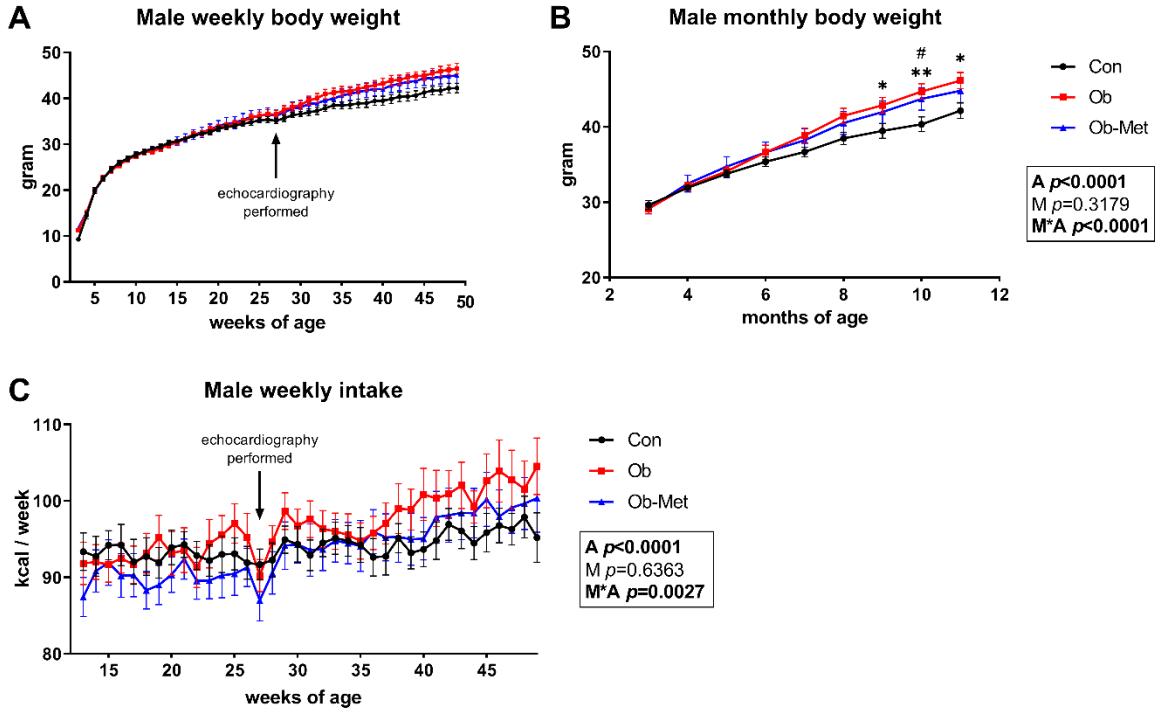

Figure 5.6: Longitudinal body weight trajectory and caloric intake in male offspring.
*A) Weekly body weight from weaning until 49 weeks of age (no statistics performed). B) Monthly body weight trajectory from 3-11 months of age. C) Male weekly caloric intake from weaning until 49 weeks of age. Box: results from repeated measures two-way ANOVA for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). *p<0.05, **p<0.01 Con vs Ob, #p<0.05 Con vs Ob-Met offspring using Tukey's multiple comparison test. Numbers are n=12 for body weight and n=12 Con, n=9 Ob, n=12 Ob-Met for caloric intake.*

Offspring showed subtly different patterns in body weight and caloric intake before and after the 6-month time-point, with groups diverging only later in life (Figure 5.6). Accordingly, no difference in body weight change was observed between 12-25 weeks of age, but between 28-49 weeks of age Ob offspring showed increased body weight gain, resulting in significantly higher weight gain in Ob offspring throughout the whole study period as well (Table 5.1). Cumulative caloric intake was not different in any of the periods investigated.

	Male offspring	Control (n=12)	Obese (n=9-12)	Ob-Met (n=12)	*p*-value
3-6m	Caloric intake 12-25w	1211 ± 27	1213 ± 29	1171 ± 32	*0.5238*
	ΔBW 12-25w (g)	6.1 ± 0.5	7.7 ± 0.4	7.2 ± 0.8	*0.1718*
	BW at 25w (g)	35.2 ± 0.6	36.2 ± 0.8	36.1 ± 1.4	*0.7478*
6-12m	Caloric intake 28-49w	1945 [1871-2066]	2063 [1948-2180]	1980 [1860-2144]	*0.3209**
	ΔBW 28-49w (g)	6.6 ± 0.5	9.0 ± 0.7 [a]	8.0 ± 0.7	***0.0250***
	BW at 49w (g)	42.2 ± 1.0	46.5 ± 1.1	45.0 ± 1.7	*0.0782*
3-12m	Caloric intake 12-49w	3479 ± 68	3578 ± 87	3466 ± 99	*0.6363*
	ΔBW 12-49w (g)	12.6 [10.7-15.5]	16.7 [15.9-19.3] [b]	16.6 [14.2-19.3]	***0.0064****

Table 5.1: Caloric intake and body weight change in male offspring between 3-6 months, 6-12 months and over the whole study period (3-12 months).
*ΔBW = change in body weight. [a]p<0.05, [b]p<0.01, [c]p<0.001 vs Con offspring, one-way ANOVA with Tukey's multiple comparison test or *Kruskal-Wallis test for nonparametric data.*

5.3.2 Body composition in 12-month male offspring

5.3.2.1 Organ weights

Unfortunately, due to the TD-NMR machine breaking mid-way through the study, TD-NMR was not performed on animals at 12 months of age. To investigate the presence of ageing-related effects on lean mass, weights of non-adipose organs were compared (Table 5.2). Brain weight (both absolute and relative to body weight) was decreased compared to Con in both Ob and Ob-Met offspring. Absolute kidney weight was unchanged but kidney weight relative to body weight was decreased in Ob and Ob-Met offspring. Muscle weight was decreased in Ob compared to Con and Ob-Met offspring. Liver weight was increased in fasted Ob-Met offspring in both absolute and relative terms.

	Absolute weights (mg)				Relative weights (% of BW)			
Organ	**Con (n=10-12)**	**Ob (n=7-8)**	**Ob-Met (n=11)**	***p*-value**	**Con (n=11-12)**	**Ob (n=7-8)**	**Ob-Met (n=10-11)**	***p*-value**
Brain	487 ± 4	468 ± 4 [a]	467 ± 6 [a]	***0.0118***	1.37 ± 0.05	1.15 ± 0.05 [a]	1.19 ± 0.06 [a]	***0.0199***
Kidneys	415 ± 9	408 ± 12	393 ± 11	*0.3436*	1.12 ± 0.03	0.98 ± 0.03 [a]	0.95 ± 0.03 [b]	***0.0020***
Vastus*	166 ± 7	125 ± 4 [b]	166 ± 6 [d]	***0.0020***	0.45 ± 0.03	0.30 ± 0.01 [a]	0.43 ± 0.04 [c]	***0.0158***
Liver	1545 ± 29	2058 ± 128	2321 ± 223 [b]	***0.0055***	4.25 ± 0.07	5.06 ± 0.18	5.37 ± 0.34 [b]	***0.0050***

Table 5.2: Non-adipose organ weights in 16-hour fasted 12-month-old male offspring.
**n=5 for vastus muscle weights in Ob offspring because muscle was accidentally not weighed for some animals. [a]p<0.05, [b]p<0.01 vs Con, [c]p<0.05, [d]p<0.01 vs Ob, one-way ANOVA with Tukey's multiple comparison test.*

5.3.2.2 Adipose tissue weights

	Absolute weights (mg)				Relative weights (% of BW)			
Depot	**Con (n=12)**	**Ob (n=8)**	**Ob-Met (n=11)**	***p*-value**	**Con (n=12)**	**Ob (n=8)**	**Ob-Met (n=11)**	***p*-value**
Body weight	37.2 ± 1.01	41.8 ± 1.88	40.5 ± 2.12	*0.1596*	-	-	-	-
Epididymal	1771 [1206-2170]	2437 [2255-2920] [b]	2288 [1569-2307]	***0.0084***	4.47 ± 0.33	5.95 ± 0.20 [b]	5.12 ± 0.20 [a]	***0.0031***
Intra-peritoneal	539 ± 44	933 ± 80 [b]	854 ± 99 [a]	***0.0031***	1.46 ± 0.09	2.21 ± 0.14 [b]	2.04 ± 0.16 [b]	***0.0012***
Retro-peritoneal	409 ± 37	410 ± 32	379 ± 32	*0.7690*	1.09 ± 0.08	0.99 ± 0.08	0.92 ± 0.05	*0.2346*
Sub-cutaneous	782 ± 111	1264 ± 115	1239 ± 186	***0.0393***	2.04 ± 0.25	3.00 ± 0.19	2.90 ± 0.35	***0.0423***
Total VAT	2603 ± 242	3834 ± 231 [a]	3237 ± 301	***0.0147***	7.01 ± 0.52	9.15 ± 0.31 [b]	8.22 ± 0.29	***0.0048***
Total WAT	3320 ± 335	5098 ± 332 [a]	4476 ± 477	***0.0154***	8.93 ± 0.74	12.15 ± 0.43 [b]	11.33 ± 0.55 [a]	***0.0031***
VAT:SAT	3.73 ± 0.19	2.95 ± 0.12	3.05 ± 0.30	*0.0635*	-	-	-	-
BAT	111 ± 8	158 ± 16	157 ± 21	*0.0579*	0.30 ± 0.02	0.37 ± 0.03	0.37 ± 0.03	***0.0435***

Table 5.3: Absolute and relative weight of excised adipose tissue depots from fasted 12-month-old male offspring.
BAT = brown adipose tissue. SAT = subcutaneous adipose tissue. VAT = visceral adipose tissue (combined weights of epididymal, intra- and retroperitoneal). WAT = total white adipose tissue. [a]p<0.05, [b]p<0.01 vs Con, one-way ANOVA with Tukey's multiple comparison test.

There were no differences in body weight or retroperitoneal WAT weight in 12-month-old offspring. Ob offspring showed increased absolute and relative weight of all other depots (Table 5.3). Ob-Met offspring had significantly increased absolute intraperitoneal WAT and relative epididymal, intraperitoneal and total WAT weights compared to Con offspring (Table 5.3). There was no difference in fat mass between Ob and Ob-Met offspring.

5.3.3 Adipose tissue biology in male offspring

5.3.3.1 Adipocyte size

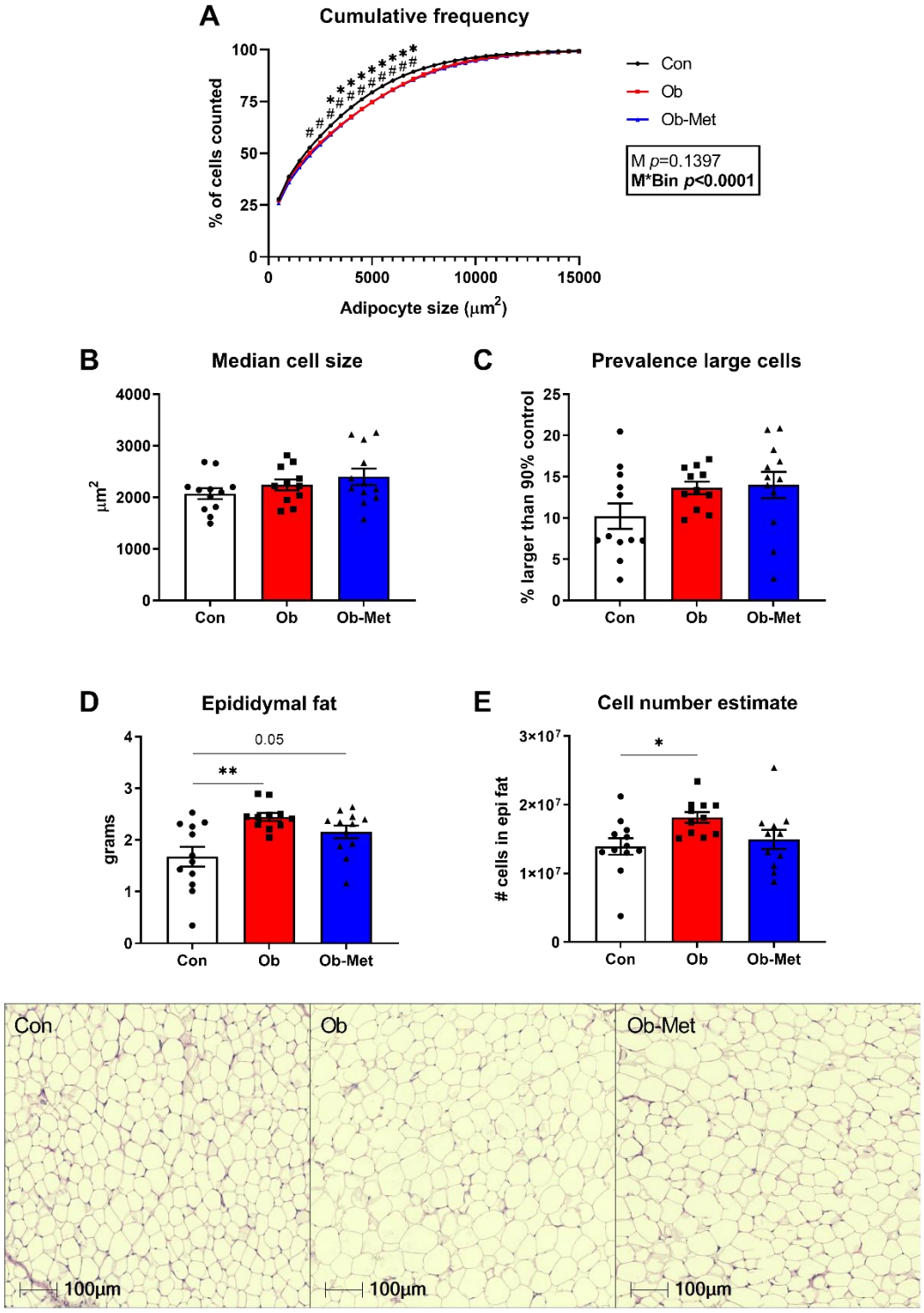

Figure 5.7: Epididymal adipocyte size in 12-month-old male fed offspring.
*A) Cumulative frequency distribution of cell size. Box: results from repeated measures two-way ANOVA for the effect of the maternal environment (M) and its interaction with adipocyte size (M*Bin). *p<0.05 (or lower) Con vs Ob, #p<0.05 (or lower) Con vs Ob-Met offspring. B) Median cell size, C) the percentage of cells larger than the 90th centile of Con offspring, D) epididymal fat depot weight of animals used for cell size analysis, E) estimated adipocyte number based on the weight of the dissected fat depot and mean adipocyte area. *p<0.05, **p<0.01, one-way ANOVA with Tukey's multiple comparison test. Bottom: representative images of male Con (n=12), Ob (n=11) and Ob-Met (n=11-12) offspring.*

Both male Ob and Ob-Met offspring showed significant hypertrophy compared to Con offspring, evidenced by a rightward shift in cumulative adipocyte size distribution (Figure 5.7A). There were no significant differences in median adipocyte size or the prevalence of large cells, but Ob offspring had increased estimated total adipocyte number indicative of eWAT hyperplasia (Figure 5.7E).

5.3.3.2 Crown-like structures

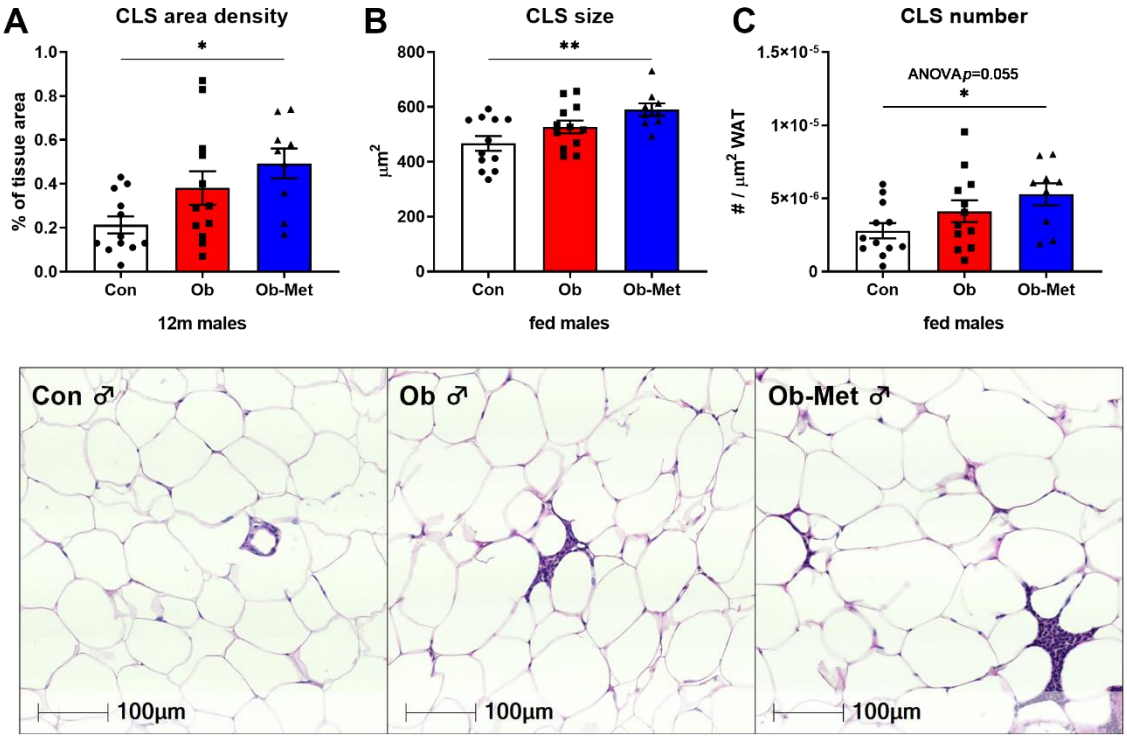

Figure 5.8: Crown-like structures in 12-month-old epididymal adipose tissue.
*A) Percentage of WAT tissue area consisting of crown-like structures (CLS), B) median size of CLS in the tissue, C) number of CLS per µ² WAT tissue. *p<0.05, **p<0.01, one-way ANOVA with Tukey's multiple comparison test. Bottom: representative images of male Con (n=12), Ob (n=12) and Ob-Met (n=9) offspring.*

Male Ob-Met offspring had increased CLS area density compared to Con offspring. Both size and number of CLS were increased, although the increase in CLS number did not reach statistical significance (Figure 5.8).

5.3.3.3 *Inflammatory gene expression*

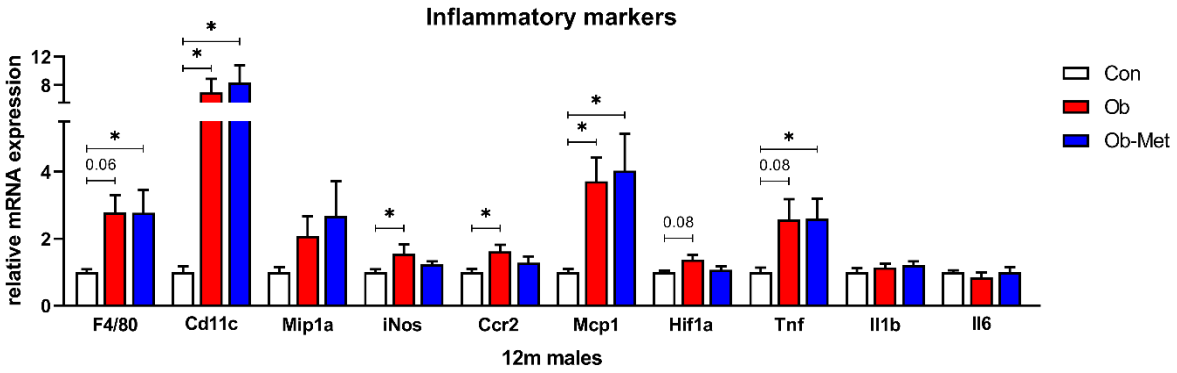

Figure 5.9: Gene expression of WAT markers in epididymal adipose tissue of 12-month-old male offspring
*Expression relative to the expression of housekeeper gene Ppia. *p<0.05, one-way ANOVA with Tukey's multiple comparison test. Numbers are n=11 Con, n=-7-8 Ob, n=10-11 Ob-Met.*

Expression of pro-inflammatory macrophage markers *Cd11c* and *iNos* and migration markers *Ccr2* and *Mcp1* were increased in Ob compared to Con offspring. In Ob-Met offspring, expression of *F4/80, Cd11c, Tnf* and *Mcp-1* was significantly increased compared to Con offspring (Figure 5.9).

There were significant relationships between markers of WAT inflammation and serum cholesterol, serum insulin and HOMA-IR. Correlations were strongest for markers of M1-style macrophage activation and *Mcp1* (Figure 5.11, other correlations in Appendix D). Markers of M1 activation were also strongly associated with epididymal fat weight, but this relationship was not linear (Figure 5.10).

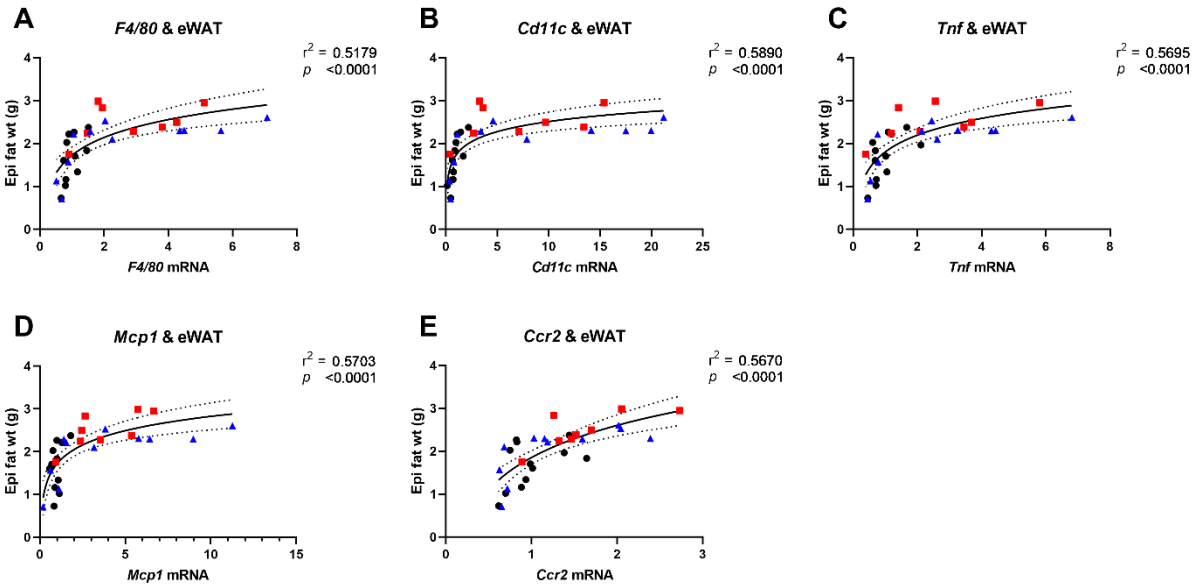

Figure 5.10: Strongest correlations between inflammatory mediators and epididymal adipose tissue weight.
Linear regression performed on log-transformed gene expression data but untransformed data is plotted on graphs with linear axes. Graphs were only included if the linear regression (using log-transformed data) showed r^2>0.4 and p<0.005. Numbers are n=31 (n=11 Con, n=8 Ob, n=11 Ob-Met offspring).

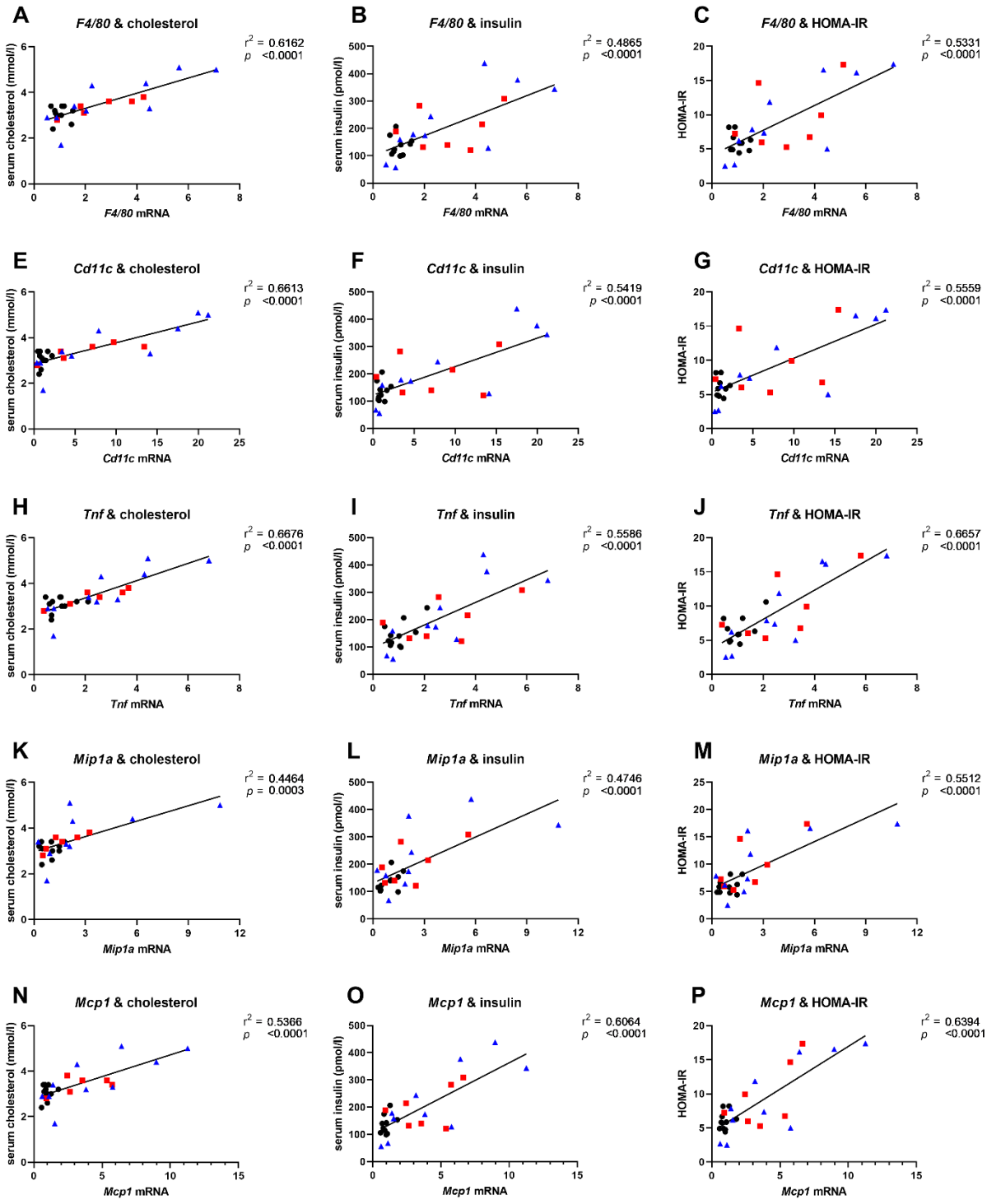

Figure 5.11: Strongest correlations between inflammatory mediators and fasted serology in aged male offspring. *Graphs were only included if the linear regression showed $r^2>0.4$ and $p<0.005$. Numbers are n=27-28 (n=11 Con, n=6-7 Ob, n=10 Ob-Met).*

5.3.3.4 Adipocyte-specific gene expression

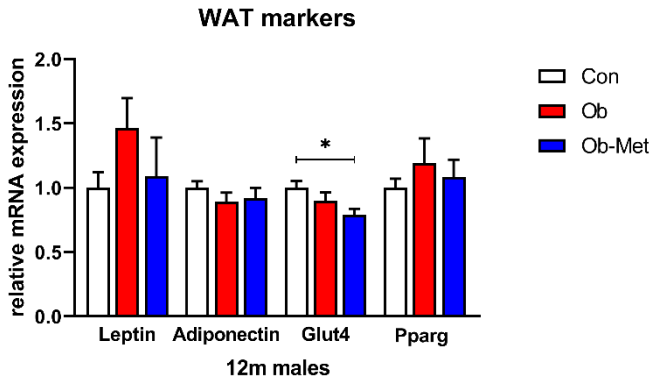

Figure 5.12: Gene expression of WAT markers in epididymal adipose tissue of 12-month-old male offspring.
*Expression relative to the expression of housekeeper gene Ppia. *p<0.05, one-way ANOVA with Tukey's multiple comparison test. Numbers are n=11-12 Con, n=8 Ob, n=11 Ob-Met.*

There was no significant difference in expression of *Leptin, Adiponectin* or *Pparg* between groups, but *Glut4* mRNA was significantly downregulated in the Ob-Met group (Figure 5.12).

5.3.4 Metabolic health of 12-month male offspring

5.3.4.1 Glucose tolerance

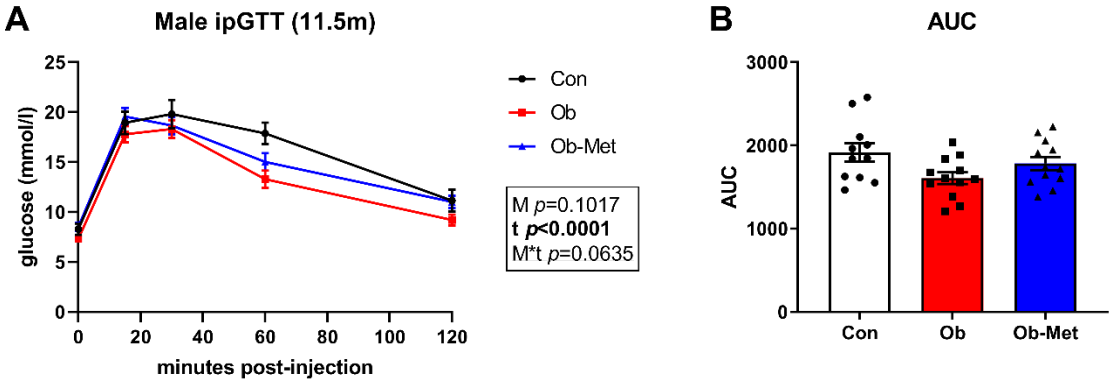

Figure 5.13: Glucose tolerance in 11.5-month-old male offspring.
*A) ipGTT curve for 10% glucose injection at t=0, with B) accompanying area under the curve. Box: results from repeated measures two-way ANOVA for the effect of time (t), the maternal environment (M) and the interaction between them (M*t). Numbers are n=11 Con, n=12 Ob, n-12 Ob-Met.*

There was no significant effect of the maternal environment on glucose levels during an ipGTT when analysed as a time-course or AUC (Figure 5.13), suggesting no impact of maternal obesity or metformin intervention on glucose tolerance at 12 months of age.

5.3.4.2 *Serum analyses*

Male offspring	Con (n=11-12)	Ob (n=7-8)	Ob-Met (n=9-11)	*p*-value
Glucose (fed, mmol/l) ***from fed siblings***	12.2 ± 0.05 (n=12)	11.9 ± 0.5 (n=12)	10.8 ± 0.3 [a] (n=10)	***0.0155***
Glucose (fasted, mmol/l) ***from fasted siblings***	6.9 ± 0.3	7.6 ± 0.4	6.8 ± 0.2	*0.2083*
Insulin (pmol/l)	146 ± 14	198 ± 28	217 ± 41	*0.2078*
HOMA-IR	6.4 ± 0.6	9.6 ± 1.8	9.4 ± 1.8	*0.2067*
HOMA-%B	136 ± 20	153 ± 19	166 ± 28	*0.6172*
Cholesterol (mmol/l)	3.1 ± 0.1	3.4 ± 0.2	3.6 ± 0.3	*0.2366*
Triglycerides (mmol/l)	1.09 ± 0.06	0.99 ± 0.07	1.09 ± 0.06	*0.4389*
FFAs (mmol/l)	1.60 ± 0.06	1.64 ± 0.12	1.61 ± 0.07	*0.9585*

Table 5.4: Serological analysis in 12-month-old male offspring.
All analysis was performed on serum from 16-hour fasted animals, except fed glucose that was taken from siblings at tissue collection. [a]p<0.05 vs Con offspring, one-way ANOVA with Tukey's multiple comparison test. FFAs = free fatty acids.

There were no differences in fasted serology in 12-month-old offspring, but Ob-Met offspring had decreased fed glucose compared to Con offspring (Table 5.4).

5.3.5 Longitudinal effects on female offspring body composition

5.3.5.1 *Short-term effects on female body composition*

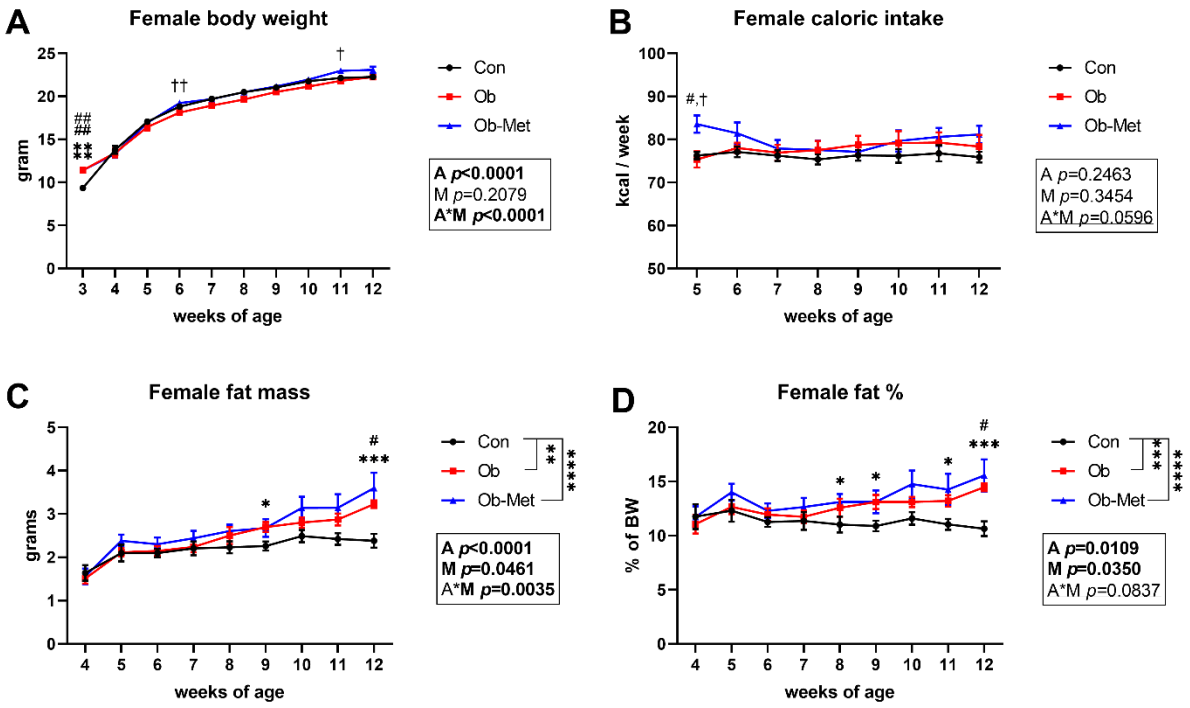

Figure 5.14: Body composition and caloric intake in female offspring until 12 weeks of age.
*A) Body weight (n=12 in all groups) and B) caloric intake trajectory until 12 weeks of age (n=9-10 Con, n=11 Ob, n=7 Ob-Met)(data are littermate averages). C) Absolute and D) relative fat mass trajectory between 4-12 weeks of age in the littermate destined for physiological phenotyping (n=11 Con, n=10-12 Ob, n=11-12 Ob-Met). Box: results from repeated measures two-way ANOVA (panel A) or mixed effects model analysis (panels B-D) for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs Con offspring, #p<0.05, ####p<0.0001 Con vs Ob-Met, †p<0.05, ††p<0.01 Ob vs Ob-Met offspring using Tukey's multiple comparison test.*

Similar to males, body weight in female Ob and Ob-Met offspring was increased compared to Con at 3 weeks but this disappeared after weaning (Figure 5.14A). There was no overt difference in body weight or weekly caloric intake until 12 weeks (Figure 5.14A-B) and total caloric intake between 4-12 weeks of age was also not different (Con 612 ± 9, Ob 623 ± 15, Ob-Met 639 ± 16 kcal, p=0.456). Although body weight gain (Con 8.5 ± 0.4, Ob 8.8 ± 0.4, Ob-Met 9.8 ± 0.5g, p=0.109) and lean mass (Con 13.9 ± 0.4, Ob 12.9 ± 0.3, Ob-Met 13.9 ± 0.4g at 12 weeks, p=0.076) were not significantly different, both Ob and Ob-Met offspring were significantly fatter than Con offspring up to and at 12 weeks of age (Figure 5.14C-D).

5.3.5.2 *Body weight and adiposity at 6 months of age*

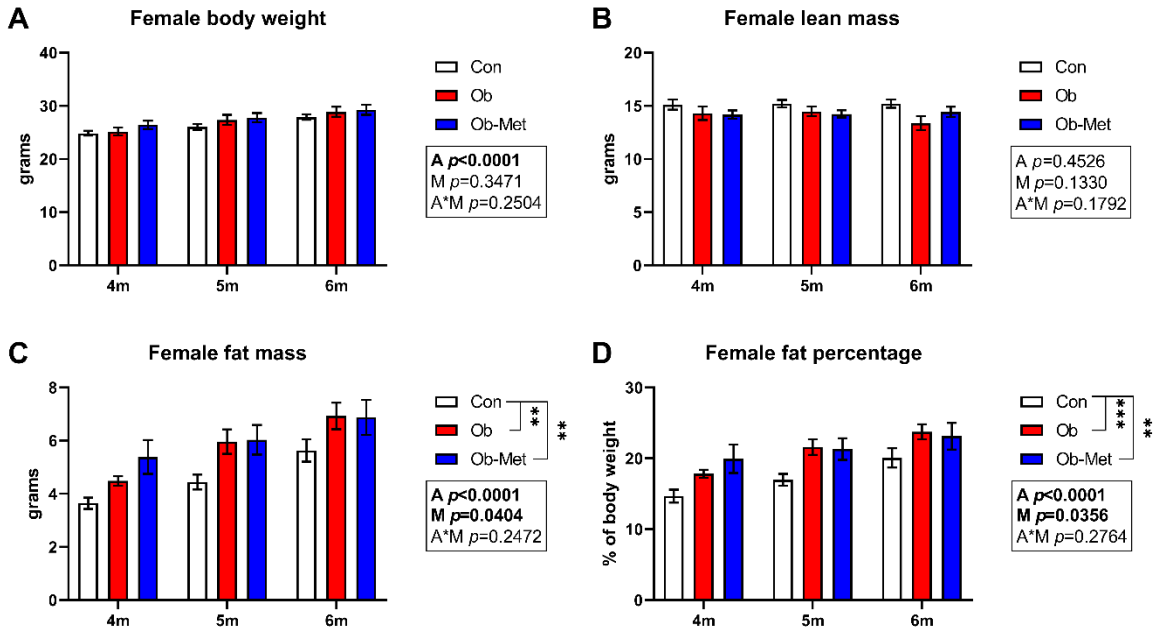

Figure 5.15: Body composition in female offspring between 4-6 months of age by TD-NMR.
*A) Body weight, B) lean mass, C) fat mass and D) fat mass relative to body weight. Box: results from repeated measures two-way ANOVA for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). **p<0.01, ***p<0.001 using Tukey's multiple comparison test. Numbers are n=10 for Con, Ob and Ob-Met offspring.*

Body weight and lean mass were not affected by the maternal environment between 4 and 6 months of age. In contrast, fat mass and fat mass percentage were significantly increased in both Ob and Ob-Met compared to Con offspring (Figure 5.15).

5.3.5.3 *Body composition and caloric intake until 12 months of age*

The maternal environment significantly affected body weight trajectory in female offspring, with Ob-Met diverging from Con and Ob offspring at 5-7 and 9 months of age, respectively (Figure 5.16A-B). Both body weight and caloric intake increased with age, although there was no effect of the maternal environment on weekly caloric intake (Figure 5.16C). As for male offspring, a drop in food intake was observed around cardiovascular phenotyping at 6 months of age.

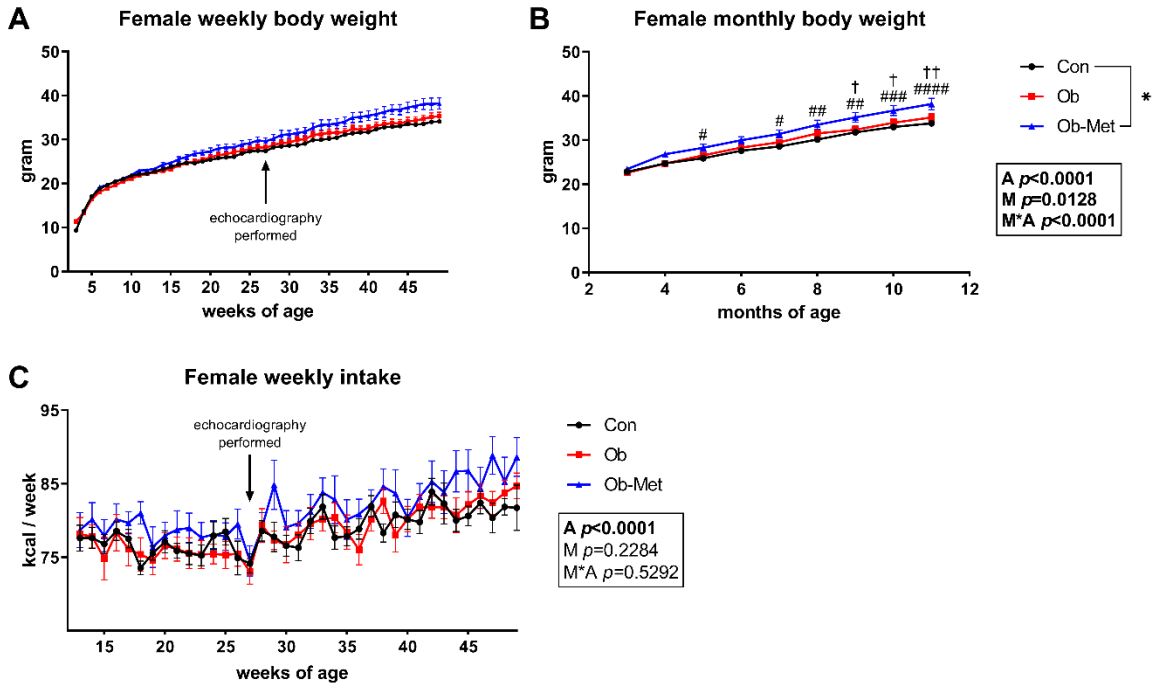

Figure 5.16: Body weight trajectory and caloric intake in female offspring.
*A) Weekly body weight from weaning until 49 weeks of age (no statistics performed). B) Monthly body weight trajectory from 3-11 months of age. C) Female weekly caloric intake from weaning until 49 weeks of age. Box: results from repeated measures two-way ANOVA for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 Con vs Ob-Met, †p<0.05, ††p<0.01 Ob vs Ob-Met offspring using Tukey's multiple comparison test. Numbers are n=12 for body weight and n=12 Con, n=12 Ob, n=11 Ob-Met for caloric intake.*

There was no detectable difference in cumulative caloric intake at any point in the study (Table 5.5). Despite this observation, female Ob-Met offspring showed significantly increased body weight gain compared to Con offspring between 6 and 12 months as well as throughout the whole study period, leading to increased body weight at 49 weeks of age (Table 5.5).

	Female offspring	Control (n=11-12)	Obese (n=12)	Ob-Met (n=11-12)	*p*-value
3-6m	Caloric intake 12-25w	987 ± 9	990 ± 22	1023 ± 22	*0.3419*
	ΔBW 12-25w (g)	5.0 ± 0.2	5.6 ± 0.4	6.1 ± 0.5	*0.1545*
	BW at 25w (g)	27.3 ± 0.3	27.9 ± 0.6	29.2 ± 0.8	*0.1031*
6-12m	Caloric intake 28-49w	1681 ± 20	1690 ± 28	1756 ± 46	*0.2220*
	ΔBW 28-49w (g)	5.9 ± 0.5	6.5 ± 0.6	8.0 ± 0.6 [a]	***0.0302***
	BW at 49w (g)	34.1 ± 0.4	35.3 ± 1.0	38.2 ± 1.3	***0.0115***
3-12m	Caloric intake 12-49w	2906 ± 35	2908 ± 52	3013 ± 69	*0.2884*
	ΔBW 25-39w (g)	11.8 ± 0.5	13.1 ± 0.7	15.2 ± 1.0 [a]	***0.0133***

Table 5.5: Caloric intake and body weight change in female offspring between 3-6 months, 6-12 months and over the whole study period (3-12 months).
ΔBW = change in body weight. [a]p<0.05 vs Con offspring, one-way ANOVA with Tukey's multiple comparison test.

5.3.6 Body composition in 12-month female offspring

5.3.6.1 Organ weights

Kidney weight in female offspring was unaltered by maternal obesity or the metformin intervention. As for male offspring, absolute brain weight was decreased in Ob and Ob-Met offspring, leading to a significant reduction when expressed relative to body weight in Ob-Met offspring only (Table 5.6). Liver weight was significantly affected by the maternal environment, with highest weights observed in Ob-Met female offspring although this failed to reach statistical significance on post-hoc testing.

	Absolute weights (mg)				Relative weights (% of BW)			
Organ	**Con (n=12)**	**Ob (n=11-12)**	**Ob-Met (n=11)**	***p*-value**	**Con (n=12)**	**Ob (n=11-12)**	**Ob-Met (n=11)**	***p*-value**
Brain	487 ± 3	462 ± 5 [b]	465 ± 5 [a]	***0.0005***	1.65 ± 0.05	1.53 ± 0.05	1.35 ± 0.06 [a]	***0.0022***
Kidneys	305 ± 6	300 ± 6	318 ± 5	*0.1033*	1.03 ± 0.02	1.00 ± 0.04	0.92 ± 0.03	*0.0561*
Vastus	117 ± 6	128 ± 7	140 ± 5	*0.0552*	0.34 ± 0.02	0.42 ± 0.03	0.41 ± 0.02	0.8320
Liver	1361 [1199-1376]	1294 [1177-1481]	1455 [1372-1602]	***0.0429****	4.42 ± 0.17	4.29 ± 0.10	4.30 ± 0.08	*0.7012*

Table 5.6: Non-adipose organ weights in 16-hour fasted 12-month-old female offspring.
*[a]p<0.01, [b]p<0.001 vs Con, one-way ANOVA with Tukey's multiple comparison test or *Kruskal-Wallis test for non-parametric data.*

5.3.6.2 Adipose tissue weights

	Absolute weights (mg)				Relative weights (% of BW)			
Depot	**Con (n=12)**	**Ob (n=12)**	**Ob-Met (n=11)**	***p*-value**	**Con (n=12)**	**Ob (n=12)**	**Ob-Met (n=11)**	***p*-value**
Body weight	29.9 ± 0.9	31.0 ± 1.1	35.1 ± 1.4 [b,e]	***0.0005***	-	-	-	-
Gonadal	1203 ± 102	1504 ± 135	2053 ± 147 [c,e]	***0.0002***	3.96 ± 0.23	4.76 ± 0.29	5.78 ± 0.21 [d,e]	***<0.0001***
Intra-peritoneal	466 ± 42	621 ± 51	809 ± 73 [c]	***0.0006***	1.53 ± 0.10	1.98 ± 0.12 [a]	2.26 ± 0.11 [c]	***0.0002***
Retro-peritoneal	244 ± 28	274 ± 21	341 ± 23 [a]	***0.0255***	0.80 ± 0.07	0.86 ± 0.05	0.97 ± 0.04	*0.1244*
Sub-cutaneous	795 ± 81	1083 ± 115	1632 ± 182 [c,e]	***0.0003***	2.61 ± 0.19	3.42 ± 0.26	4.53 ± 0.33 [d,e]	***<0.0001***
Total VAT	1913 ± 165	2376 ± 208	3203 ± 235 [c,e]	***0.0004***	6.29 ± 0.38	7.87 ± 0.33 [c]	9.01 ± 0.32 [d]	***<0.0001***
Total WAT	2708 ± 242	3641 ± 282	4835 ± 412 [c,e]	***0.0002***	8.90 ± 0.56	11.39 ± 0.56 [a]	13.53 ± 0.62 [d,e]	***<0.0001***
VAT:SAT	2.33 [2.20-2.69]	2.13 [1.95-2.66]	2.01 [1.68-2.24] [a]	***0.0444***				
BAT	74 ± 5	80 ± 6	114 ± 10 [b,f]	***0.0070***	235 [220-275]	240 [220-310]	300 [a,e] [280-370]	***0.0070****

Table 5.7: Absolute and relative weight of excised adipose tissue depots from fasted 12-month-old female offspring.
*BAT = brown adipose tissue. SAT = subcutaneous adipose tissue. VAT = visceral adipose tissue (combined weights of gonadal, intra- and retroperitoneal fat). WAT = total white adipose tissue. [a]p<0.05, [b]p<0.01, [c]p<0.001, [d]p<0.0001 vs Con, [e]p<0.05, [f]p<0.01 vs Ob, one-way ANOVA with Tukey's multiple comparison test or *Kruskal-Wallis test for non-parametric data.*

Ob-Met offspring had the highest body weight at 12 months, while there was no difference between Con and Ob offspring. Moreover, weight of all collected WAT depots was significantly increased in Ob-Met offspring compared to Con and Ob offspring (with the exception of intraperitoneal and retroperitoneal depots compared to Ob offspring, Table 5.7). The same pattern was seen for visceral WAT, BAT and total WAT, indicating a global adiposity phenotype. Despite the apparent lack of depot-specificity, the ratio between visceral and subcutaneous WAT (VAT:SAT) was decreased in Ob-Met offspring.

5.3.7 Adipose tissue biology in female offspring

5.3.7.1 Adipocyte size

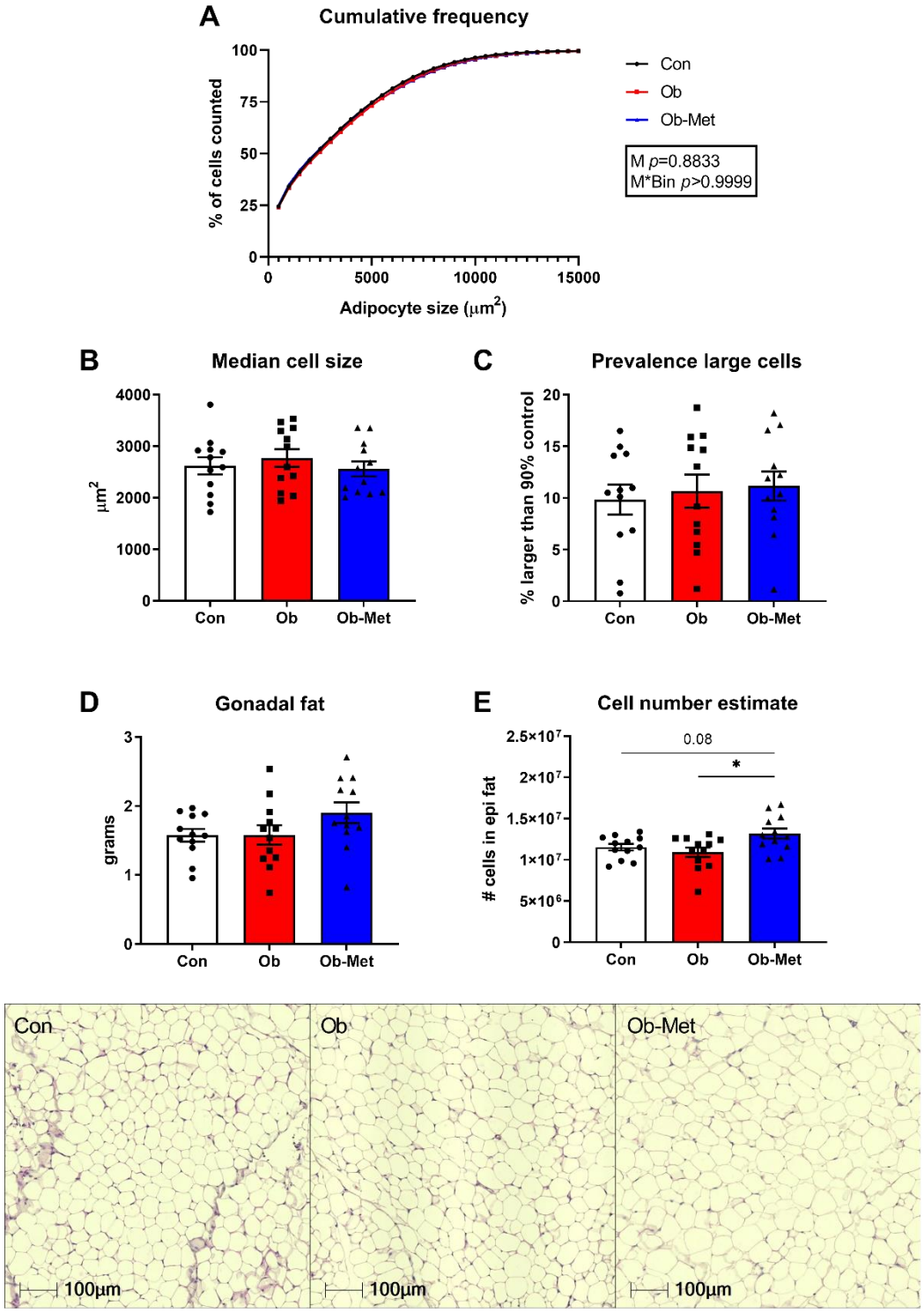

Figure 5.17: Gonadal adipocyte size in 12-month-old female offspring.
*A) Cumulative frequency distribution of cell size. Box: results from repeated measures two-way ANOVA for the effect of the maternal environment (M) and its interaction with adipocyte size (M*Bin). B) Median cell size, C) the percentage of cells larger than the 90th centile of Con offspring, D) gonadal fat depot weight of animals used for cell size analysis, E) estimated adipocyte number based on the weight of the dissected fat depot and mean adipocyte area. *p<0.05 one-way ANOVA with Tukey's multiple comparison test. Bottom: representative images of female Con, Ob and Ob-Met offspring (n=12 in all groups).*

There was no difference in adipocyte size distribution, median cell size or the prevalence of large adipocytes in 12-month-old female offspring. However, Ob-Met offspring had significantly increased estimated total adipocyte number in gWAT compared to Ob offspring (Figure 5.17D) suggesting that increased adiposity resulted from hyperplasia rather than hypertrophy in female offspring at this age.

5.3.7.2 Crown-like structures

Female Ob-Met offspring had increased CLS density compared to Con and Ob offspring, which was related to increased number of CLS but not CLS size (Figure 5.18). No difference was found between Con and Ob offspring.

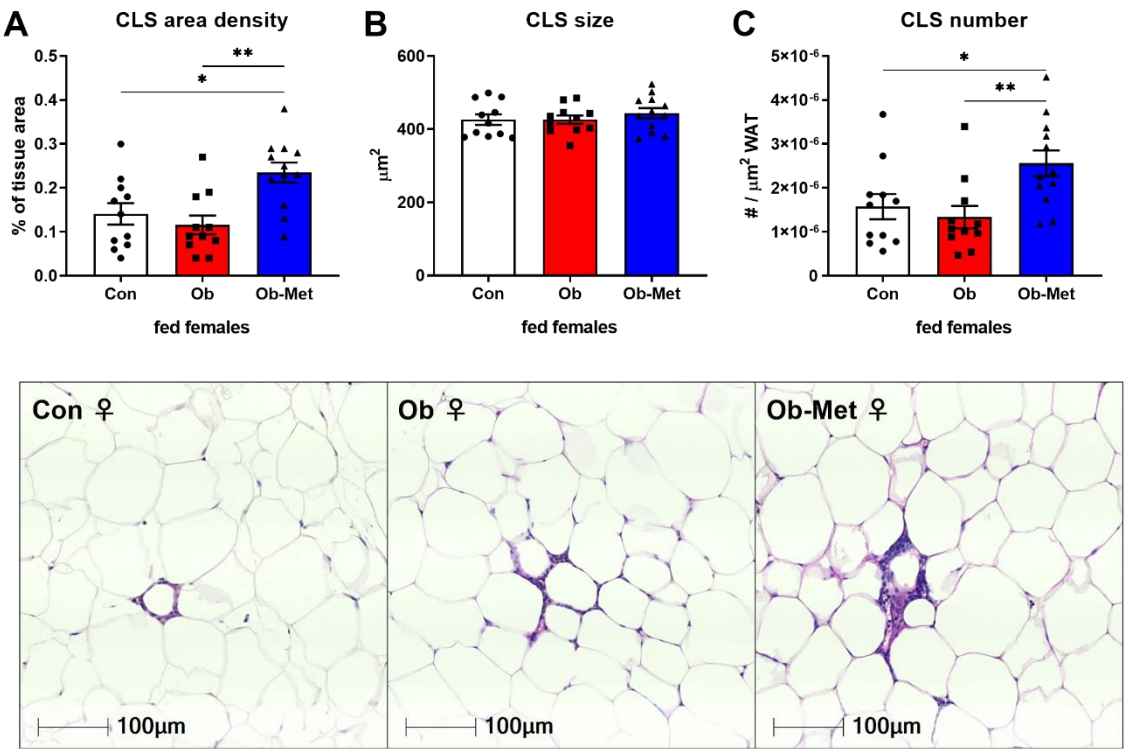

Figure 5.18: Crown-like structures in 12-month-old gonadal adipose tissue.
*A) percentage of WAT tissue area consisting of crown-like structures (CLS), B) median size of CLS in the tissue, C) number of CLS per µ² WAT tissue. *p<0.05, **p<0.01, one-way ANOVA with Tukey's multiple comparison test. Bottom: representative images of male Con, Ob and Ob-Met offspring. Numbers are n=11 Con, n=11 Ob, n=12 Ob-Met.*

5.3.7.3 Inflammatory gene expression

The expression of chemokine *Mcp1* and pro-inflammatory markers *Cd11c*, *Mip1a* and *iNos* (with a trend for *Tnf*) was increased in Ob-Met offspring compared to controls (Figure 5.19). *Hif1a* expression was instead downregulated in Ob-Met females. In Ob offspring, only *Mip1a* and *iNos* were significantly elevated compared to control, although a trend for decreased *Il6* expression was observed (Figure 5.19).

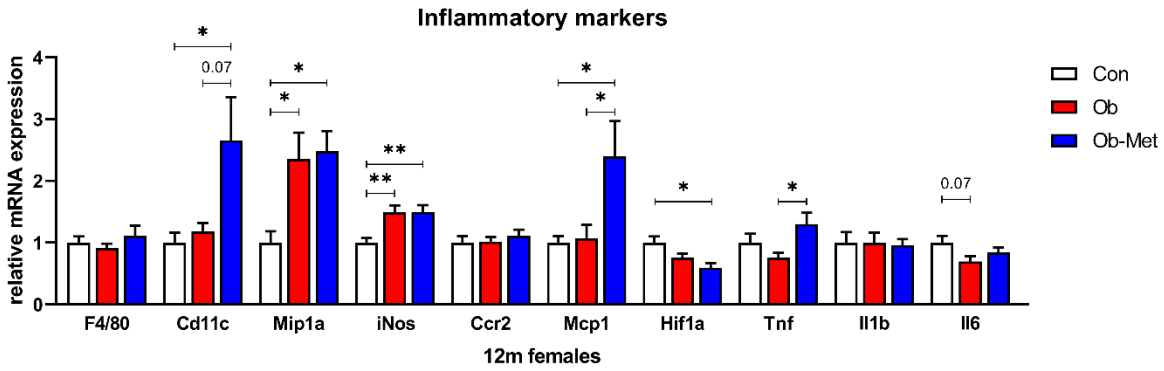

Figure 5.19: Gene expression of WAT markers in gonadal adipose tissue of 12-month-old female offspring
*Expression relative to the expression of housekeeper gene Ppia. *p<0.05, one-way ANOVA with Tukey's multiple comparison test. Numbers are n=11-12 Con (n=10 for Mip1a), n=11-12 Ob, n=11 Ob-Met (n=10 for Il1b).*

In contrast to male offspring, inflammatory genes did not correlate with serum cholesterol (Appendix E), but significant relationships were found between *Cd11c* and serum insulin and HOMA-IR (Figure 5.20A-B). Both *Cd11c* and *Mcp1* related to gWAT weight in a non-linear fashion (Figure 5.20C-D). For full overview of correlations please refer to Appendix E.

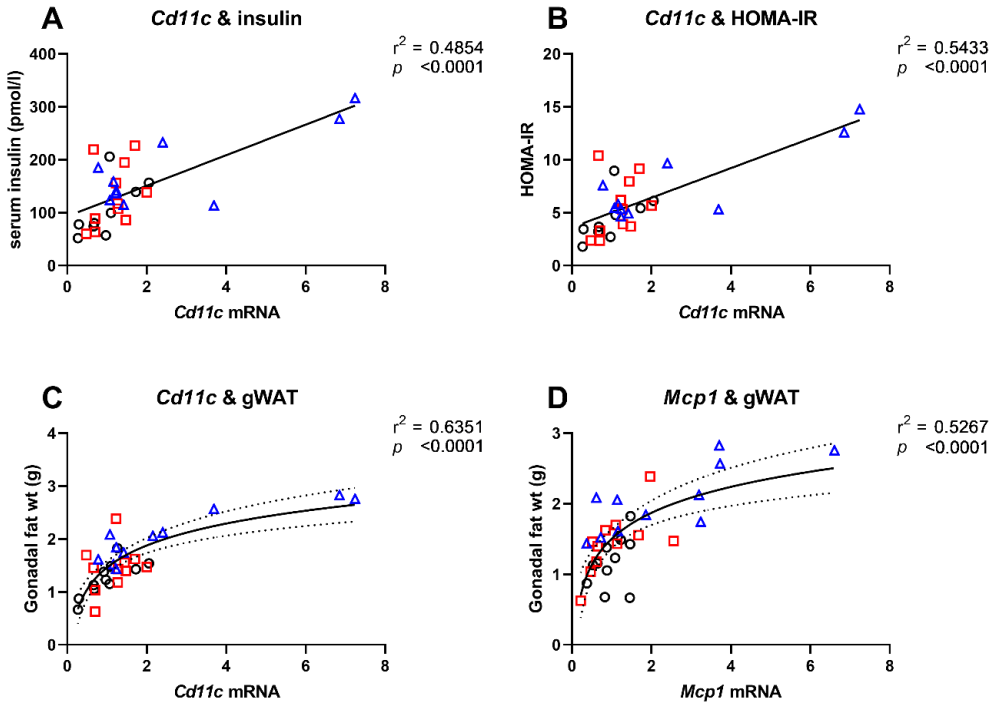

Figure 5.20: Strongest correlations of inflammatory mediators with fasted serology and adiposity in female offspring.
Graphs were only included if the linear regression (using untransformed or log-transformed data) showed r^2>0.4 and p<0.0001. Linear regression for gonadal adipose tissue weight was performed on log-transformed gene expression data but untransformed data is plotted on graphs with linear axes. Numbers are n=30 for panels A-B (n=9 Con, n=11 Ob, n=10 Ob-Met offspring) and n=33 for panels C-D (n=11 in all groups).

5.3.7.4 Adipocyte-specific gene expression

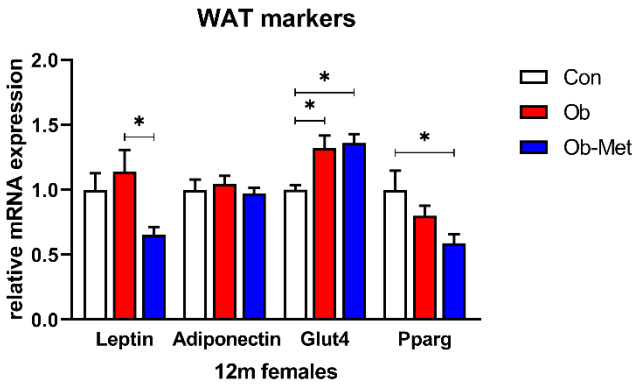

Figure 5.21: Gene expression of WAT markers in gonadal adipose tissue of 12-month-old female offspring.
*Expression relative to the expression of housekeeper gene Ppia. *p<0.05, one-way ANOVA with Tukey's multiple comparison test. Numbers are n=12 Con (n=11 for Glut4), n=12 Ob, n=11 On-Met.*

Both female Ob and Ob-Met offspring had elevated *Glut4* mRNA compared to Con offspring (Figure 5.21). The expression of *Glut4* significantly correlated with serum insulin, HOMA-IR and β-cell activity (Figure 5.22) suggesting compensatory upregulation of *Glut4* mRNA in response to IR. *Pparg* mRNA was downregulated in Ob-Met offspring only (Figure 5.21).

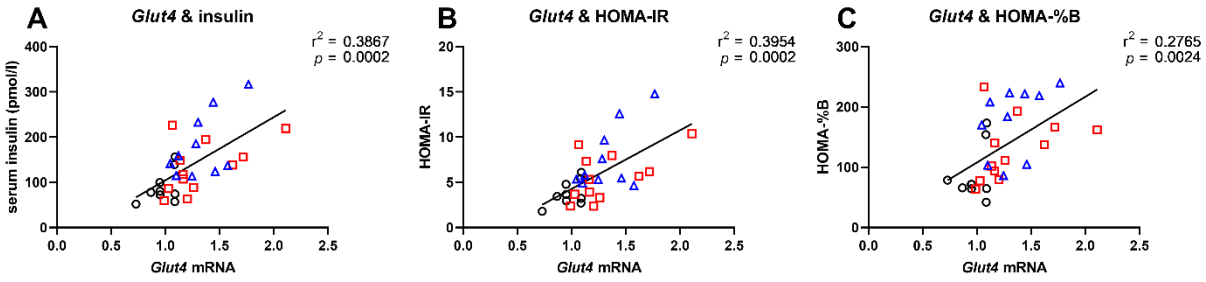

Figure 5.22: Correlations between Glut4 expression and fasted serology in aged female offspring. *Numbers are n=32 (n=9 Con, n=12 Ob, n=10 Ob-Met offspring).*

Ob-Met offspring showed lower *Leptin* expression compared to Ob offspring (Figure 5.21). Leptin expression across all groups did not correlate with gWAT weight (Figure 5.23A). However, when groups were analysed separately, a relationship was found between *Leptin* and gWAT weight for Con and Ob offspring (Figure 5.23A). In Ob-Met offspring, there was no correlation, indicating dysregulation of leptin production in this group.

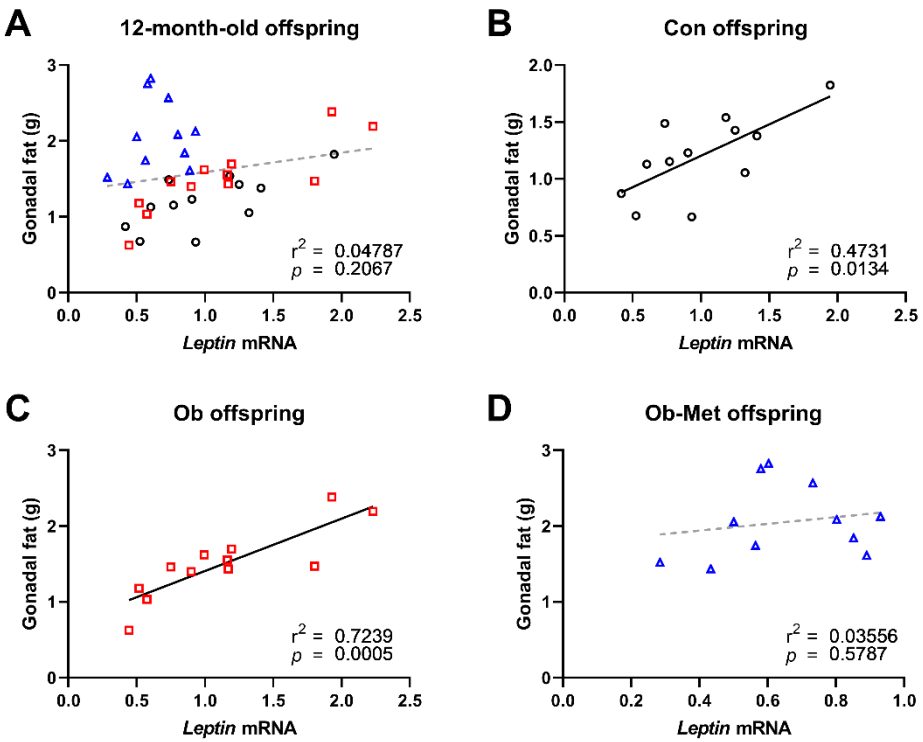

Figure 5.23: The relationship between weight and Leptin expression in gonadal WAT of 12-month-old female offspring. *A) All offspring, B) Con offspring (black circles, n=12), C) Ob offspring (red circles, n=12), D) Ob-Met offspring (blue triangles, n=11). Solid lines represent correlations with signifiant linear regression (p<0.01), dotted lines are non-significant linear regressions.*

5.3.8 Metabolic health of 12-month female offspring

5.3.8.1 Glucose tolerance

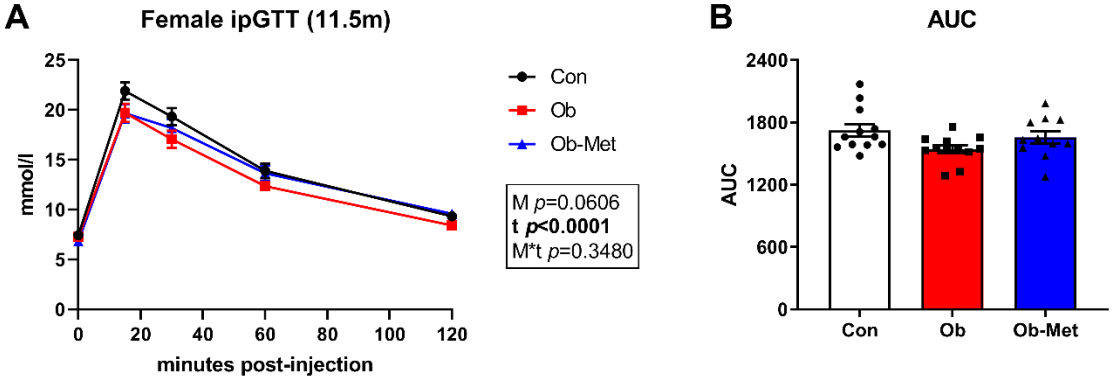

Figure 5.24: Glucose tolerance in 11.5-month-old female offspring.
*A) ipGTT curve for 10% glucose injection at t=0, with B) accompanying area under the curve. Box: results from repeated measures two-way ANOVA for the effect of time (t), the maternal environment (M) and the interaction between them (M*t). Numbers are n=12 Con, n=12 Ob, n=11 Ob-Met.*

There was no significant effect of the maternal environment on glycaemia during an ipGTT (Figure 5.24), suggesting no effect of maternal obesity or the metformin intervention on glucose tolerance.

5.3.8.2 *Serum analyses*

There were no differences in fed or fasted serology between 12-month-old Con and Ob offspring, but Ob-Met offspring showed fasting hyperinsulinaemia, systemic IR and enhanced β-cell activity compared to Con offspring (Table 5.8).

Female offspring	Con (n=11-12)	Ob (n=11-12)	Ob-Met (n=10-11)	*p*-value
Glucose (fed, mmol/l) ***from fed siblings***	11.7 ± 0.3 (n=12)	11.2 ± 0.3 (n=12)	11.5 ± 0.7 (n=12)	*0.7223*
Glucose (fasted, mmol/l) ***from fasted siblings***	6.6 ± 0.2	6.5 ± 0.2	6.5 ± 0.2	*0.8968*
Insulin (pmol/l)	101 ± 16	134 ± 17	180 ± 23 [a]	***0.0227***
HOMA-IR	4.30 ± 0.66	5.63 ± 0.76	7.61 ± 1.13 [a]	***0.0298***
HOMA-%B	97 ± 16	130 ± 15	176 ± 18 [b]	***0.0070***
Cholesterol (mmol/l)	2.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	*0.8665*
Triglycerides (mmol/l)	0.97 ± 0.05	1.06 ± 0.06	1.12 ± 0.05	*0.1545*
FFAs (mmol/l)	1.35 ± 0.05	1.28 ± 0.05	1.31 ± 0.05	*0.6229*

Table 5.8 Serological analysis in 12-month-old female offspring.
All data reflects serum from fasted animals, except fed glucose that was taken from siblings at tissue collection. [a]p<0.05, [b]p<0.01 vs Con offspring, one-way ANOVA with Tukey's multiple comparison test. FFAs = free fatty acids.

5.3.9 Age-related changes in WAT cellularity

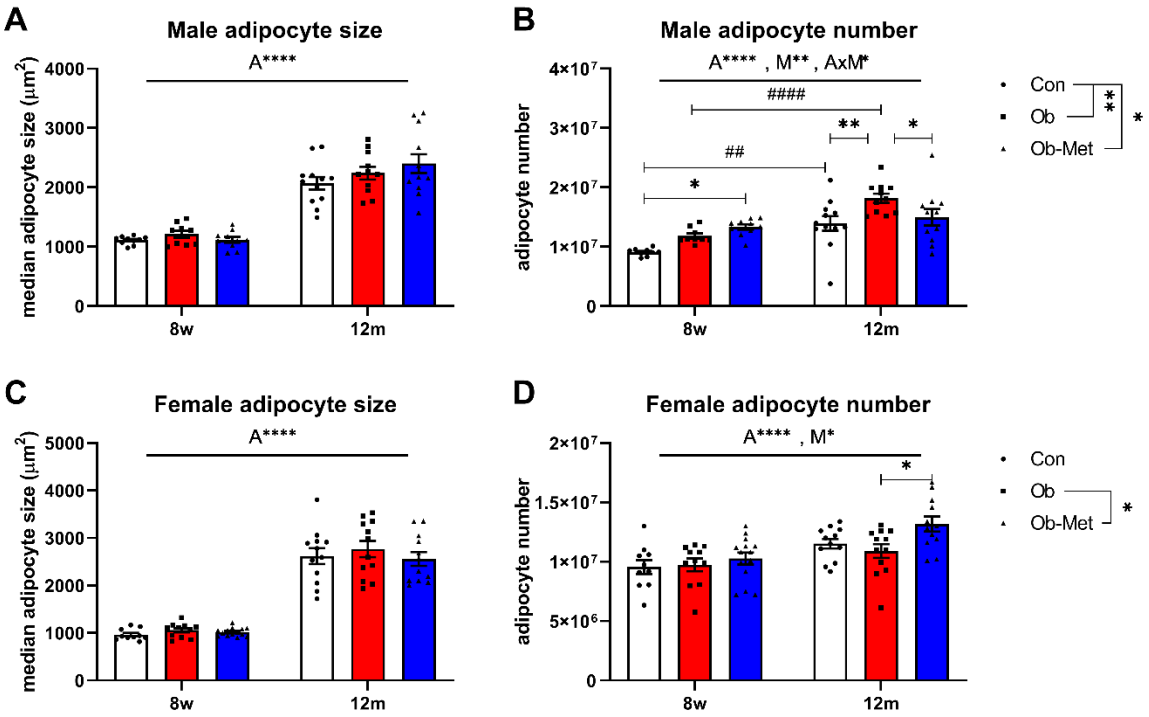

Figure 5.25: Differences in adipose tissue cellularity between 8 weeks and 12 months of age.
*Right: adipocyte size in A) male eWAT and C) female gWAT. Left: estimated adipocyte number in A) male eWAT and D) female gWAT. Box: results from two-way ANOVA for the effect of age (A), the maternal environment (M) and the interaction between them. *p<0.05, **p<0.01, ****p<0.001 for group effects, main effects and interactions. ##p<0.01, ####p<0.0001 for age effects using Tukey's multiple comparison test. White bars = Con, Red bars = Ob, Blue bars = Ob-Met offspring. Numbers for male 8w offspring are n=9-10 Con, n=9 Ob, n=10 Ob-Met; for male 12m offspring n=12 Con, n=11 Ob, n=11-12 Ob-Met; for female 8w offspring n=10 Con, n=11 Ob, n=14 Ob-Met; and for female 12m offspring n=12 in all groups.*

Ageing increased both median size and number of adipocytes in male and female offspring, indicating WAT expansion with age involves both adipocyte hypertrophy and hyperplasia (Figure 5.25).

The maternal environment significantly affected adipocyte number. In males, cell number increased with age in Con and Ob offspring, more drastically so in Ob offspring leading to significantly increased cell number at 12 months. In contrast, male Ob-Met offspring showed hyperplasia already at 8 weeks of age which did not increase further with age, indicating an early hyperplasia phenotype in these offspring (Figure 5.25B).

There were no differences in adipocyte number in young female offspring, but female Ob-Met offspring showed exaggerated hyperplasia with age leading to the highest adipocyte number at 12 months (Figure 5.25D), coinciding with the development of excessive adiposity in these offspring. Prenatal metformin exposure thus promotes an early hyperplastic response in male offspring but late hyperplastic effect in female offspring.

5.4 Discussion

The aim of this chapter was to determine the long-term adiposity and metabolic phenotype of offspring exposed to maternal obesity and metformin intervention during gestation. Exposure to maternal obesity caused increased adiposity in male but not female offspring which was not prevented by the metformin intervention. Although both male and female Ob-Met offspring exhibited adiposity during the life-course, the intervention had sex- and age-specific effects on WAT biology and metabolic health, with the most pronounced effects observed in female Ob-met offspring at 12 months of age.

5.4.1 Effects of maternal obesity on aged offspring

Few studies report on the effects of maternal obesity in aged animal offspring. In this study, both male and female mice (irrespective of experimental group) continued to gain weight up to 49 weeks of age. Maternal obesity led to increased fat mass in both male and female offspring up to 6 months of age prior to changes in body weight. Consistent with observations in Chapter 4, the development of adiposity occurred slightly earlier in male offspring. While until 12 weeks of age body weight gain was largely explained by an increase in lean mass, after 12 weeks offspring from all groups accumulated mostly fat mass. This age-related increase in body weight and fat mass is consistent with data in both rodents and humans (396,456,468,471), and the accompanying increase in weekly caloric intake with ageing most likely reflects a compensatory increase in energy required to sustain the metabolic needs of larger older mice. In male offspring aged beyond 6 months, maternal obesity caused excessive weight gain and increased WAT expansion characterised by adipocyte hypertrophy and hyperplasia. The finding of increased adiposity in Ob offspring compared to controls is in accordance with a study by Rodríguez-González *et al.* that found increased body weight and fat mass at day 450 in male rat offspring of obese dams (468). WAT expansion in Ob males was associated with increased pro-inflammatory gene expression at 12 months but not 8 weeks of age (Chapter 4), which is consistent with the roles of ageing and obesity in promoting a pro-inflammatory environment (472,473).

In contrast, no difference in adiposity or WAT cellularity was observed in aged female offspring in this study. Instead, female offspring seemed relatively protected against the development of obesity later in life, indicating sex-specific effects of programming by maternal obesity on aged mouse offspring that cannot currently be explained. Liang *et al.* described dilution of the overweight phenotype in female offspring of HFD-fed dams between 6 and 12 months, which was ascribed to accelerated ageing (93). Alternatively, as female rats in the Rodríguez-González *et al.* study were not heavier until 450 days of age (468), further ageing might be required for body weight to diverge in our study. Female Ob offspring were also relatively protected from WAT dysfunction in the current study as indicated by

absence of increased CLS (although some pro-inflammatory changes were observed at the molecular level). This is consistent with the sexually dimorphic propensity to WAT inflammation discussed in Chapter 4.4.6.

Our group previously reported accelerated ageing in offspring exposed to maternal low-protein diet with or without cross-fostering to control-fed dams (465–467). The current study was not designed to assess accelerated ageing directly, but the development of adiposity and WAT inflammation in male Ob offspring could be phenotypic signs of accelerated ageing. The age at which animals reach their maximum weight before an age-related decline in body weight can be used as a marker for premature ageing. However, as C57BL/6J mice reportedly reach their maximum weight around 50 weeks of age (89), this analysis could not be carried out in the current study as body weight was only measured until 49 weeks of age. However, previous studies with maternal HFD-feeding found no obvious difference using this parameter when offspring were fed chow (89). The observation of decreased brain weight in both male and female offspring could be a sign of accelerated brain ageing as brain weight is shown to decline with age (474). Decreased brain weight was also observed in 18-month-old rat offspring of sucrose-fed dams, which was accompanied by hippocampal oxidative stress and cognitive defects (475). However, altered brain weight may also reflect programmed impairments in neurogenesis as observed in 3- and 6-month-old mouse offspring of obese HFD-fed dams (463). Unilateral vastus muscle weight was also decreased in Ob male offspring, potentially indicative of sarcopenia. Nevertheless, future experiments investigating molecular markers of cellular ageing are required to conclusively determine if maternal obesity introduces an accelerated ageing phenotype in this model.

5.4.2 Metformin intervention effect on male offspring

The metformin intervention did not correct the early adiposity phenotype observed in male offspring following exposure to maternal obesity. Ageing did dampen the excessive adiposity phenotype as observed in 8-week-old Ob-Met offspring, since most WAT depot weights in Ob-Met were no longer significantly increased compared to Con offspring by 12 months of age. This dilution of WAT phenotype in aged male metformin-exposed offspring has previously been reported by Gregg *et al.* in both their lactational and gestational metformin studies (307,392), although in these studies metformin had a protective effect on offspring adiposity. Nevertheless, in the current study the metformin intervention introduced an inflammatory WAT phenotype associated with CLS and pro-inflammatory gene expression indicative of WAT dysfunction. The specific upregulation of M1 (*Cd11c, Tnf*) and migratory markers (*Mcp1*) alongside the general macrophage marker *F4/80* indicates recruitment of pro-inflammatory macrophages to hypertrophied WAT. The expression of pro-inflammatory genes increased with eWAT mass, consistent with previous reports of correlations between inflammatory gene expression and adiposity (403). In accordance with studies in genetically

obese mice (433), this relationship was not linear suggesting that at high adiposity levels absolute fat mass is no longer the main determinant of pro-inflammatory gene expression. This agrees with the notion that after macrophage infiltration is initiated in hypertrophic WAT, inflammation can be self-sufficient and factors produced by dysfunctional WAT and activated macrophages are more important for the propagation of inflammation (433). This could explain why inflammation is most severe in Ob-Met male offspring even though WAT depot weights are highest in Ob offspring. Moreover, a degree of inflammatory memory is observed in formerly obese mice (476), suggesting potential for the increased inflammation in 8-week-old Ob-Met offspring to persist until old age.

The finding of excessive WAT inflammation in Ob-Met offspring is important because of its relationship with IR and metabolic health (reviewed in Chapter 4). Indeed, *Glut4* expression was downregulated in eWAT from Ob-Met males indicative of local IR. Moreover, the strong correlations between pro-inflammatory gene expression and serum cholesterol, insulin, HOMA-IR and β-cell activity suggest WAT inflammation could be detrimental to metabolic health at the systemic level. The relationship between WAT inflammation and insulin homeostasis is well-described (see Chapter 4), with WAT inflammation thought to contribute to the development of IR while consequent hyperinsulinaemia acts to exaggerate the pre-existing pro-inflammatory state (404). Interestingly, the highly significant correlation between insulin, insulin-derived indices and the adipocyte-produced chemokine *Mcp1* supports the finding that MCP-1 production retains responsiveness to insulin even in insulin resistant adipocytes, while simultaneously able to contribute to WAT IR (432). A relationship between plasma cholesterol and pro-inflammatory macrophages was previously reported in humans although the mechanism remains unknown (477). Rather than reflecting a causal relationship, elevated cholesterol and inflammation might reflect the same underlying adiposity phenotype. This is supported by the absence of correlations between serum cholesterol and inflammatory gene expression in genetic models of hyperlipidaemia except for when serum lipids correlated to fat mass, suggesting there may be no causative role of hyperlipidaemia per se and increased lipids may instead be a sign of WAT expansion (433).

Despite increased inflammation and accompanying correlations, metabolic abnormalities were not observed in 12-month-old male offspring and no effect of the metformin intervention was seen. This is in accordance with previous work from our laboratory in 12-month-old male offspring of obese dams where serum cholesterol, insulin and glycaemia during an ipGTT were not different. As outlined in section 5.1.2, the age of offspring heavily influences outcomes. Normalisation of IGT between 9-12 months of age has previously been reported in offspring of maternal HFD-fed mouse pregnancy (464). Moreover, age-related effects of metformin intervention during chow-fed gestation or lactation were previously shown to dilute with age in male offspring (307,392). Since in our model male offspring of

obese dams show glucose intolerance at 6 months of age (470), metformin intervention might affect offspring metabolic health at this age instead. Further experiments investigating serum metabolites or glucose tolerance at either younger (6 or 9 months) or older ages (18 months) are thus required to conclusively determine intervention effects on metabolic health.

While Con and Ob offspring showed significant increases in estimated epididymal adipocyte number between 8 weeks and 12 months of age, this was not observed in Ob-Met offspring. Adipocyte turnover from differentiating preadipocytes has been demonstrated to occur throughout life and a degree of hyperplastic capacity is maintained in adult WAT as an adaptative mechanism when hypertrophy is insufficient (395). Because Ob-Met offspring already showed increased adipocyte number by 8 weeks of age, this suggests they reach their limit of WAT hyperplastic expansion capacity relatively early in life. This inability to elicit compensatory hyperplasia in old age may be a sign of restricted WAT expansion. When accompanied by increasing body weight (as seen in this study), this could lead to ectopic lipid deposition, potentially explaining the significant increase in fasted liver and muscle weights compared to Con and Ob offspring, respectively (Figure 5.26, left side). Accordingly, increased liver weight was previously observed in 20-week-old offspring of metformin-treated chow-fed dams accompanied by increased hepatic expression of the *Insig1* gene involved in lipogenesis (301). Female offspring of obese or OE-NPY dams also showed hepatomegaly by 60 days and 7 months of age, respectively (302,359). In contrast, decreased liver weight was seen in metformin-exposed offspring of mice fed HFD exclusively in pregnancy (303), although as outlined in 5.1.4 the results of this study contrast heavily with ours. Tong *et al.* described decreased rather than increased lipid deposition in skeletal muscle of 60-day-old offspring born to obese dams treated with metformin during gestation and lactation (304), although they partly attributed these beneficial effects to lactational metformin exposure which is an important difference in study design between their work and ours. Moreover, these offspring were much younger therefore complicating extrapolation of these findings to data in the current study. It would be important in the future to investigate lipid content in offspring livers and skeletal muscle to test this hypothesis of restricted WAT expansion and ectopic lipid deposition with prenatal metformin in male offspring (Figure 5.26).

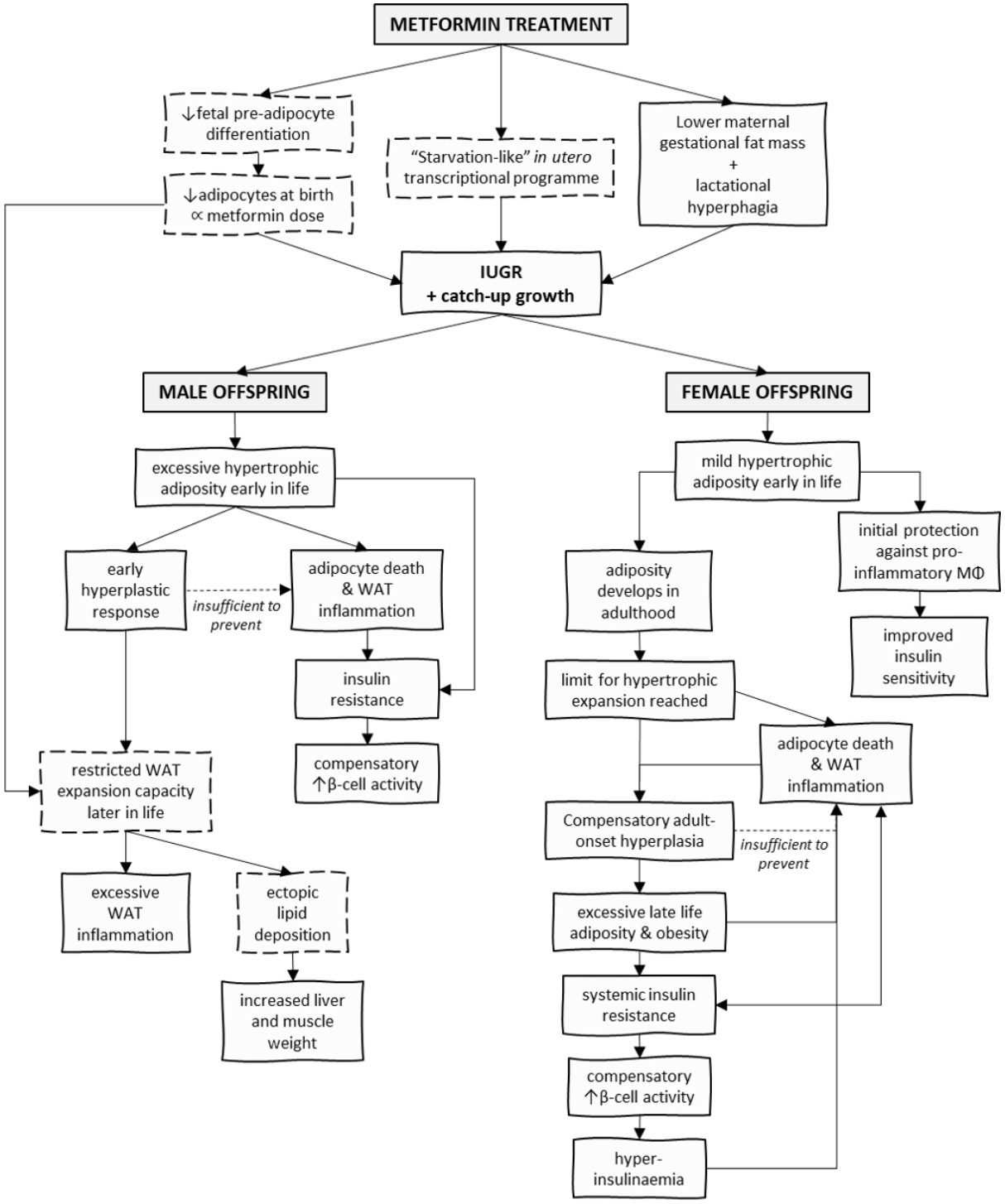

Figure 5.26: Hypothesised action of prenatal metformin exposure on offspring adipose tissue and metabolic health. *Maternal metformin treatment during gestation may accidentally introduce an intrauterine growth restriction (IUGR) and catch-up growth phenotype in exposed offspring. In males this leads to early life hypertrophic and hyperplastic adiposity with white adipose tissue (WAT) inflammation, preventing late-life hyperplasia and resulting in restricted WAT expansion, excessive WAT inflammation and ectopic lipid deposition. In female offspring adiposity is introduced later in life, resulting in hyperplastic obesity with WAT inflammation and metablic abnormalities in aged offspring. Solid lines: evidence presented in this study. Dashed lines: not directly assessed in this study.*

5.4.3 Metformin intervention effect on female offspring

As for male offspring, the metformin intervention did not affect the increased fat mass in female offspring of obese dams up to 6 months of age. However, from 6 months of age female Ob-Met offspring diverged in body weight, gaining significantly more weight and ultimately becoming heavier than both Con and Ob offspring. Weights of WAT and BAT depots were also significantly increased in Ob-Met offspring at 12 months of age. The metformin intervention thus introduced an adiposity phenotype separate to that associated with maternal obesity. This was not related to a detectable change in caloric intake, making it unlikely that basal hyperphagia underlies the drastic difference in adiposity. The increased adiposity could result from alterations in energy expenditure or nutrient assimilation in the intestines. Neither were assessed in this study, but alterations in BAT thermogenic capacity and intestinal microbiota have previously been reported with early life metformin exposure (302,345).

The adiposity phenotype in the current study is stronger than previously reported by Salomäki *et al.* in female offspring from chow-fed pregnancies, where only the mesenteric WAT depot was increased in weight (301). Instead, the data look more similar to those obtained for female offspring of OE-NPY dams who show exaggerated body weight gain and a global adiposity phenotype with elevated weight of all visceral WAT depots as well as BAT (302). Specific expansion of subcutaneous depots following prenatal metformin with putative beneficial effects on offspring health was proposed by Rowan *et al.* based on initial follow-up results from the MiG trial (298). This claim however is not substantiated with any evidence, and results from the current study as well as longer-term follow-up from the MiG trial (388) do not support this. There was no apparent depot-specificity with regard to adiposity in Ob-Met offspring in the current study with increases observed in both visceral and subcutaneous depots as well as BAT. Although we see a decrease in VAT:SAT ratio (indicative of more drastic subcutaneous WAT expansion) this is unlikely to explain the phenotype in Ob-Met offspring, that besides adiposity is characterised by hyperinsulinaemia and WAT dysfunction.

From 8 weeks to 12 months of age, gWAT expansion was associated with both hypertrophy and hyperplasia in all groups. However, the excessive WAT expansion in Ob-Met females specifically resulted from exaggerated hyperplasia rather than hypertrophy as cell size was not different in aged offspring. As the capacity for hypertrophic expansion is restricted after a certain adipocyte size is reached (395), this suggests that in Ob-Met offspring the hypertrophic limit has been exceeded and compensatory hyperplasia was induced (Figure 5.26, right side). Nevertheless, this may not have been sufficient to cope with expansion demands, resulting in WAT dysfunction as evidenced by the increased pro-inflammatory signature of female Ob-Met gWAT (CLS density and pro-inflammatory gene expression). Increased macrophage invasion was related to an increase in CLS number (not size)

indicative of increased adipocyte death in female Ob-Met WAT. Strissel *et al.* neatly illustrated the temporal relationship between adipocyte size, WAT inflammation and hyperplastic remodelling (409). In their study, HFD-feeding in adult mice was associated with a progressive increase in adipocyte size and the number of CLS-surrounded adipocytes for the first 12 weeks, followed by a drastic drop in adipocyte number coinciding with the highest CLS prevalence (i.e. adipocyte death rate) after 16 weeks, leading ultimately to a striking increase in newly formed small adipocytes and partial resolution of the CLS phenotype after 20 weeks of HFD (409). Therefore, increased adipocyte death in Ob-Met WAT may in fact have initiated the hyperplastic response leading to WAT hypercellularity observed in these offspring. Moreover, a higher contribution of both larger (hypertrophic) and smaller than average (necrotic and/or newly formed) adipocytes would explain the lack of differences in median adipocyte size in Ob-Met offspring.

As WAT function was only investigated at two ages, it is currently unclear at what age the hyperplastic response occurred in Ob-Met WAT. The lack of difference in adipocyte size distribution would argue that hyperplasia took place earlier in life. However, due to the great abundance of cells investigated over a large size range we cannot exclude that more subtle differences in cellularity were masked by this approach. Since Ob-Met offspring only diverged in body weight from Ob offspring later in life it is likely that the excessive hyperplasia took place between 9-12 months of age. Recent hyperplasia may be supported by the decreased expression of *Leptin* and *Pparg* mRNA in Ob-Met gWAT compared to Ob and Con offspring. These markers of late stage adipogenesis may be downregulated in immature adipocytes that have not yet progressed to the terminal differentiation stage following recent hyperplasia (478). This would also explain why the well-established relationship between *Leptin* production and gWAT weight is dysregulated exclusively in Ob-Met females. On the other hand, as PPARγ also acts as an early determinant for commitment to the adipocyte lineage, the decreased expression of *Pparg* could reflect exhaustion of PPARγ-dependent adipogenic capacity (as is seen in aged obesity)(479), suggesting hyperplastic gWAT expansion may have occurred earlier in life. Alternatively, since targeted deletion of *Pparg* in mature adipocytes results in WAT inflammation, adipocyte death and concomitant hyperplasia (480), the downregulation of *Pparg* may also be related to the pro-inflammatory gWAT phenotype, although expression of *Pparg* and pro-inflammatory genes did not correlate in female offspring (not shown). Lastly, absolute weights of liver and vastus muscle tended to be increased in female Ob-Met offspring. As in male offspring, this might indicate the beginning of ectopic lipid deposition outside of WAT. Since PPARγ is known to promote lipid storage in adipose tissue and deletion of *Pparg* induces a lipodystrophy-like phenotype (400), the decreased expression of *Pparg* in Ob-Met gWAT may simply indicate restricted hypertrophic WAT expansion

consistent with a necessity to switch to hyperplasia. Further research is required to determine the timing and regulatory pathways of the hyperplastic response in Ob-Met females.

Ob-Met females also displayed systemic IR and hyperinsulinaemia associated with enhanced β-cell activity at 12 months of age, which was not observed in Ob offspring. Interestingly, this is a reversal of the phenotype at 8 weeks of age where an improvement in insulin sensitivity was observed in Ob-Met offspring. Obesity and increased fat mass are strongly associated with IR and hyperinsulinaemia (5). Therefore, it is likely that the abnormalities in insulin homeostasis in 12-month-old Ob-Met offspring are secondary to the development of obesity (Figure 5.26). This may be at least partly mediated by the pro-inflammatory signature of gWAT as expression of the M1-type macrophage marker *Cd11c* (and to a lesser extent *Tnf* and *Mcp1*, see Appendix E) was highly correlated with insulin, HOMA-IR and gWAT mass. Both Ob and Ob-Met offspring had increased expression of *Glut4* mRNA, which strongly correlated with serum insulin, HOMA-IR and HOMA-%B suggesting compensatory upregulation of *Glut4* mRNA to compensate for gWAT IR. In rodent adipocytes, glucose uptake and metabolism in response to insulin stimulation were shown to promote *Leptin* expression and secretion (481). Moreover, *Leptin* mRNA expression in subcutaneous WAT was related to glucose disposal during euglycaemic-hyperinsulinaemic clamping in humans, indicating a relationship between peripheral insulin sensitivity and leptin expression (482). The decreased and dysregulated expression of *Leptin* in Ob-Met females could therefore also be the result of local adipose tissue IR. Alternatively, autoregulation of *Leptin* mRNA expression involving a negative feedback loop has been demonstrated in rodent WAT (483), suggesting that potential hyperleptinaemia resulting from excessive adiposity in Ob-Met offspring may have contributed as well.

5.4.4 Sexual dimorphism in the effect of metformin

Until 6 months of age there were no sex-specific effects of metformin intervention during obese pregnancy, with both male and female Ob-Met offspring showing similar adiposity as their Ob counterparts. However, sexually dimorphic responses to maternal metformin treatment emerged after 6 months of age, indicating that long-term follow-up is crucial when investigating early life interventions. In contrast to the phenotype at 8 weeks of age, adiposity in aged females was more strongly affected by the metformin intervention than in males, and deterioration of metabolic health was observed in aged female Ob-Met offspring only. Interestingly, although the metformin intervention introduced hyperplasia in both sexes at some point in life, sex-specific differences were observed in the timing of when hyperplasia occurred (which may reflect different mechanisms as described in sections 5.4.2 and 5.4.3). Prenatal metformin exposure therefore had adverse effects on long-term body composition and metabolic health in female offspring, but whether the intervention is beneficial, deleterious or without effect in male offspring remains to be determined. Sexual

dimorphism has often been described in the context of developmental programming and could be related to biologically underpinned differences in vulnerability of relevant tissues during development or differential effects of sex steroids in postnatal life (306,484,485). Alternatively, as values for insulin and derived indices tended to be higher in male compared to female offspring regardless of experimental group, additional maternal environment effects might be masked by a stronger ageing effect in male offspring. Although not directly assessed, ageing increased values for serum insulin, HOMA-IR and β-cell activity as well as glucose excursion curves following ipGTT challenge in 12-month-old offspring compared to the 8-week-old mice in the previous chapter, consistent with reported age-related increases in IR and IGT in both humans and rodents (471,486). Interestingly, a delayed glucose peak (at 30 rather than 15min) was observed in male but not female aged offspring, suggesting relative protection against age-related glucose intolerance in female offspring. This was previously suggested in a study of C57BL/6J mice where ageing induced glucose intolerance between 6 and 18 months of age in male but not female mice (471). It should also be mentioned that our study period coincides with the onset of estropause in females, which is initiated between 9-12 months of age leading to complete anovulation between 12-18 months of age (484). A recent loss of the protective effects of oestrogen might thus have exacerbated the phenotype in female Ob-Met offspring.

Most studies investigating the effects of early life metformin intervention see similar (yet not identical) outcomes in both sexes, although few have investigated offspring at 12 months of age (307,392). Adiposity was similarly increased with metformin in both sexes in the chow-fed study by Salomäki *et al.*, although in contrast to the current study glucose tolerance was only impaired in male offspring (301). In studies where prenatal metformin had a protective effect on fat mass following a HFD challenge, this protection was stronger in 4/6-month-old female compared to male offspring (303,307), suggesting adult female WAT may be more sensitive to programming by metformin exposure. Gregg *et al.* report effects on insulin homeostasis in young males that dilute with age, while females were less affected at a young age with changes in metabolic function appearing with increasing age (392). Although in this study metformin was beneficial for offspring metabolic health, the temporal pattern is similar to the data presented in this thesis. Interesting, the 2016 study investigating gestational metformin treatment to OE-NPY dams is the only study to date that sees clear sex differences (in addition to ours). In fact, these are similar to those reported in this thesis: prenatal metformin introduced global adiposity and glucose intolerance in female offspring but no difference in adiposity or metabolic health was observed in male offspring (302). The OE-NPY dams used to generate offspring were normoglycaemic at conception, but age-matched non-pregnant mice showed tendency for IGT at the age of mating. Therefore, a diabetic environment initiated during (but not before) pregnancy may be responsible for the sex-specific effects of maternal metformin

treatment. Interestingly, it was shown that repeated metformin injections in the first week of life shortened lifespan in female mice (indicative of accelerated ageing) whereas male mice showed increased longevity, indicating clear opposing age-related sex-specific effects of neonatal metformin exposure (487).

Explanation of the sex-specific findings in this thesis are unclear at present, but the data clearly supports the importance of investigating both sexes. Interestingly, the finding of WAT inflammation in Ob-Met offspring was consistent across ages and sexes, suggesting this may be an effect intrinsic to fetal metformin exposure. This phenotype could be related to restricted WAT expansion in metformin-exposed offspring although this remains to be determined.

5.4.5 Strengths and limitations

The main strength of this study is the long-term follow-up of both male and female offspring exposed to maternal obesity and metformin intervention, thus providing evidence about the development of obesity across the life-course. One major limitation was the unfortunate problems with the TD-NMR machine in our facility, which became defective mid-way through the study. This prevented longitudinal analysis of adiposity and complicated investigation into the timing of whole-body adiposity development. Another limitation was the absence of metabolic phenotyping at time-points earlier than 12 months as this was not feasible due to time constraints associated with the cardiovascular phenotyping carried out simultaneously (Chapter 6). Further studies are therefore required to address these questions.

5.5 Conclusion

Maternal metformin intervention during obese pregnancy does not correct the increased adiposity observed in mouse offspring of obese dams. In male offspring, prenatal metformin exposure was not associated with changes in metabolic phenotype at 12 months of age. In contrast, female offspring exposed to maternal metformin treatment exhibited excessive adiposity, WAT dysfunction and hyperinsulinaemia indicative of systemic insulin resistance at the same age. Moreover, WAT inflammation was observed in both male and female offspring at 12 months of age. Therefore, instead of 'correcting' the adiposity phenotype in offspring of obese pregnancy, metformin treatment may increase metabolic risk factors in a sex- and time-dependent manner. As sex-specific phenotypes emerged beyond 6 months of age, this study highlights the importance of following up offspring of both sexes and throughout the life-course.

5.6 Key findings

- Maternal obesity leads to increased adiposity in aged male but not female offspring
- Metformin intervention does not correct adiposity in aged (12-month-old) male offspring but instead leads to a more severe pro-inflammatory WAT phenotype
- Metformin intervention introduces late-life adiposity associated with WAT inflammation, hyperinsulinaemia and insulin resistance in aged (12-month-old) female offspring
- Follow-up of offspring into old age is crucial to fully understand programmed phenotypes

6 Longitudinal effects on cardiovascular health

6.1 Introduction

6.1.1 The burden of CVD

Cardiovascular disease remains the number one killer in the world, attributing to over 30% of deaths world-wide in 2017 with rates even higher for the developed world (488). Despite improvements in health care leading to some decline in death rates from CVD in the developed world, this decline has slowed in recent years and may even increase due to the increasingly ageing population and the rise in obesity (488). Presence of cardiovascular risk factors (including hypertension), heart disease and cardiovascular mortality increases with advanced age (127,488–492).

6.1.2 Principles of cardiovascular physiology

6.1.2.1 Blood pressure regulation

Arterial blood pressure depends on flow and total peripheral resistance (TPR), with both higher flow and higher resistance leading to increased BP. Cardiac output (CO), the main determinant of systemic blood flow, is regulated through changes in HR, force of contraction and blood volume. TPR is mainly controlled through vasoconstriction and vasodilation regulated by hormonal signalling, sympathetic and parasympathetic innervation and the balance of local vasoconstrictors (e.g. noradrenaline) and vasodilators (e.g. nitric oxide). Vasoconstriction causes an increase in TPR and consequently BP if HR remains constant (indeed, a negative chronotropic response can be a compensatory mechanism to maintain BP in case of increased TPR). TPR is also influenced by blood composition: higher viscosity increases resistance to flow (493).

Normal BP in humans is around 120mmHg for SBP and 80mmHg for DBP. Elevated BP increases afterload on the LV, which is defined as LV wall stress during ejection and reflects the pressure gradient against which the LV has to work to generate sufficient pressure to open the aortic valve (Figure 6.1). In case of hypertension, the LV undergoes compensatory hypertrophy in order to maintain CO. Although initially protective, ultimately pathological LV hypertrophy impairs cardiac function, predisposing to heart failure (HF)(493–495). Indeed, hypertension is a strong predictor for HF in humans (491).

6.1.2.2 Cardiac structure and function

In order to investigate cardiac function *in vivo*, the heart can be imaged using echocardiography (495). Echocardiographic assessment generates both structural and functional data providing information about cardiac function in a physiological setting. This technique is also used in the clinic and therefore a clinically relevant method to research the impact of maternal obesity and metformin intervention in

exposed offspring. Moreover, the technique is non-invasive and therefore allows longitudinal analysis of cardiac function across the life-course in the same animal (495).

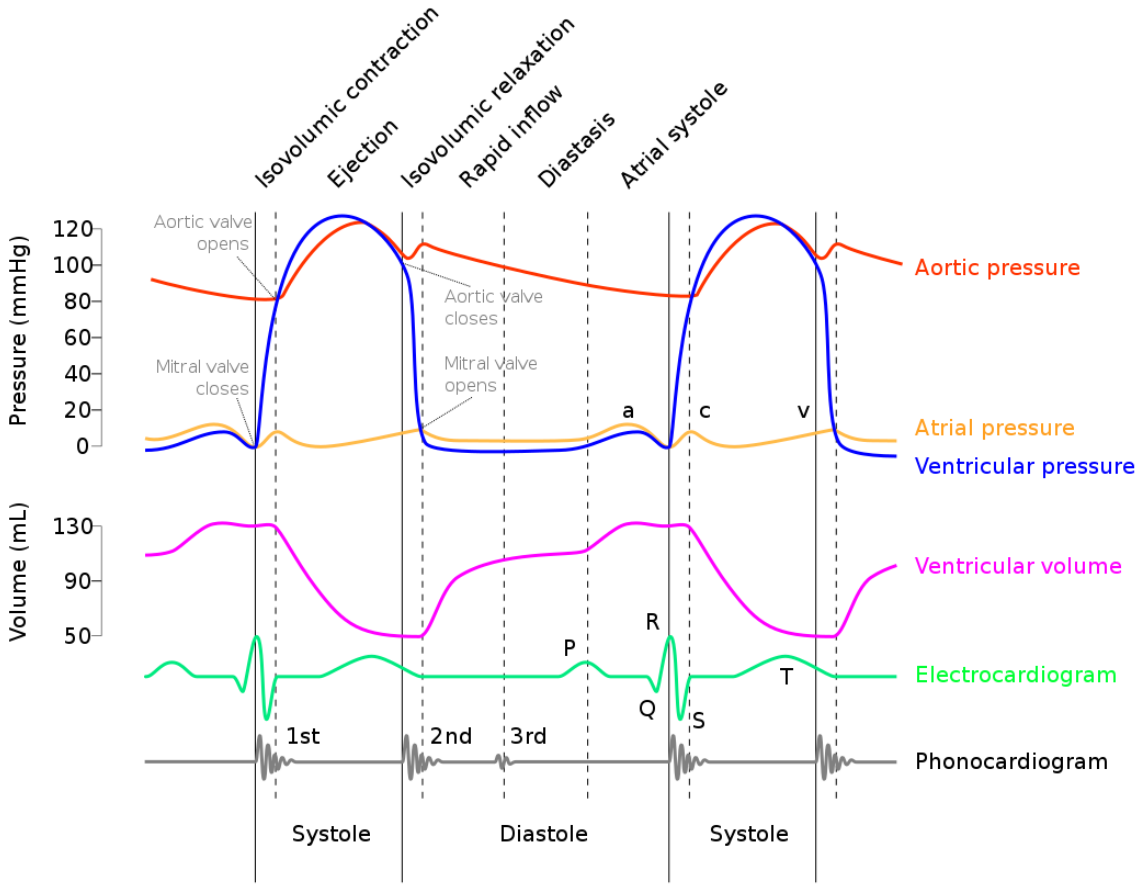

Figure 6.1: Wiggers diagram detailing changes in LV pressure, volume and electrical signal during the cardiac cycle. *Reproduced with permission from https://commons.wikimedia.org/wiki/File:Wiggers_Diagram_2.svg on 20th February 2021 under a CC-BY-SA-4.0 licence.*

6.1.2.2.1 Systolic function

Systole is the phase in the cardiac cycle when ventricles contract. When LV pressure generated during contraction exceeds aortic pressure, the aortic valve opens and blood is ejected from the heart. LV pressure decreases as a result of ventricular emptying and the start of myocardial relaxation, leading to closure of the aortic valve and the start of diastole (Figure 6.1)(493). The LV can easily be visualised by echocardiography and allows measurement of LV dimensions (495). Several parameters are used to assess LV function during systole: ejection fraction (EF) refers to the percentage of LV end-diastolic volume (EDV) that is ejected during systole and can be used as a measure of contractility (493). Similarly, fractional shortening (FS) reflects how much the LV diameter (radial) or length (longitudinal FS) is decreased during contraction (495). Lastly, the difference between EDV and end-systolic volume is the stroke volume (SV) which is used to compute CO (493).

6.1.2.2.2 Diastolic function

Diastole is the phase of the cardiac cycle characterised by ventricular relaxation and filling. Following systole, relaxation of the LV decreases LV pressure. This is not accompanied by a change in volume until LV pressure decreases to below the level of atrial pressure (isovolumetric relaxation), after which the mitral valve opens and filling begins. This consists of a passive early filling phase, followed by a short period of diastasis where little filling occurs after which atrial contraction generates the final push of blood into the LV (Figure 6.1). Ventricular depolarisation then initiates (initially isovolumetric) LV contraction.

Cardiac catheterisation is the gold standard method to measure diastolic pressures (494). However, mitral valve flow dynamics are used to assess diastolic function non-invasively. Pulsed wave (PW) mitral valve Doppler echocardiography (Figure 6.4) generates a waveform from which the peak velocity during early (E-wave) as well as atrial filling (A-wave) can be measured. The ratio between these peaks provides information on filling dynamics: a decreased E/A ratio is indicative of 'impaired relaxation', leading to poor filling and increased reliance on atrial contraction. This often results from a decrease in ventricular compliance. Proposed mechanisms include LV hypertrophy, increased collagen deposits or altered calcium reuptake following contraction, thus preventing efficient relaxation and ultimately filling (494). Worsening diastolic function is accompanied by a compensatory increase in left atrial pressure (LAP) which initially restores the atrio-ventricular pressure gradient leading to 'pseudonormalisation' of the E/A ratio. Ultimately, continued elevation of LAP and progressive LV stiffness lead to an increased E/A, characteristic of progression to 'restrictive' diastolic dysfunction with increased filling pressures and exaggerated passive filling (494). Mitral valve Doppler also allows measurement of the isovolumetric contraction and relaxation times (IVCT and IVRT, respectively). Besides informing on the dynamics of contraction and relaxation, these parameters can be used to calculate the Myocardial Performance Index (MPI) which assesses both systolic and diastolic function (496). Lastly, the deceleration slope and deceleration time (DT) of the E-wave are also used to assess diastolic function in clinical settings (497).

While HF accompanied by systolic dysfunction normally presents with reduced EF (HFrEF), HF of diastolic origin is often characterised by preserved EF (HFpEF)(498). HFpEF is associated with lower mortality than HFrEF but mortality remains increased compared to healthy age-matched controls (499). In most cases of HF with systolic dysfunction, diastolic dysfunction is also observed, although the reverse does not hold true (498). Indeed, isolated diastolic dysfunction without systolic impairment is relatively common (20-30% of the general population show asymptomatic diastolic dysfunction) and increases with known cardiovascular risk factors such as age, hypertension, T2DM

and the metabolic syndrome (498). Pre-clinical diastolic dysfunction is associated with increased risk of progressing to overt HF and ultimately cardiovascular mortality (498).

6.1.2.2.3 The right heart

Left heart failure and right heart failure can but do not always coincide. The presence or absence of right ventricular (RV) dysfunction can provide essential information on the pathology underlying LV dysfunction. Moreover, RV systolic pressures are closely related to LAP due to the low resistance in the pulmonary circulation (500). Although a view of the right heart can be obtained using echocardiography, the easiest way to assess RV function is by generating a PW Doppler waveform at the level of the pulmonary artery (Figure 6.5). This provides information on pulmonary pressures, total flow as well as flow velocities from the RV (501).

6.1.3 Maternal obesity and CVD risk

As outlined in Chapter 1.4.3.3, offspring hypertension is a common finding in animal studies of pre-conception maternal obesity (105). However, *in vivo* cardiac function remains less extensively studied. Echocardiographic evidence of LV hypertrophy and systolic dysfunction have been found in 8-week-old male and female mouse offspring of obese pregnancy, respectively (131). Others have recently reported isolated diastolic dysfunction in 3- and 6-month-old male (but not female) mouse offspring of HFHS-fed dams (502). In a mouse model of maternal HFD-feeding in pregnancy and lactation, cardiac function and structure using echocardiography at 5 months of age was only impaired when offspring were weaned onto a postnatal HFD, although altered isolated cardiomyocyte function was also present in chow-fed offspring exposed to maternal HFD (503). Echocardiographic indices of cardiac dysfunction have also been reported in E16.5 mouse fetuses from HFD-fed obese dams (504). To our knowledge no studies have investigated *in vivo* cardiac function in aged offspring or across the life-course.

In our model, maternal obesity leads to pathological LV hypertrophy in male offspring from as early as 3 weeks of age, characterised by increased cardiomyocyte size and re-expression of fetal genes (128,129,132,133). This was associated with hypertension (increased SBP using tail cuff plethysmography), impaired systolic function (EF, FS and CO using M-mode echocardiography) and cardiac fibrosis in 8-week-old male offspring (128,129). These changes occurred in the absence of differences in adiposity and were of the same magnitude as those introduced by weaning onto postnatal HFD (129). In fact, hypertension and cardiac hypertrophy were highest in offspring exposed to an obesogenic diet both *in utero* and postnatally indicating offspring of obese dams are at high risk of HF if exposed to a 'second hit' (129). By 12 weeks of age, heart weight had normalised but both systolic (left ventricular developed pressure and trend for dP/dt_{max}) and diastolic (left ventricular end-

diastolic pressure [LVEDP] and dP/dt_{min}) impairments remained apparent when primary cardiac function was investigated using the isolated Langendorff heart perfusion preparation (132). These hearts also showed sympathetic dominance and decreased expression of cardiac contractile proteins (132). Proposed mechanisms underlying the cardiovascular effects of an obese intrauterine milieu include increased sympathetic tone, oxidative stress and enhanced mitogenic insulin signalling following selective peripheral IR (132,133). In contrast to young male offspring, cardiac outcomes in female offspring or aged offspring of either sex have not yet been investigated in our model.

In accordance with these animal studies, maternal obesity predisposes offspring to hypertension and increases risk of premature mortality of cardiovascular origin in humans (70,75), consequently contributing to the global burden of CVD. Hence it is vital to investigate the long-term efficacy of clinically relevant interventions (such as maternal metformin treatment) on cardiovascular outcomes in exposed offspring.

6.1.4 Cardiovascular follow-up with prenatal metformin intervention

As described in section 4.1.1, offspring from human RCTs investigating prenatal metformin interventions remain young and therefore cardiovascular follow-up is limited. The first study reporting on cardiovascular outcomes was the small PregMet pilot study, where a trending increase in SBP was found in 8-year-old offspring of PCOS pregnancies treated with metformin (299). However, follow-up from the larger PregMet trial did not replicate these findings as SBP, DBP and HR were unaltered in 5- to 10-year-old offspring, although an increase in HR was found when looking only at boys or at pregnancies complicated by obesity (335). Moreover, a trending increase (p=0.06) in metabolically abnormal obesity was found, a composite outcome that included hypertension and dyslipidaemia as risk factors (335). Follow-up from the MiG trial found no difference in SBP or DBP at 2 years of age (505). The most detailed phenotyping of prenatally metformin-exposed children originates from a relatively recent report from the MOP study looking at 4-year-old offspring of obese non-GDM women treated with metformin or placebo in the UK. The study reported decreased IVRT and left atrial area (without changes in E/A ratio or DT) suggesting improved diastolic function in the absence of differences in systolic function (391). Moreover, even though SBP and DBP were not different between the metformin and placebo arms, metformin-exposed offspring had decreased aortic SBP and pulse pressure, as well as a significant decrease in the augmentation index (a marker of arterial stiffness associated with CVD risk in adults)(391). These data suggest a putative protective effect of metformin exposure in obese pregnancy on offspring cardiovascular health in early childhood.

Very few studies report cardiovascular outcomes in animal models of early life metformin exposure. Tain *et al.* found that metformin treatment during pregnancy attenuated the hypertension observed

in 12-week-old HFD-fed rat offspring of dams fed a high-fructose diet in pregnancy and lactation. This protection was mediated at least partly by downregulating renin-angiotensin system (RAS) activity and oxidative stress in the kidney (380). Metformin also downregulated plasma markers of nitric oxide bioavailability, which is counterintuitive as nitric oxide is normally associated with vasodilation and reduced BP (380). Despite this, other studies reported no difference in relaxation or contractile response in thoracic or abdominal aortas from 11-week-old offspring of chow-fed dams exposed to metformin either in pregnancy alone or in both pregnancy and lactation (343,382). Lastly, a follow-up study using the chow-fed pregnancy cohort generated by Salomäki *et al.* (301) found that at 20 weeks of age (following 10 weeks of HFD challenge), no change was found in aortic intima-media thickness (marker of atherosclerosis) but aortic rings showed a decrease in hyaluronan stain intensity (506). As hyaluronan production increases with vascular pathologies such as diabetic vasculopathy and atherosclerosis, this might be a favourable outcome (506). The results described in the literature therefore suggest a potential vascular phenotype, but no studies have looked into cardiac function. Moreover, the above studies were all performed exclusively in male offspring and effects of a metformin intervention in female offspring therefore remain unknown. It is thus imperative that both male and female offspring exposed to metformin during obese diabetic pregnancy (such as presented in this thesis) are followed up into adulthood to determine the intervention effect on cardiovascular outcomes.

6.1.5 Aims of the chapter

The long-term effect of *in utero* metformin exposure and maternal obesity on the cardiovascular system of offspring remains poorly investigated, especially in female offspring. This chapter therefore aims to characterise the cardiovascular phenotype of male and female mouse offspring of obese diabetic pregnancy exposed to a metformin intervention *in utero.* In order to achieve this aim, BP measurements and echocardiography were performed in male and female offspring of control, obese and obese metformin-treated dams longitudinally at 3, 6 and 12 months of age.

6.2 Methods

Cardiovascular phenotyping was performed longitudinally in the same mouse at 3, 6 and 12 months of age, providing information on changes in cardiovascular outcomes within a single animal across life. Total n-numbers are n=37 males from 37 independent litters (n=12 Con; n=13 Ob [of which one was only included at the 3-month time-point as he did not survive to 6 months of age]; n=12 Ob-Met offspring) and n=36 females from 36 independent litters (*n*=12 in each group). Breeding was continued until cardiovascular phenotyping was performed in *n*=12 animals in each group.

6.2.1 Echocardiography

For *in vivo* assessment of cardiac function, transthoracic echocardiography was performed (Vevo 3100 system with MX400 probe, Fujifilm Visualsonics)[17] under short-term general anaesthesia (1.5-2% isoflurane inhalation following induction at 2%). After induction of anaesthesia, the mouse was placed on a heated platform and anaesthesia was confirmed by pinching the feet. Core temperature and HR were recorded using a rectal thermometer and electrodes. Eyes were covered with lubricant to avoid drying and fur on the chest was removed using hair removal cream. Images were obtained from the mouse in the parasternal long-axis view (PSLAX), modified parasternal short-axis view (mSAX) and apical four-chamber view. Data from recorded clips was analysed using the Cardiac Package from the VevoLAB software version 3.2.6 (Fujifilm Visualsonics).

6.2.1.1 PSLAX B-mode

Cineloops of beating hearts were obtained in PSLAX using B-mode 2D imaging (Figure 6.2). Systolic function parameters were obtained using the B-mode LV Trace tool and measurements from the Generic and Cardiac Packages. For LV Trace measurements, marker points were placed on the left ventricular outflow tract (LVOT) and apex after which the endocardial wall was traced (Figure 6.2, marked by A-C). This was done at the end-diastolic and end-systolic points for several cardiac cycles per clip. The aim was to trace at least 6 cycles in each animal at each age: this was achieved in the vast majority of cases.

To assess aortic distension, the diameter of the ascending aorta was measured in end-diastole and end-systole (Figure 6.2E). Information about the intracardiac dimension of the aorta was obtained by tracing the LVOT in end-diastole (Figure 6.2A, top image).

[17] Echocardiographic imaging was performed by Dr H. Blackmore at 12 weeks whilst JMS was being trained in the technique. JMS performed the echocardiographic imaging at 6 and 12 months, and carried out the preparatory work, image capture and analysis at all ages.

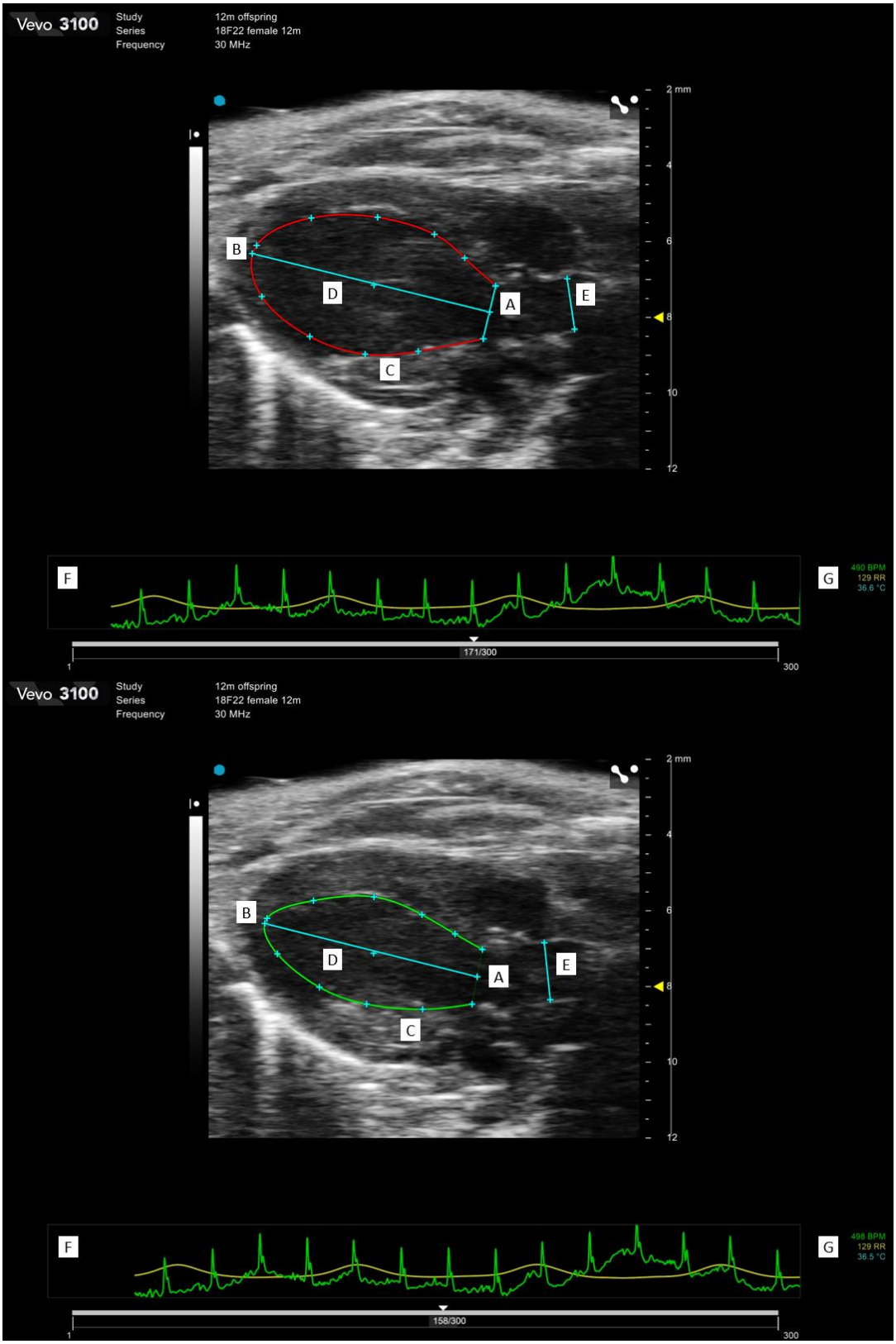

Figure 6.2: PSLAX B-mode analysis.

Top: end-diastole, bottom: end-systole. A) LVOT, first anchor point for LV Trace and measured in diastole. B) apex, second anchor point for LV Trace. C) LV Trace at maximum size in end-diastole (red) and at minimum size in end-systole (green). D) LV length measured between the centre of the LVOT and the apex. E) Ascending aortic diameter. F) electrocardiogram used for respiration gating and determination of end-diastole and end-systole. G) physiologic information from external electrodes and probes (heart rate, respiration rate and core temperature) used to monitor wellbeing and to exclude clips based on heart rate and core temperature.

6.2.1.2 PSLAX M-mode

M-mode clips have higher temporal resolution and are thus superior to B-mode for vertical measurements, provided these are perpendicular to the aorta-apex axis. The internal LV diameter was obtained by tracing the endocardial walls in end-systole and -diastole using the M-mode LV Trace tool (Figure 6.3, top panels). When respiration was too high for an LV Trace, the internal diameters could be traced manually. To prevent overestimation of internal diameter in tilted hearts, the angle of the LV was determined in the B-mode clip closest to the M-mode measurement (Figure 6.3D-E). Hearts with angles >17° were excluded from analysis as this would influence the results.

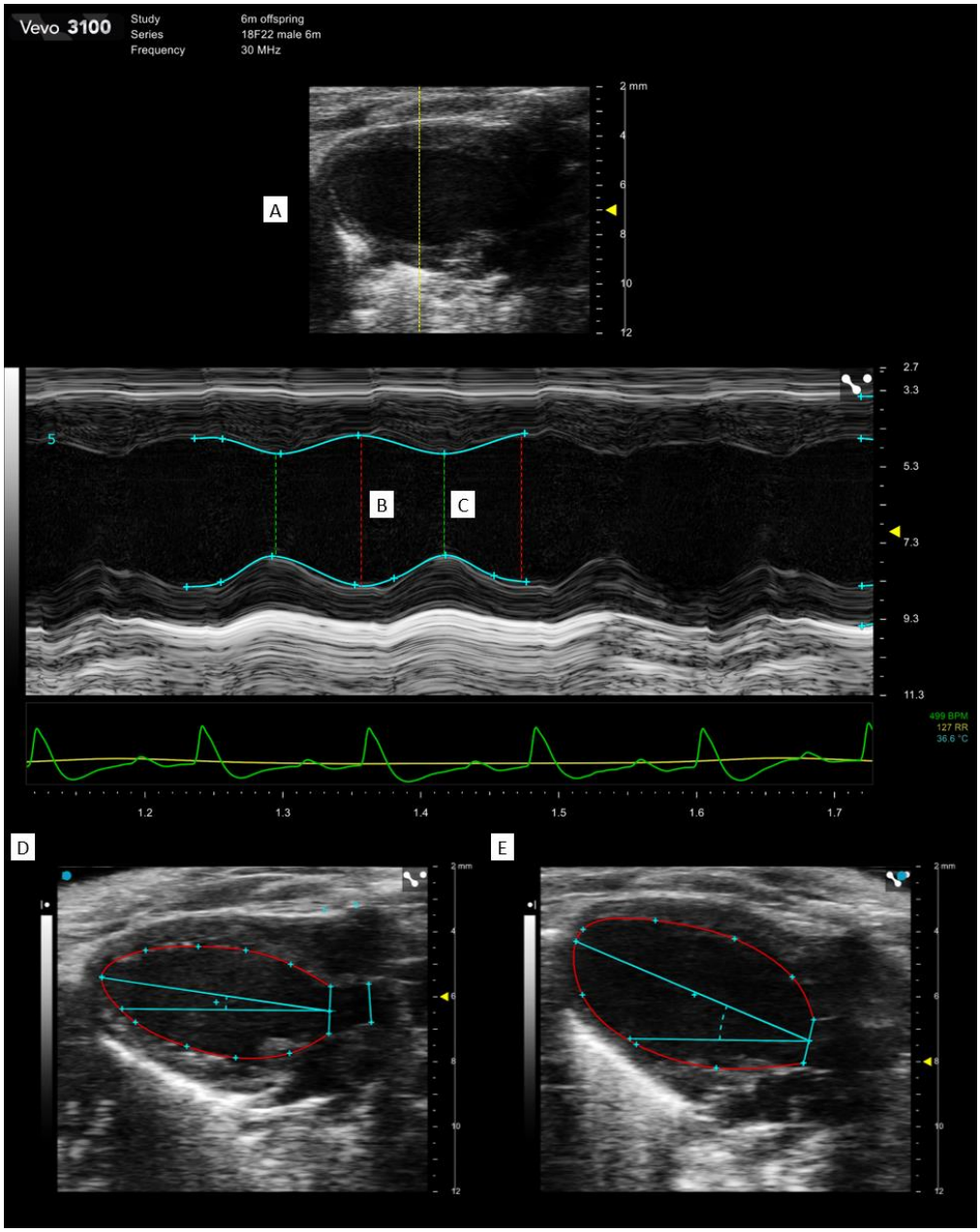

Figure 6.3: PSLAX M-mode analysis using the LV trace tool.
A) Representative B-mode image with yellow line indicating where M-mode was taken. B) End-diastolic and C) end-systolic diameter as determined by the LV Trace measurement. D-E) Determination of heart angle with representative B-mode images of a heart tilted <17° (D, included) and >17° (E, excluded).

6.2.1.3 Mitral valve flow

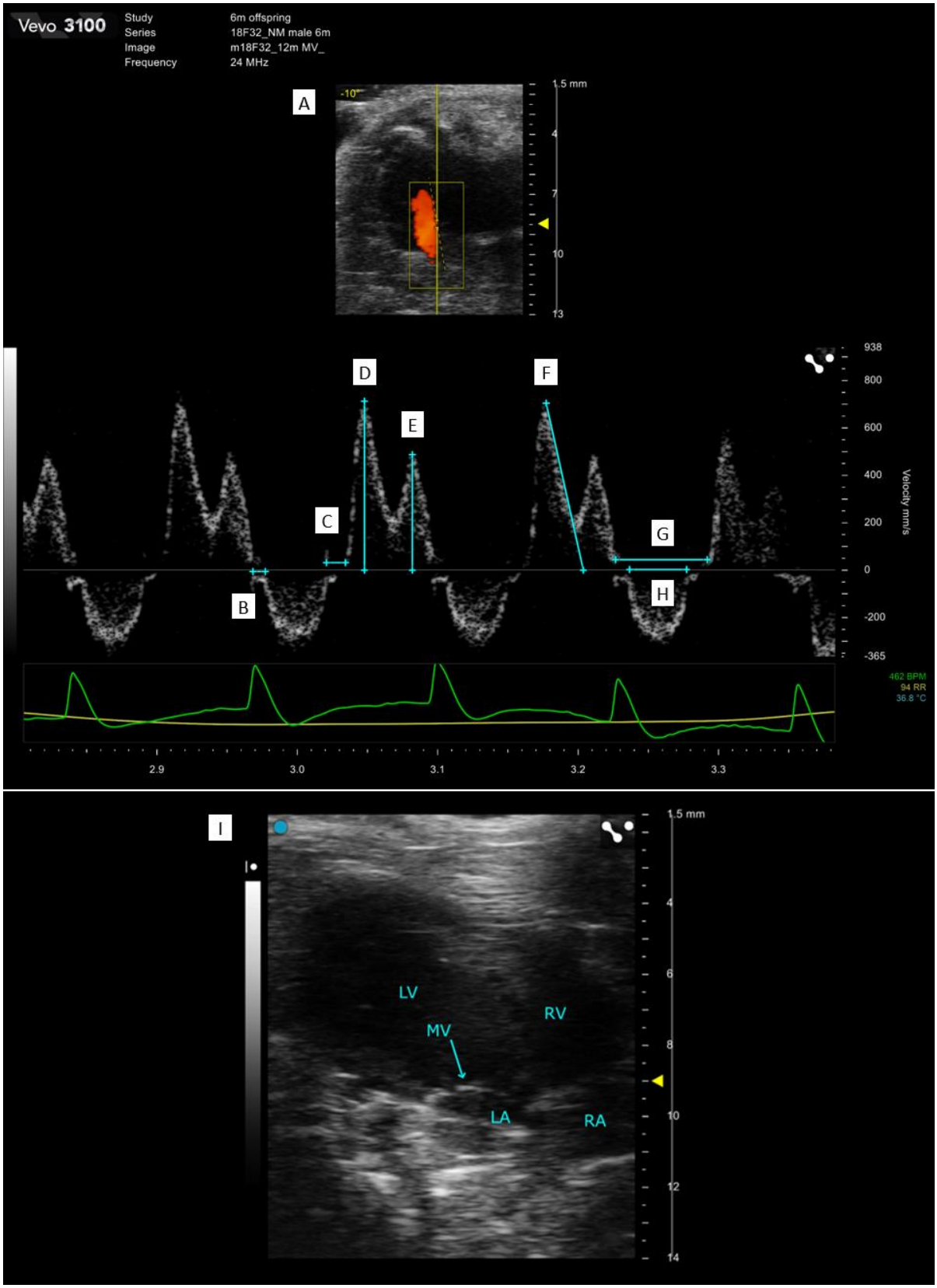

Figure 6.4: Top: mitral valve pulsed wave Doppler.
A) Representative image with colour Doppler. B-H) Measurement of B) isovolumetric contraction time, C) isovolumetric relaxation time, D) E-wave peak velocity, E) A-wave peak velocity, F) E-wave deceleration, G) non-filling time and H) aortic ejection time. I) apical four chamber view corresponding to A). LA = left atrium. LV = left ventricle. MV = mitral valve. RA = right atrium. RV = right ventricle.

Mitral valve flow was used to assess diastolic function (Figure 6.4). First, the apical four chamber view was obtained (Figure 6.4I). Then, colour Doppler was introduced to guide placement of the PW Doppler (Figure 6.4A). From the resulting waveform, several measurements were performed including IVCT, IVRT, E- and A-wave peak velocities, E-wave deceleration slope and DT. The myocardial performance index (MPI) can be derived either from IVCT and IVRT measures or by measuring AET and NFT. Since the intra-animal variability of AET and NFT was lower (and as longer measurements they are less prone to error), these values were used to calculate the MPI.

6.2.1.4 Pulmonary artery valve flow

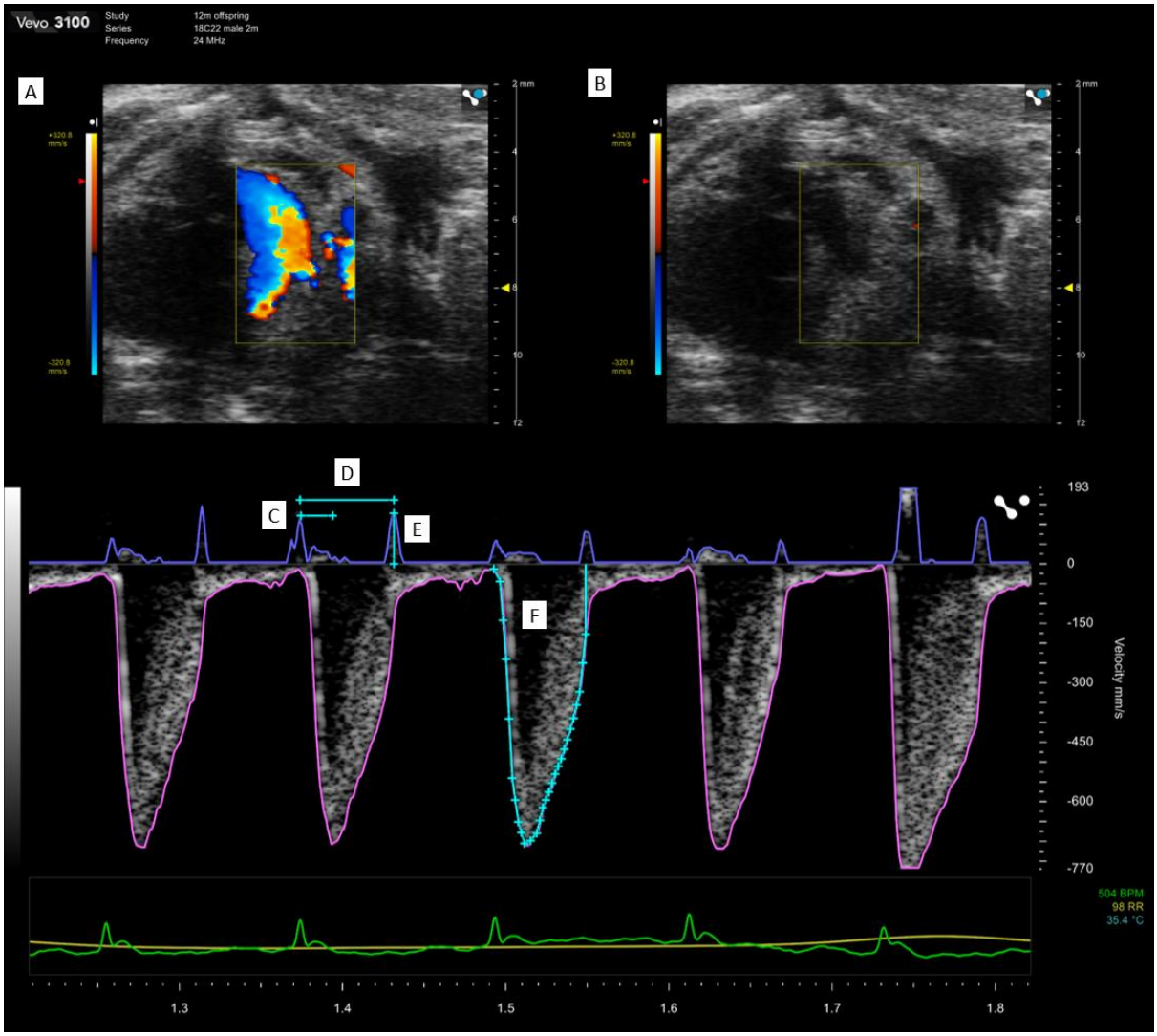

Figure 6.5: Top: pulmonary artery pulse-wave Doppler.
A) Colour Doppler at the level of the pulmonary artery and B) accompanying B-mode view in the modified short axis. Bottom panels: measurement of C) pulmonary artery acceleration time, D) pulmonary artery ejection time, E) pulmonary artery regurgitation peak and F) pulmonary artery velocity time integral, from which mean and peak flow velocity and pressure gradient are derived.

The SAX mode is obtained by rotating the probe 90° while in PLSAX mode. Then, the probe is shifted upwards towards the head of the mouse until the pulmonary artery is in view (Figure 6.5A-B). The PW Doppler measurement is taken at the point of highest flow (in the centre of the jet seen in Figure 6.5A)

and in the direction of flow. This generates the waveform in Figure 6.5, allowing for the measurement of pulmonary artery acceleration time (PAT), ejection time (PET) and regurgitation peak as well as the pulmonary artery velocity time integral (VTI), from which mean and peak flow velocity and pressure gradients are derived. Since alterations in RV morphology in young female offspring of obese dams from a different cohort was observed recently (University of Cambridge PhD thesis Dr Jessica Beeson), pulmonary artery flow was included in the echocardiography protocol at 12 months of age only.

6.2.1.5 Calculations

The measurements derived from the analyses described can be used to compute other parameters (Table 6.1). The VevoLAB system generates most of these calculations automatically, but for this report some calculations were performed manually to ensure consistency across the cohort. These calculations are given below (Equations 11-15).

View	Tool	Measurements	Calculations
PSLAX	**B-mode** **LV Trace**	Area (diastole) Area (systole) Cardiac output (CO) Heart rate (HR) Stroke volume (SV) Volume (diastole) Volume (systole)	
PSLAX	**B-mode** **Linear**	LV endocardial length (diastole) LV endocardial length (systole) LV angle	Sphericity index Fractional shortening (FS, longitudinal)
PSLAX	**M-mode** **LV Trace**	Diameter (diastole) Diameter (systole)	Fractional shortening (radial)
A4C	**Mitral valve** **PW Doppler**	Aortic ejection time (AET) A wave peak velocity E wave peak velocity Isovolumetric contraction time (IVCT) Isovolumetric relaxation time (IVRT) Deceleration slope Deceleration time (DT) Non-filling time (NFT)	E/A ratio Myocardial performance index (MPI)
mSAX	**Pulmonary artery (PA)** **PW Doppler**	PA acceleration time (PAT) PA ejection time (PET) PA velocity time integral (VTI) PA regurgitation peak	PAT/PET Mean PA pressure (MPAP) Mean/peak PA pressure gradient Mean/peak PA flow velocity

Table 6.1: Echocardiographic measures and calculations used in this chapter.

Equation 11: Sphericity index = EDV / (4/3 · πr^3) x 100%

Equation 12: FS (longitudinal) = (diastolic length – systolic length) / diastolic length x 100%

Equation 13: FS (radial) = (diastolic diameter – systolic diameter) / diastolic diameter x 100%

Equation 14: MPI = (NFT – AET) / AET

Equation 15: MPAP = 90 – (0.62 · PAT)

6.2.1.6 *Data processing*

Image quality was assessed at image capture and image analysis, and data from poor quality images was excluded based on observer experience. A subset of images taken at all three ages were independently analysed by another blinded researcher for quality control, and data was not different between observers. Exported data was checked for intra-animal outlier testing using GraphPad Prism 8 as previously described. Data was excluded when obtained from images/cineloops where HR was <400bpm or body temperature deviated by more than 1.5°C from 37.0°C (exclusion limits were set prior to data analysis following optimisation using this and previous cohorts).

6.2.2 Non-invasive tail cuff plethysmography

For *in vivo* assessment of BP, mice were subjected to non-invasive restraint tail cuff plethysmography (BP-200 system, Visitech) for three days in a row (2 training days and 1 measurement day). The mice were placed onto a heated platform and allowed to acclimatise for a few minutes before inflation of the cuff. Following 5 acclimatisation cycles, 15 measurements were taken per animal (Figure 6.6). Data was only included if more than 10 successful readings could be obtained and within-animal variation was less than 10%. In the rare case measurements data within such limits could not be obtained on day 3, data from the second training day was used if this fulfilled the criteria. As previous work using our model demonstrated changes in BP via telemetry during the night-time active phase only (87), tail cuff plethysmography was performed between 4-5PM to obtain data at the start of the waking phase.

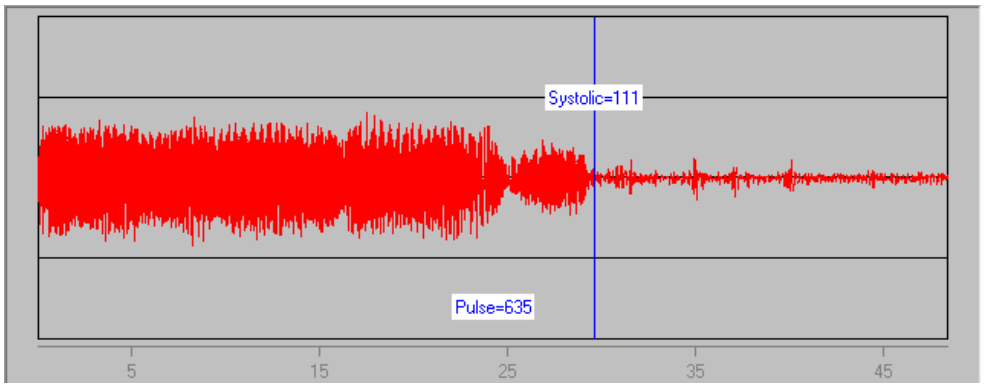

Figure 6.6: Representative image of pulse rate and SBP measurement using tail cuff plethysmography.

6.2.3 Statistical analysis

Data were analysed using Prism 8.0 (GraphPad). For data plotted as a time-course, repeated measures mixed effect modelling was used testing the effects of age, maternal environment and the interaction between them. Post-hoc testing was performed if $p<0.1$ for either main effects or interactions. When one time-point was assessed, one-way ANOVA (parametric) or Kruskal-Wallis test (non-parametric) was used. Data are presented as mean ± SEM or median [interquartile range]. A p-value <0.05 is considered statistically significant. For linear regression, $p<0.01$ is considered statistically significant.

6.3 Results

6.3.1 Cardiac function in male offspring

6.3.1.1 Left ventricular systolic function

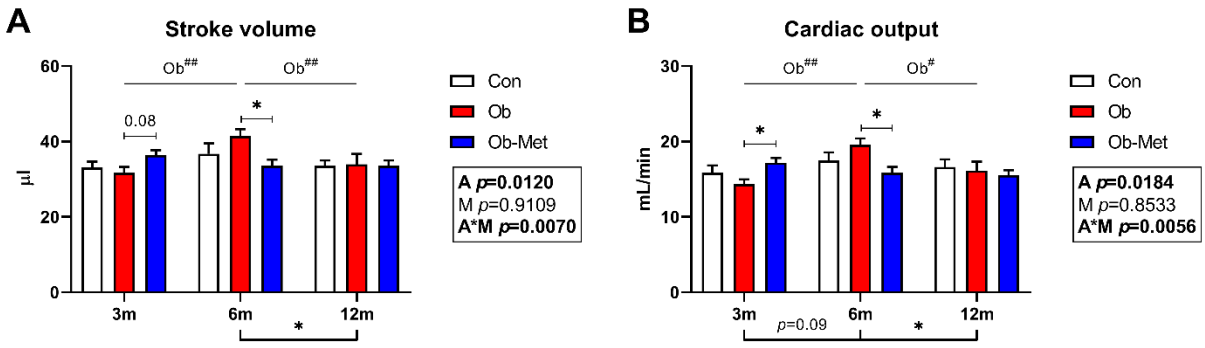

Figure 6.7: Left ventricular function in male offspring.
*A) Stroke volume, B) cardiac output. Box: results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). *p<0.05 using Tukey's multiple comparison test. #within-group ageing effect. Numbers are n=8-12 for Con, n=10-11 for Ob and n=9-12 for Ob-Met offspring.*

There was a significant effect of age on SV, CO and radial FS (and trends for EF and longitudinal FS), with lower systolic function observed at 12 months (Figure 6.7, Table 6.2). There was an interaction between the maternal environment and age for SV and CO: Ob offspring showed increased SV and CO at 6 months compared to at 3 or 12 months, as well as compared to Ob-Met offspring at the same age. At 3 months of age, CO (p<0.05) and SV (p=0.08) were increased in Ob-Met compared to Ob offspring. HR and longitudinal FS were not significantly affected by either age or maternal diet (Table 6.2).

	Age	Con (n=8-12)	Ob (n=11-12)	Ob-Met (n=8-12)	A	M	A*M	post-hoc
HR (bpm)	**3m**	480 ± 21	457 ± 11	473 ± 9	*ns*	*ns*	*ns*	-
	6m	480 ± 12	474 ± 12	472 ± 14				
	12m	492 ± 16	479 ± 14	462 ± 10				
EF (%)	**3m**	53.8 ± 1.3	54.0 ± 1.9	58.8 ± 1.7	*0.0831*	*ns*	*0.0985*	*6 vs 12m p=0.06*
	6m	58.2 ± 2.0	59.4 ± 1.6	53.8 ± 2.9				
	12m	55.4 ± 2.4	51.8 ± 2.9	52.1 ± 2.1				
FS radial (%)	**3m**	36.6 ± 1.4	34.3 ± 0.6	38.2 ± 1.5	***0.0254***	*ns*	*ns*	*3 vs 12m p=0.06*
	6m	37.5 ± 1.2	34.2 ± 1.5	34.1 ± 2.1				
	12m	35.0 ± 1.8	32.0 ± 1.9	30.8 ± 2.1				
FS longitudinal (%)	**3m**	13.2 ± 0.5	13.6 ± 0.6	14.7 ± 0.3	*0.0501*	*ns*	*ns*	***6 vs 12m p=0.03***
	6m	14.6 ± 0.9	15.4 ± 0.8	13.4 ± 1.0				
	12m	13.2 ± 0.6	12.9 ± 1.2	12.7 ± 0.7				

Table 6.2: Other systolic parameters in male offspring.
*Right-hand columns reflect results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M) with Tukey's multiple comparison test. EF = ejection fraction, FS = fractional shortening, HR = heart rate, ns = not significant.*

There was a significant (diastolic, p=0.033) and trending (systolic, p=0.078) effect of the maternal environment on ascending aortic width, driven by an increase in Ob compared to Con offspring which was prevented by maternal metformin treatment (Figure 6.8A-B). Advanced age was associated with a stepwise increase in aortic diameter. The LVOT diameter increased in diameter by 12 months of age consistent with previous reports of increased LVOT in 12-month aged mice (507), but there was no difference between groups (Figure 6.8C).

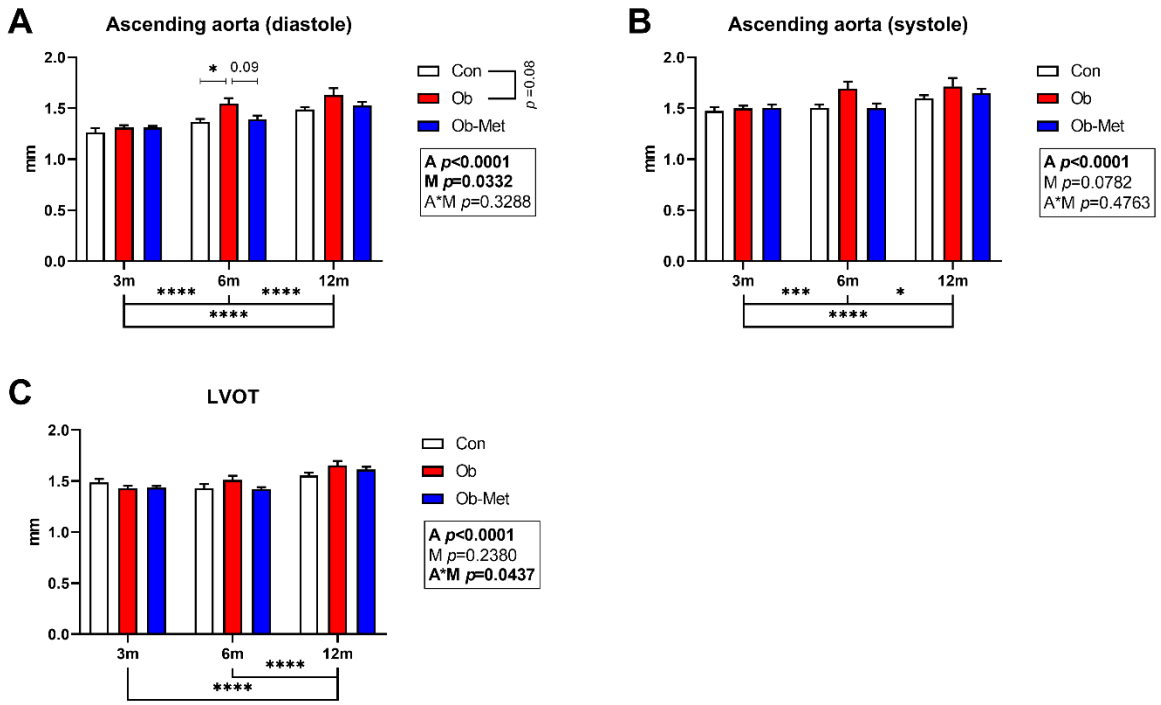

Figure 6.8: Aortic width in male offspring.
*A) Aortic width in diastole and B) in systole, C) diameter of the left ventricular outflow tract. Box: results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). *p<0.05, ***p<0.001, ****p<0.0001 using Tukey's multiple comparison test. #within-group ageing effect. Numbers are n=7-12 for Con, n=9-10 for Ob and n=5-11 for Ob-Met offspring.*

6.3.1.2 Left ventricular diastolic function

There was an effect of the maternal environment on E-wave peak velocity, driven by a decrease in Ob-Met compared to Ob offspring throughout the life-course (Figure 6.9A). A-wave velocity was significantly decreased in both Ob and Ob-Met compared to Con offspring (Figure 6.9B). The E/A ratio was significantly increased compared to Con in both Ob and Ob-Met offspring (main effect of maternal environment). However, while both Ob and Ob-Met offspring had increased E/A compared to controls at 6 months of age, by 12 months of age E/A in Ob-Met offspring was not significantly different from either Con or Ob offspring (Figure 6.9C). DT was decreased in Ob-Met compared to Con and Ob offspring (main effect of the maternal environment, Figure 6.9D). All filling parameters were influenced by age, being significantly different at 12 months compared to earlier timepoints.

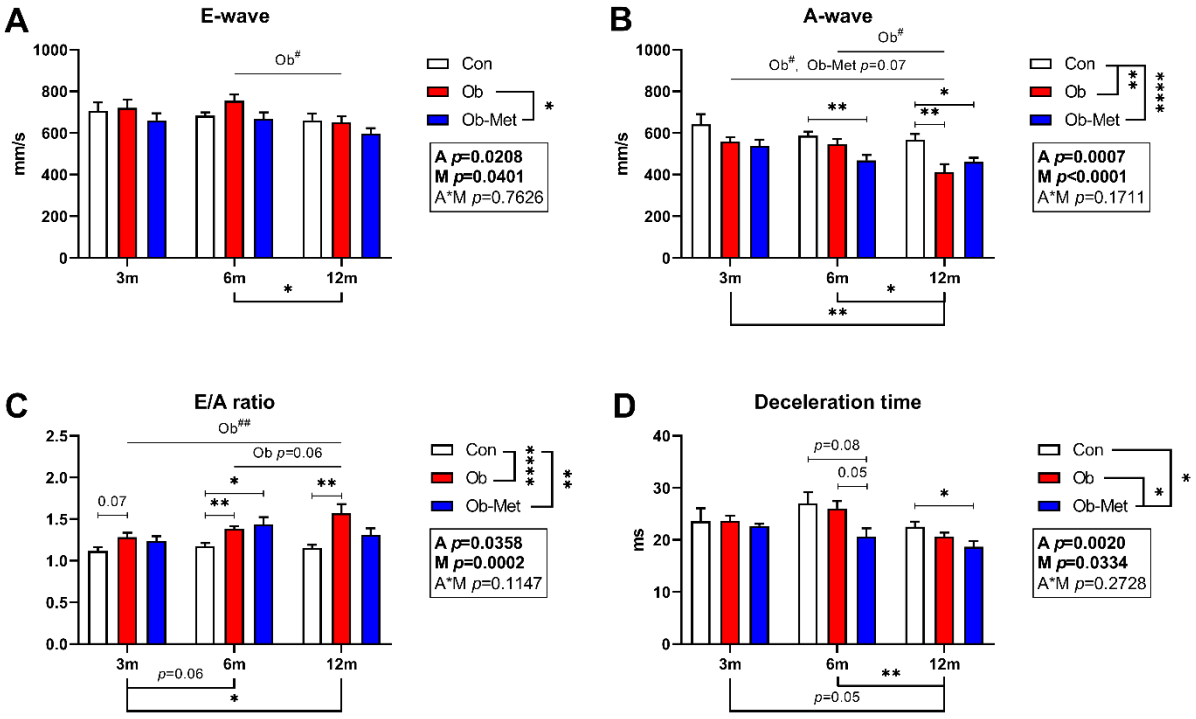

Figure 6.9: Diastolic function in male offspring.

*A) Early filling velocity, B) atrial filling velocity, C) ratio between early and atrial filling velocities, D) E-wave deceleration time. Box: results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). *p<0.05, **p<0.01, ****p<0.0001 using Tukey's multiple comparison test. #within-group ageing effect. Numbers are n=8-12 for Con, n=9-11 for Ob and n=7-11 for Ob-Met offspring.*

	Age	Con (n=8-12)	Ob (n=8-11)	Ob-Met (n=7-11)	A	M	A*M	post-hoc
IVRT (ms)	**3m**	14.2 ± 0.7	13.7 ± 0.6	13.0 ± 0.8	***0.0391***	*ns*	*ns*	***6 vs 12m p=0.04***
	6m	13.0 ± 0.6	12.9 ± 0.7	12.9 ± 1.0				
	12m	14.1 ± 1.0	14.3 ± 1.1	15.4 ± 0.8				
IVCT (ms)	**3m**	9.9 ± 0.5	11.6 ± 0.8	11.6 ± 0.7	*ns*	***0.0355***	*ns*	***Con vs Ob-Met p=0.04***
	6m	9.9 ± 0.3	9.5 ± 0.49	10.4 ± 0.64				
	12m	9.4 ± 0.6	9.5 ± 0.6	11.6 ± 1.1				
MPI	**3m**	0.62 ± 0.03	0.65 ± 0.03	0.66 ± 0.04	***0.0008***	*ns*	*ns*	***3 vs 6m p=0.004 6 vs 12m p=0.005***
	6m	0.57 ± 0.02	0.51 ± 0.04	0.55 ± 0.03				
	12m	0.59 ± 0.03	0.63 ± 0.04	0.70 ± 0.03				
Decel (mm /s^2)	**3m**	-31650 ± 3538	-30495 ± 1336	-29134 ± 12346	*ns*	*ns*	*ns*	-
	6m	-26550 ± 1742	-29885 ± 2076	-34167 ± 2615				
	12m	-29633 ± 2007	-30759 ± 1194	-33910 ± 1830				

Table 6.3: Other diastolic parameters in male offspring

*Right-hand columns reflect results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M) with Tukey's multiple comparison test. Decel = deceleration slope. IVCT = isovolumetric contraction time. IVRT = isovolumetric relaxation time. MPI = myocardial performance index.*

There were no group differences in IVRT or MPI, but IVCT was increased in Ob-Met compared to Con offspring across the life-course (main effect of maternal environment p=0.04, Table 6.3). Across the

cohort there was an age-related increase in IVRT between 6 and 12 months of age, whereas MPI was significantly lower at 6 months compared to the other time-points studied (Table 6.3).

6.3.1.3 Left ventricular dimensions

	Age	Con (n=8-12)	Ob (n=10-12)	Ob-Met (n=8-12)	A	M	A*M	post-hoc
EDA	**3m**	23.4 ± 0.5	23.2 ± 0.6	23.7 ± 0.4	*ns*	*ns*	*ns*	-
	6m	23.8 ± 0.9	24.6 ± 0.5	23.9 ± 0.5				
	12m	23.3 ± 0.6	24.0 ± 0.9	24.2 ± 0.6				
ESA	**3m**	15.0 ± 0.3	14.7 ± 0.5	14.0 ± 0.4	*ns*	*ns*	*ns*	-
	6m	14.3 ± 0.7	14.8 ± 0.8	15.1 ± 0.7				
	12m	14.5 ± 0.6	15.5 ± 0.8	15.6 ± 0.7				
EDV	**3m**	61.4 ± 1.7	61.1 ± 2.9	61.9 ± 1.6	*ns*	*ns*	*ns*	-
	6m	63.2 ± 4.4	66.8 ± 2.5	63.4 ± 2.3				
	12m	61.2 ± 2.3	65.5 ± 4.0	65.0 ± 2.6				
ESV	**3m**	28.3 ± 0.7	28.1 ± 1.8	25.5 ± 1.3	*0.0990*	*ns*	*ns*	-
	6m	26.5 ± 2.3	26.7 ± 1.8	29.3 ± 2.4				
	12m	27.6 ± 2.1	31.6 ± 2.6	31.4 ± 2.3				
EDD	**3m**	3.80 ± 0.08	3.88 ± 0.07	3.89 ± 0.05	***0.0072***	*ns*	*ns*	***3 vs 6m p=0.007*** ***3 vs 12m p=0.01***
	6m	3.92 ± 0.14	4.12 ± 0.07	4.01 ± 0.07				
	12m	3.92 ± 0.05	4.13 ± 0.11	4.08 ± 0.09				
ESD	**3m**	2.41 ± 0.09	2.56 ± 0.08	2.40 ± 0.06	***0.0047***	*0.0984*	*ns*	*3 vs 12m p=0.07* ***3 vs 12m p=0.006*** ***Con vs Ob p=0.03***
	6m	2.46 ± 0.12	2.72 ± 0.09	2.65 ± 0.12				
	12m	2.55 ± 0.10	2.82 ± 0.12	2.84 ± 0.14				
EDL	**3m**	7.67 ± 0.11	7.62 ± 0.09 [b]	7.86 ± 0.05	***0.0070***	*ns*	*ns*	*3 vs 12m p=0.08* ***6 vs 12m p=0.0002***
	6m	7.76 ± 0.10	7.82 ± 0.09	7.73 ± 0.03				
	12m	7.51 ± 0.10	7.41 ± 0.15 [b]	7.60 ± 0.09				
ESL	**3m**	6.65 ± 0.10	6.58 ± 0.09	6.57 ± 0.10	*ns*	*ns*	*ns*	-
	6m	6.62 ± 0.07	6.61 ± 0.11	6.69± 0.06				
	12m	6.52 ± 0.09	6.46 ± 0.13	6.64 ± 0.10				
SpI	**3m**	26.1 ± 0.8	26.2 ± 0.7	25.0 ± 0.5	***0.0002***	*ns*	*ns*	***3 vs 12m p=0.01*** ***6 vs 12m p=0.0002***
	6m	26.0 ± 1.7	27.2 ± 0.8	27.2 ± 1.0				
	12m	27.6 ± 0.7	30.6 ± 1.5 [a,b]	28.3 ± 1.1 [a]				

Table 6.4: Left ventricular dimensions in male offspring.
*Right-hand columns reflect results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M) with Tukey's multiple comparison test. EDA = end-diastolic area, EDD = end-diastolic diameter, EDL = end-diastolic length, EDV = end-diastolic volume, ESA = end-systolic area, ESD = end-systolic diameter, ESL = end-systolic length, ESV = end-systolic volume (mm). ns = not significant. SpI = Sphericity Index (%). [a]p<0.05 vs 3m, [b]p<0.05 vs 6m.*

There were no overt changes in LV dimensions between groups (Table 6.4). Age significantly affected end-diastolic and end-systolic diameter, increasing after 3 months of age. In contrast, end-diastolic LV length decreased between 6 and 12 months of age. This was accompanied by a significant increase in sphericity index by 12 months of age (Table 6.4), consistent with age-related rounding of the LV in all groups.

6.3.1.4 *Right ventricular function*

There were no significant differences in RV function at 12 months of age (Table 6.5).

12m males	Con (n=9-10)	Ob (n=9)	Ob-Met (n=11)	*p*-value
PA VTI (mm)	28.2 ± 0.8	29.4 ± 1.1	29.6 ± 0.9	*0.4862*
Mean velocity (mm/s)	-529 ± 11	-544 ± 19	-547 ± 15	*0.6647*
Peak velocity (mm/s)	-749 ± 14	-772 ± 26	-774 ± 18	*0.6001*
Mean gradient (mmHg)	1.12 ± 0.04	1.19 ± 0.08	1.21 ± 0.07	*0.6289*
Peak gradient (mmHg)	2.25 ± 0.08	2.41 ± 0.16	2.41 ± 0.11	*0.5633*
PAT (ms)	23.4 ± 0.7	24.7 ± 0.9	24.9 ± 0.4	*0.2151*
PET (ms)	56.4 ± 1.3	57.7 ± 1.9	58.0 ± 1.6	*0.7389*
PAT/PET	0.42 ± 0.01	0.43 ± 0.01	0.43 ± 0.01	*0.6145*
MPAP (mmHg)	75.5 ± 0.5	74.7 ± 0.5	74.6 ± 0.3	*0.2097*
Regurgitation (mm/s)	131 ± 18	138 ± 12	117 ± 18	*0.6398*

Table 6.5: Right ventricular function assessed by pulmonary artery PW Doppler in 12-month-old male offspring.
MPAP = mean pulmonary artery pressure. PA = pulmonary artery. PAT = pulmonary artery acceleration time. PET = pulmonary artery ejection time. VTI = velocity time integral. The p-values reflect outcomes of one-way ANOVA testing.

6.3.2 Blood pressure in male offspring

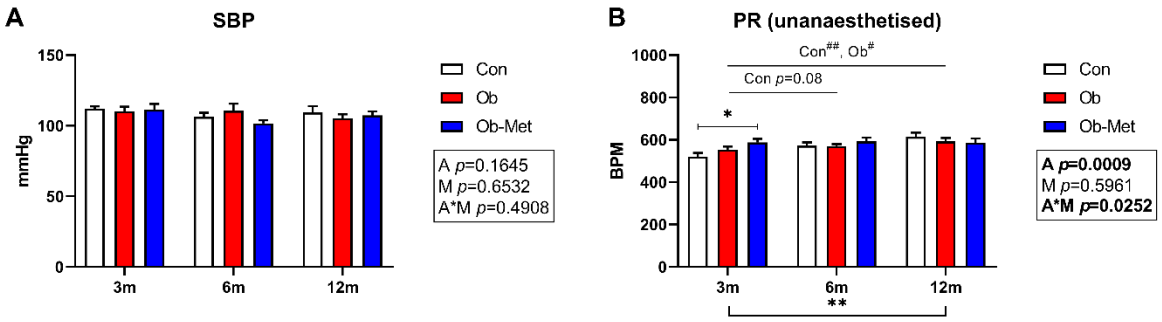

Figure 6.10: Blood pressure and pulse rate in male offspring.
*A) Systolic blood pressure (SBP), B) pulse rate (PR) measured by tail cuff plethysmography. Box: results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). *p<0.05 using Tukey's multiple comparison test. #within-group ageing effect. Numbers are n=9-12 for Con, n=10-13 for Ob and n=9-12 for Ob-Met offspring.*

There was no significant effect of maternal environment or age on SBP (Figure 6.10A). There was a significant effect of age to increase PR. Although there was no overall effect of the maternal environment, there was an interaction between maternal environment and age, with the Ob-Met offspring having significantly elevated pulse rate (PR) at 3 months of age only (Figure 6.10B).

6.3.3 Heart and ventricular weight

	Con (n=11-12)	Ob (n=7)	Ob-Met (n=10-11)	*p*-value
Heart weight (mg)	170 ± 4	183 ± 4	190 ± 7 [a]	***0.0392***
Heart weight (%/BW)	0.46 ± 0.01%	0.44 ± 0.01%	0.46 ± 0.01%	*0.6853*
Ventricular weight (mg)	156 ± 3	171 ± 4	177 ± 7 [a]	***0.0119***
Ventricular weight (%/BW)	0.43 ± 0.01%	0.41 ± 0.01%	0.43 ± 0.02%	*0.4543*
Body weight (g)	37.2 ± 1.0	41.8 ± 1.9	40.5 ± 2.1	*0.1596*

Table 6.6: Whole heart and ventricular tissue weights from male offspring following a 16-hour fast.
The p-values reflect outcomes of one-way ANOVA testing. [a]p<0.05 vs Con using Tukey's multiple comparison test. BW = body weight.

Whole heart and ventricular weights were collected from the 16h-fasted sibling not exposed to cardiovascular phenotyping at 12 months of age. There was an increase in absolute weight of the collected ventricles as well as the whole heart in Ob-Met compared to Con offspring. However, no significant differences were found when tissue weights were expressed relative to body weight (Table 6.6).

6.3.4 Cardiac function in female offspring

6.3.4.1 Left ventricular systolic function

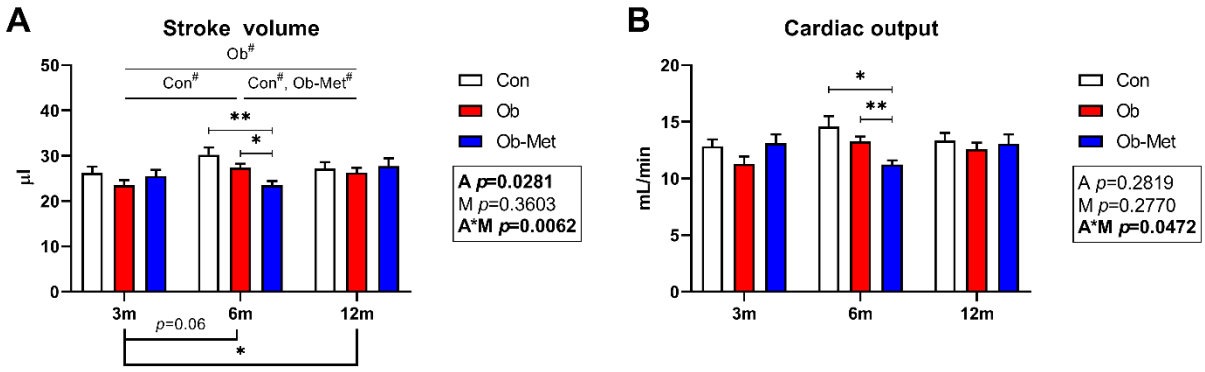

Figure 6.11: Left ventricular function in female offspring.
*A) Stroke volume, B) cardiac output. Box: results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). *p<0.05, **p<0.01 using Tukey's multiple comparison test. #within-group ageing effect. Numbers are n=10-12 for Con, n=8-12 for Ob and n=8-11 for Ob-Met offspring.*

Age significantly affected SV (Figure 6.11) and radial FS (Table 6.7), specifically between 3 and 12 months of age. There was a significant interaction between the maternal environment and age for SV and CO. At 6 months of age, Ob-Met offspring showed decreased SV and CO compared to both Con and Ob offspring (Figure 6.11). No maternal environment effects were found for other systolic functional parameters (Table 6.7). As for males, ageing led to a stepwise increase in ascending aortic diameter, but no effect of the maternal environment was observed (Appendix F).

	Age	Con (n=9-12)	Ob (n=8-12)	Ob-Met (n=9-11)	A	M	A*M	post-hoc
HR (bpm)	**3m**	492 ± 13	476 ± 14	513 ± 15	*ns*	*ns*	*ns*	-
	6m	481 ± 11	484 ± 8	493 ± 13				
	12m	493 ± 9	477 ± 8	472 ± 11				
EF (%)	**3m**	54.3 ±2.1	54.5 ± 2.2	56.2 ± 1.6	*ns*	*ns*	*ns*	-
	6m	58.1 ± 1.5	58.9 ± 1.0	54.1 ± 1.8				
	12m	55.6 ± 1.7	55.4 ± 1.0	55.1 ± 1.6				
FS radial (%)	**3m**	36.7 ± 2.4	34.5 ± 1.9	39.2 ± 1.3	***0.0469***	*ns*	***0.0421***	***3 vs 12m p=0.023***
	6m	35.6 ± 1.6	38.4 ± 2.0	33.6 ± 2.0 [a]				
	12m	33.8 ± 1.6	31. ± 0.9	34.6 ± 1.5 [a]				
FS longitudinal (%)	**3m**	14.4 ± 0.5	14.8 ± 0.8	13.6 ± 0.6	*ns*	*ns*	*ns*	
	6m	14.8 ± 0.7	15.9 ± 0.4	13.5 ± 0.8				
	12m	14.3 ± 0.8	15.0 ± 0.5	14.9 ± 1.0				

Table 6.7: Heart rate, ejection fraction and fractional shortening in female offspring.
*Right-hand columns reflect results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M) with Tukey's multiple comparison test. EF = ejection fraction, FS = fractional shortening, HR = heart rate, ns = not significant. [a]p<0.05 vs Ob-Met 3m.*

6.3.4.2 *Left ventricular diastolic function*

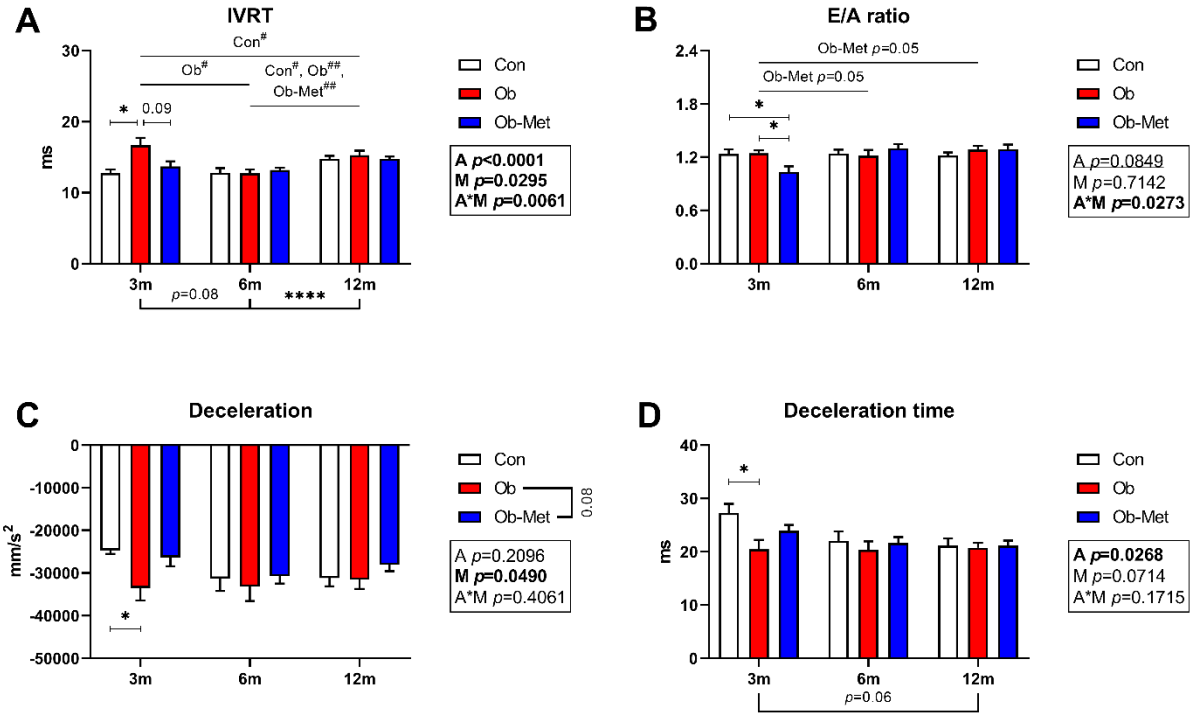

Figure 6.12: Diastolic function in female offspring.
*A) IVRT = isovolumetric relaxation time, B) ratio between early and atrial filling velocities, C) E-wave deceleration slope and D) deceleration time. Box: results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). *p<0.05 using Tukey's multiple comparison test. #within-group ageing effect. Numbers are n=7-12 for Con, n=6-12 for Ob and n=6-12 for Ob-Met offspring.*

There was an effect of age to increase IVRT between 6 and 12 months in all three groups (Figure 6.12A). There was also an interaction between age and the maternal environment with female Ob offspring showing increased IVRT compared to Con offspring at 3 months of age (which was not seen in Ob-Met offspring), and consequently IVRT decreased between 3 and 6 months in Ob offspring only. The maternal environment tended to increase IVCT in Ob and Ob-Met offspring, but this relationship did not reach statistical significance (Table 6.8). There was a non-significant (p=0.077*)* effect of the maternal environment to increase MPI (Table 6.8).

There was an interaction between the maternal environment and age for the E/A ratio, characterised by decreased E/A in 3-month-old Ob-Met offspring which then tended to increase with age exclusively in this group (p=0.05, Figure 6.12B). There was a significant effect of the maternal environment on E-wave deceleration with the slope tending to be steeper in Ob compared to Con offspring (p=0.08 on post-hoc, Figure 6.12C), which was not seen in Ob-Met offspring. A similar effect of the maternal environment was seen for DT (p=0.07, Figure 6.12D).

	Age	Con (n=8-12)	Ob (n=6-12)	Ob-Met (n=7-12)	A	M	A*M	Post-hoc
IVCT (ms)	**3m**	10.6 ± 0.6	12.7 ± 0.8	11.7 ± 1.0	*0.500*	*0.0779*	*ns*	*Con v Ob p=0.05 Con v Ob-Met p=0.07*
	6m	9.2 ± 0.4	10.9 ± 0.7	11.4 ± 0.7				
	12m	10.0 ± 0.5	11.4 ± 1.0	10.7 ± 0.7				
MPI	**3m**	0.59 ± 0.03	0.73 ± 0.05	0.68 ± 0.03	***0.0443***	*0.0769*	*ns*	*6 vs 12m p=0.07*
	6m	0.59 ± 0.04	0.60 ± 0.03	0.62 ± 0.02				
	12m	0.62 ± 0.02	0.65 ± 0.03	0.65 ± 0.02				

Table 6.8: Other diastolic parameters in female offspring
*Right-hand columns reflect results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M) with Tukey's multiple comparison test. IVCT = isovolumetric contraction time. MPI = myocardial performance index. ns = not significant*

6.3.4.3 Left ventricular dimensions

	Age	Con (n=9-12)	Ob (n=8-12)	Ob-Met (n=9-11)	A	M	A*M	post-hoc
EDA	**3m**	19.9 ± 0.6	19.5 ± 0.5	19.2 ± 0.7	*ns*	*ns*	*0.0677*	-
	6m	21.0 ± 0.6	19.8 ± 0.4	18.8 ± 0.4 [b]				
	12m	20.0 ± 0.6	19.8 ± 0.3	20.4 ± 0.6				
ESA	**3m**	12.5 ± 0.6	12.0 ± 0.4	11.8 ± 0.6	*ns*	*ns*	*ns*	-
	6m	12.5 ± 0.4	11.7 ± 0.4	12.1 ± 0.6				
	12m	12.4 ± 0.6	12.3 ± 0.1	12.5 ± 0.4				
EDV	**3m**	48.8 ± 2.8	45.6 ± 1.9	45.7 ± 2.9	*ns*	*ns*	*ns*	-
	6m	51.9 ± 2.2	46.7 ± 1.7	43.5 ± 1.6				
	12m	49.2 ± 2.7	47.5 ± 1.3	50.2 ± 2.3				
ESV	**3m**	22.6 ± 1.9	20.7 ± 1.2	20.2 ± 1.8	*ns*	*ns*	*ns*	-
	6m	21.6 ± 1.0	19.3 ± 1.0	21.1 ± 1.6				
	12m	22.1 ± 1.6	21.1 ± 0.6	22.4 ± 1.1				
EDD	**3m**	3.77 ± 0.10	3.50 ± 0.08	3.69 ± 0.09	*0.0604*	*ns*	*ns*	*3 vs 12m p=0.06* *6 vs 12m p=0.09*
	6m	3.68 ± 0.05	3.58 ± 0.06	3.79 ± 0.12				
	12m	3.80 ± 0.08	3.68 ± 0.05	3.86 ± 0.11				
ESD	**3m**	2.40 ± 0.14	2.30 ± 0.11	2.25 ± 0.09	***0.0317***	*ns*	*ns*	***3 vs 12m p=0.02***
	6m	2.37 ± 0.06	2.21 ± 0.10	2.54 ± 0.15 [a]				
	12m	2.52 ± 0.10	2.51 ± 0.04	2.53 ± 0.12				
EDL	**3m**	6.89 ± 0.08 [c]	7.03 ± 0.13	6.90 ± 0.10	***0.0013***	*ns*	*ns*	***3 vs 6m p=0.009*** ***6 vs 12m p=0.02***
	6m	7.27 ± 0.10	7.12 ± 0.09	6.95 ± 0.10				
	12m	6.83 ± 0.08 [c]	6.99 ± 0.07	7.01 ± 0.08				
ESL	**3m**	5.95 ± 0.04 [b]	5.99 ± 0.10	5.96 ± 0.08	***0.0299***	*ns*	*ns*	*3 vs 6m p=0.09* *6 vs 12m p=0.06*
	6m	6.19 ± 0.09	6.02 ± 0.10	6.01 ± 0.11				
	12m	5.86 ± 0.10 [b]	5.94 ± 0.07	5.97 ± 0.12				
SpI	**3m**	27.8 ± 1.2	25.6 ± 1.6	26.5 ± 1.2	***0.0003***	*ns*	*ns*	*3 vs 12m p=0.09* ***6 vs 12m p=0.0004***
	6m	25.7 ± 0.8	24.8 ± 0.8	26.1 ± 1.5				
	12m	29.3 ± 1.1 [b]	26.8 ± 0.8	27.7 ± 1.0				

Table 6.9: Left ventricular dimensions in female offspring.
*Right-hand columns reflect results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M) with Tukey's multiple comparison test. EDA = end-diastolic area, EDD = end-diastolic diameter, EDL = end-diastolic length, EDV = end-diastolic volume, ESA = end-systolic area, ESD = end-systolic diameter, ESL = end-systolic length, ESV = end-systolic volume (mm). ns = not significant. SpI = Sphericity Index (%). [a]p<0.05 vs 3m Ob-Met, [b]p<0.05 and [c]p<0.01 vs 6m Con.*

There were no significant differences in LV dimensions between groups, except for trending decrease in end-diastolic area and length in 6-month-old Ob-Met compared to Con offspring (Table 6.9). Ageing increased LV diameter and decreased LV length at 12 months of age. There was no group effect on sphericity index, but as for male offspring a rounding of the LV was observed between 6 and 12 months of age (Table 6.9).

6.3.4.4 Right ventricular function

12m females	Con (n=9-10)	Ob (n=9)	Ob-Met (n=11)	*p-value*
PA VTI (mm)	26.8 ± 1.0	24.3 ± 0.5	28.9 ± 1.2	*0.1241*
Mean velocity (mm/s)	-513 ± 18	-456 ± 14 [a]	-508 ± 12	***0.0227***
Peak velocity (mm/s)	-722 ± 21	-651 ± 19 [a]	-720 ± 18	***0.0235***
Mean gradient (mmHg)	1.07 ± 0.08	0.84 ± 0.05 [a]	1.04 ± 0.05	***0.0295***
Peak gradient (mmHg)	2.11 ± 0.12	1.71 ± 0.10 [a]	2.09 ± 0.10	***0.0284***
PAT (ms)	23.2 ± 0.5	24.4 ± 0.7	23.0 ± 0.6	*0.2468*
PET (ms)	55.0 ± 0.9	56.8 ± 0.9	55.3 ± 1.8	*0.5398*
PAT/PET	0.42 ± 0.01	0.43 ± 0.01	0.42 ± 0.01	*0.7005*
MPAP (mmHg)	75.6 ± 0.3	74.9 ± 0.4	75.7 ± 0.4	*0.2457*
Regurgitation (mm/s)	122 [75-131]	120 [72-140]	144 [91-170]	*0.3810**

Table 6.10: Right ventricular function assessed by pulmonary artery PW Doppler in 12-month-old male offspring.
*MPAP = mean pulmonary artery pressure. PA = pulmonary artery. PAT = pulmonary artery acceleration time. PET = pulmonary artery ejection time. VTI = velocity time integral. The p-values reflect outcomes of one-way ANOVA or *Kruskal-Wallis testing for non-parametric data.*

There was no difference in pulmonary artery VTI [a marker of blood volume passing through the vessel that reflects RV CO (508)] between groups. However, there was a decrease in velocity of blood flow and pressure gradient across the pulmonary artery in Ob offspring which was not seen in Ob-Met offspring (Table 6.10), suggestive of decreased pulmonary pressures in Ob offspring. In contrast, there was no significant difference in PAT, the PAT/PET ratio used to normalise PAT for HR (508) or MPAP.

6.3.5 Blood pressure in female offspring

There was a significant effect of the maternal environment on SBP and PR. Post-hoc testing revealed a significant increase in SBP in Ob compared to Con offspring across the life-course, and increased SBP in Ob-Met offspring that was apparent at 12 months of age (Figure 6.13B). SBP was also significantly affected by age, with an age-related decrease in SBP observed after 3 months of age (Figure 6.13A). PR was significantly decreased in Ob-Met offspring compared to Con and Ob offspring throughout the life-course (Figure 6.13B). At 12 months of age, SBP was positively correlated to body weight, caloric intake between 6 and 12 months, and total fat mass at 12 months of age (Figure 6.13C-F). SBP at 3 months was positively correlated to IVRT (Figure 6.13G).

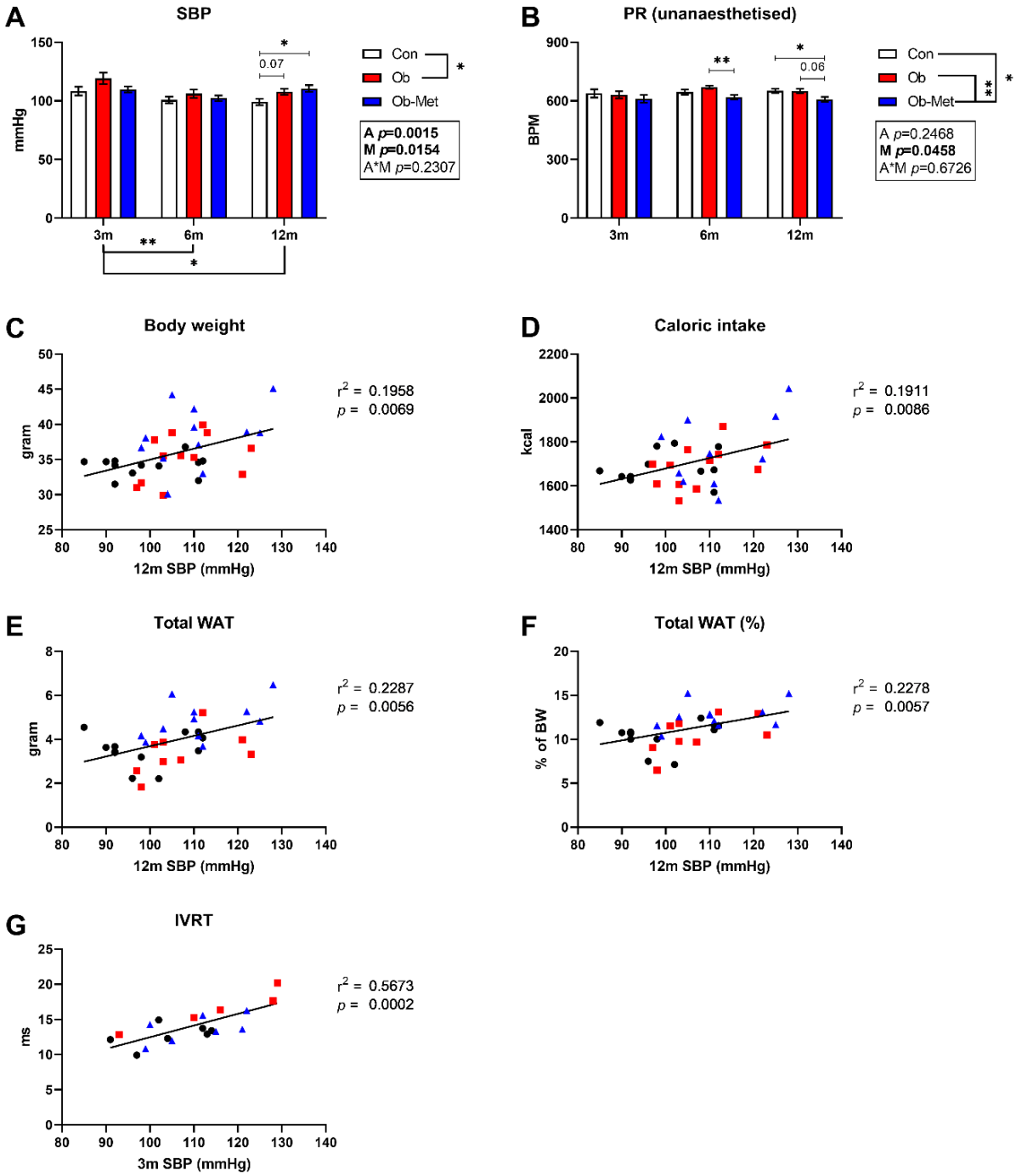

Figure 6.13: Blood pressure and pulse rate in female offspring.

*A) Systolic blood pressure (SBP), B) pulse rate (PR) measured by tail cuff plethysmography. Box: results from repeated measures mixed effects model for the effect of age (A), the maternal environment (M) and the interaction between them (A*M). *p<0.05, **p<0.01 using Tukey's multiple comparison test. Numbers are n=11-12 for Con, n=10-12 for Ob and n=11-12 for Ob-Met offspring. C-F) correlations between SBP at 12 months and adiposity parameters, specifically C) littermate average body weight at 49 weeks (n=36 total, n=12 in all groups), D) caloric intake between 28-49 weeks (n=35 total, n=12 Con, n=12 Ob, n=11 Ob-Met), E) total WAT collected in grams and F) as a percentage of body weight at 12 months of age (n=32 total, n=12 Con, n=9 Ob, n=11 Ob-Met). G) correlation between SBP and IVRT at 3 months (n=19 total, n=7 Con, n=5 Ob, n=7 Ob-Met offspring).*

6.3.6 Heart and ventricular weight

There was no significant difference in absolute heart or ventricular weight in 16-hour fasted siblings at 12 months of age (Table 6.11). However, ventricular weight relative to body weight was significantly decreased in Ob-Met offspring compared to controls related to a significant increase in body weight at 12 months of age.

	Con (n=12)	**Ob (n=11)**	**Ob-Met (n=11)**	***p*-value**
Heart weight (mg)	134 ± 3	139 ± 3	145 ± 3	*0.0942*
Heart weight (%/BW)	0.45 ± 0.01%	0.45 ± 0.01%	0.42 ± 0.02%	*0.1481*
Ventricular weight (mg)	126 ± 3	131 ± 3	133 ± 3	*0.1812*
Ventricular weight (%/BW)	0.42 ± 0.01%	0.42 ± 0.01%	0.39 ± 0.02% [a]	***0.0429***
Body weight (g)	29.9 ± 0.9	31.0 ± 1.1	35.1 ± 1.4 [b,c]	***0.0065***

Table 6.11: Whole heart and ventricular tissue weights from female offspring following a 16-hour fast.
The p-values reflect outcomes of one-way ANOVA testing. [a]p<0.05, [b]p<0.01 vs Con, [c]p<0.05 vs Ob offspring using Tukey's multiple comparison test. BW = body weight.

6.4 Discussion

In this chapter the cardiovascular phenotype of offspring of obese dams and the effect of prenatal metformin intervention thereon was investigated longitudinally from 12 weeks to 12 months of age. Echocardiography and BP assessment revealed clear sex- and age-specific effects for both the maternal obesogenic environment and the metformin intervention. This shows the importance of following up offspring of both sexes at several ages in order to comprehensively phenotype CVD risk associated with intrauterine insults.

6.4.1 Male offspring cardiovascular health

6.4.1.1 Maternal obesity

Male offspring of obese dams showed no difference in cardiovascular parameters at 3 months of age. However, by 6 months of age they displayed significantly increased diameter of the ascending aorta. They also showed signs of diastolic dysfunction indicated by an increase in E/A ratio that became more apparent with age (whereas no significant age-related increase was seen for Con offspring). These changes were not accompanied by any differences in SBP or HR.

An increase in E/A ratio indicates disproportionate reliance on passive early filling in these offspring, which is characteristic of a restrictive filling pattern with increased filling pressures. 'Overfilling' of the LV is achieved by either volume or pressure overload. Since LVEDV was unaltered in male Ob offspring, it is likely that LVEDP is increased instead. Although not assessed in this study, increased LVEDP in male Ob offspring was previously described in our model at 12 weeks of age using the isolated Langendorff heart perfusion preparation (132). Pressure overload increases LV preload, which according to the Frank-Starling mechanism would result in enhanced systolic function. Although systolic function parameters were not significantly different compared to Con, the width of the aortic diameter was increased in 6-month-old Ob offspring. Since LVOT diameter (reflecting the intracardiac portion of the aortic root) was not different, the widening of the aorta suggests exaggerated distension in response to blood flow. Together, this suggests hyperdynamic LV function in response to pressure overload (Figure 6.14). Interestingly, increased LVEDP coupled with increased contractile function was also observed in 4-month-old rat offspring exposed to prenatal hypoxia (509), suggesting chronic fetal hypoxia in obese pregnancy may have contributed to these findings.

The hypothesised increase in LVEDP is likely to result from ventricular stiffening. LV stiffening can occur secondary to compensatory hypertrophy in arterial hypertension (494). However, SBP was not increased in male Ob offspring. Therefore, it is more likely that maternal obesity programmed an increase in ventricular stiffness independently of SBP. Indeed, the normalisation of cardiac function but not SBP by maternal exercise in our model indicates that maternal obesity programs cardiac

dysfunction and hypertension in male offspring through distinct mechanisms (128). Work from our laboratory also showed that pathological hypertrophy was programmed as early as 3 weeks of age thus likely reflecting a primary cardiac effect (132). Maternal obesity could thus have direct programming effects on male offspring hearts, likely leading to increased collagen deposits and fibrosis as previously seen in our model at 8 weeks of age (129). Interestingly, although cardiac weight was normalised by 12 weeks of age, ventricular stiffening was maintained (132) suggesting increased stiffness may not necessarily be accompanied by hypertrophy at the ages investigated in the current study. Similarly, SBP may have normalised between 8 and 12 weeks as well. However, it should be mentioned that although no differences in SBP were detected in these offspring at 4-5PM, radiotelemetry experiments in offspring generated using the same model showed elevated SBP at 3 and 6 months of age in the night-time only (87). Therefore, although no hypertension was detected in our offspring in the late afternoon, we cannot definitively exclude an influence of mild hypertension in the night-time. Similarly, it has been suggested that although data using the tail cuff technique is similar to telemetry data at rest (510,511), it may lack sensitivity to detect the changes in aortic arch BP observed with telemetry in response to restraint stress (510). Therefore, differences in central BP might be underestimated by the technique.

Diastolic dysfunction with ventricular fibrosis (but in absence of changes in EF or FS) was previously seen in 3- and 6-month-old mouse offspring of HFHS-fed dams (502), consistent with data in this study. This occurred independent of cardiomyocyte size (502). LV stiffening may be an early programming effect since impaired relaxation (decreased E/A) was already seen in rat weanlings from HFD-fed dams (94). Similarly, cardiac fibrosis was observed in late gestation fetuses of obese ewes. Although diastolic function was not yet impaired this was associated with decreased contractile function in isolated hearts upon higher workload, suggesting an inability to cope with increased demand and potential predisposition to HF as adults (512,513). Notably, cardiac fibrosis was also observed in adult offspring from this model (203) suggesting these changes were long-term. Hence, a direct fibrotic effect of maternal obesity on the developing heart may also underlie the diastolic dysfunction in the current study. However, LV stiffening could also have developed later in life accompanying the hypertrophy observed at younger ages in our model (132), since extracellular matrix remodelling is an essential component of LV hypertrophy and re-expression of fetal genes may alter sensitivity to calcium recycling in diastole (502,512). Additionally, ageing is associated with increased ventricular stiffening and deterioration of diastolic function (489,507,514) as was also seen in the current study evidenced by the age-related increase in IVRT and decrease in DT across the cohort. Therefore, the development of diastolic dysfunction in Ob offspring may also be a sign of accelerated ageing.

The finding of a restrictive phenotype in male Ob offspring is clinically relevant since this advanced (and potentially irreversible) grade of diastolic dysfunction is associated with increased risk of HF, worse prognosis and lower survival in humans even if EF is maintained (515,516). This suggests that these offspring may be at risk of developing HF.

6.4.1.2 Metformin intervention

In male offspring, metformin intervention corrected the hyperdynamic LV systolic function as indicated by significantly decreased SV, CO and aortic width by 6 months of age. Moreover, although the E/A ratio remained elevated compared to controls at 6 months, by 12 months of age E/A in Ob-Met offspring was attenuated to more closely resemble Con offspring. Together, these data suggest that the metformin intervention slowed the progression of filling defects programmed by maternal obesity. This was associated with a decrease in early passive filling velocity (likely reflecting decreased LAP) compared to Ob offspring across the life-course, suggesting an attenuation of filling pressures in Ob-Met offspring (Figure 6.14).

It is unclear what might underlie this protective effect of the metformin intervention. Previous work from the laboratory suggested that hypertrophy might be related to hyperinsulinaemia resulting in enhanced mitogenic insulin signalling in 8-week-old male offspring hearts (133). Since maternal insulin correlated to offspring insulin and correction of maternal insulin with exercise prevented cardiac hypertrophy and dysfunction (128,136), this suggests that metformin (an antidiabetic medication) may have attenuated maternal insulin and consequently slowed LV stiffening and progression of diastolic dysfunction. Similarly, insulin signalling was altered in fetuses of obese ewes that also showed myocardial fibrosis and altered contractile function, leading the authors to hypothesise that insulin might have programmed this in the sheep (512,513).

DT was decreased in Ob-Met compared to Con and Ob offspring, most clearly at 6 and 12 months of age. As an increase in DT is associated with impaired relaxation (514), this finding could indicate improved LV relaxation in aged Ob-Met animals compared to the other groups. However, this is unlikely since the increased E/A ratio indicates that animals have progressed beyond impaired relaxation to restrictive diastolic dysfunction. Decreased DT normally accompanies increased E/A because both reflect an increased atrioventricular pressure gradient (517), indicating a disconnect between DT and E/A in our male offspring that does not follow the classic diastolic dysfunction pattern. It is thus unclear why decreased DT is seen in 12-month-old Ob-Met offspring when E/A is normalised. In humans, E/A is less variable than DT and may be a better determinant of diastolic function (517). Moreover, questions have been raised about the feasibility of E-wave deceleration measurement in murine echocardiography where high HR and short time interval between E and A

peaks are often observed (495,497). Indeed, the DT and deceleration slope showed relatively high intra-animal variation compared to other parameters in the current study. It would be interesting to investigate if any changes in LVEDP, left atrial diameter and LV remodelling are observed in 12-month-old male Ob-Met compared to Ob hearts to determine whether the intervention has had beneficial or detrimental effects on diastolic function in aged male offspring.

Ob-Met offspring showed an increase in unanaesthetised HR (versus Con), SV and CO (versus Ob) at 3 months of age. Interestingly, increased HR in childhood was also observed in male offspring of PCOS-complicated pregnancies treated with metformin (335). Metformin has been associated with vascular improvements in human offspring exposed to maternal obesity (391), and did not adversely affect vasculature in rodent models of non-obese pregnancy (343,382,506). Moreover, pilot data from our lab suggests potential improvement of vascular reactivity in 8-week-old Ob-Met offspring (personal correspondence with Dr H. Blackmore, manuscript in preparation). Hence, the increase in HR and CO (in the conscious and anaesthetised state, respectively) may be related to decreased TPR. Alternatively, enhanced function at 3 months followed by deteriorating function at 6 months of age might reflect adaptation to programmed changes that cannot be maintained long-term. This would be consistent with the observed increase in IVCT reflecting an impairment of the LV to efficiently generate sufficient pressure required to open the aortic valve (493). Mdaki *et al.* reported that maternal HFD but not diabetes increased IVCT in exposed rat offspring (94), perhaps partly explaining why maternal treatment with glucose-lowering medication like metformin did not prevent an increase in IVCT in our offspring. However, in absence of hypertension or defects in systolic function compared to Con offspring the isolated finding of increased IVCT may also be unrelated to contractile dysfunction.

6.4.2 Female offspring cardiovascular health

6.4.2.1 Maternal obesity

Female offspring of obese dams showed increased SBP across the life-course, indicating maternal obesity programmed hypertension in these offspring. Consistently, SBP was increased in 8-week-old female offspring of obese dams in a previous cohort generated in our laboratory (University of Cambridge PhD Thesis Dr Jessica Beeson) but BP had not been assessed before in female offspring beyond that age. Our findings are consistent with results from groups reporting increased night-time SBP via radiotelemetry at 3 and 6 months using the same maternal DIO model (87) as well as elevated BP in 6- and 12-month old female offspring in a rat model of maternal HFD-feeding (90,117).

Since no difference in HR or SV was observed in this study, the increased SBP is hypothesised to result from increased TPR instead. Several mechanisms for a programmed increase in TPR and consequent

hypertension have been suggested, including sympathetic hyperactivity, endothelial dysfunction and overactivation of the RAS (518). Accordingly, exaggerated contractile response to noradrenaline and impaired relaxation response to acetylcholine were reported in 3-month-old female offspring using the same mouse model of maternal DIO as the current study (87), and endothelial dysfunction has also been reported in rat offspring up to 6 months of age (90,117). In order to determine whether vascular dysfunction plays a role in the current study, reactivity of isolated vessels could be tested. Indeed, preliminary experiments from the laboratory suggest blunted response to parasympathetic stimulation in femoral arteries obtained from 12-month-old Ob females (personal correspondence with P. Wilsmore, unpublished), consistent with increased sympathetic tone in these offspring. Other future experiments to address this include histological and molecular assessment of major blood vessels to investigate endothelial morphology and function as well as balance of local vasodilatory and -constrictor signals. In addition, impaired kidney development may also contribute to the development of hypertension (518). In fact, reduced nephron number was observed in male offspring in our model (University of Cambridge PhD thesis, Dr A. Pinnock) but this remains to be assessed in female offspring. Notably, the absence of pulmonary arterial hypertension indicates the arterial hypertension in the current study is not a biventricular defect secondary to right heart failure. In fact, the lower pressure gradient and flow velocity across the pulmonary artery suggest that pulmonary pressures were decreased rather than increased in Ob offspring.

At 3 months of age female Ob offspring also showed increased IVRT, a sign of impaired LV relaxation. Maternal obesity may not decrease LV compliance in female offspring via the same mechanism as in males since aortic width was unaltered in female offspring. Since hypertension is commonly associated with compensatory LV remodelling (494), it is likely that the increased SBP indirectly led to diastolic dysfunction via ventricular stiffening. This is supported by the finding that IVRT was positively correlated with SBP at 3 months of age. Indeed, an increase in IVRT that correlates to SBP has previously been found in hypertensive humans (517). However, the additional influence of direct cardiac programming effects by maternal obesity cannot definitively be excluded.

In addition to increased IVRT, a steeper deceleration slope and trending decrease in DT was observed in 3-month-old female Ob offspring. This seems unusual since decreased DT is usually associated with a more progressive restrictive diastolic dysfunction phenotype often accompanied by an increased E/A ratio and/or decreased IVRT (514). However, in humans hypertension and LV hypertrophy are able to program diastolic dysfunction independently and distinctly influence echocardiographic parameters, with IVRT relating more strongly to LV wall thickness and concentric hypertrophy, while E/A and DT depend more on the atrio-ventricular pressure gradient and DBP (not measured in the current study) than compliance (517). This might partly explain the disconnect in our animals between

IVRT and DT in context of the 'grade' of diastolic dysfunction used for diagnosis in humans. Hence, although increased IVRT normally occurs prior to elevation of LAP, in Ob females LAP may have recently begun to increase, shortening DT and pseudonormalising E/A while the atrioventricular pressure gradient is not sufficiently high to prematurely initiate filling and shorten IVRT (hence remaining prolonged by the initial relaxation defect). In any case, the decreased DT and increased IVRT in 3-month-old female Ob offspring both reflect alterations in diastolic function compared to controls.

At 6 and 12 months of age, no group differences in echocardiographic diastolic parameters were observed between Con and Ob offspring. This could reflect normalisation of diastolic function with age. Indeed, reversal of mild diastolic dysfunction is described following antihypertensive treatment (519). Since there was a decline in SBP between 3 and 6 months of age across the cohort, this may have returned SBP in Ob females from hypertensive back into the normal range (despite remaining elevated compared to controls), consequently attenuating afterload and improving diastolic function. In contrast, the lack of difference in diastolic function parameters at 6 and 12 months could also reflect progression to the pseudonormal stage following months of sustained hypertension. Similarly, pseudonormalisation might explain why E/A was not different from controls at 3 months of age. Tissue Doppler or estimation of filling pressures are required to distinguish between these two patterns.

Systolic function was not different between Con and Ob offspring at any age investigated. This suggests that hearts in female Ob offspring are able to compensate for the increased afterload associated with hypertension up to 12 months of age, for instance through compensatory LV hypertrophy. Indeed, isolated diastolic dysfunction without systolic impairment is often seen with chronic hypertension in humans (517). However, previous work from our lab showed that maternal and postnatal obesogenic diet have additive effects on hypertension in 8-week-old male offspring (129) suggesting that female offspring of obese pregnancy may be susceptible to severe hypertension and associated CVD risk when exposed to a calorie-rich environment postnatally. Indeed, hypertension remains a major predictor for coronary heart disease independent of hypertrophy, especially in women (127). Moreover, even elevated BP in the 'high normal' range is associated with increased risk of CVD including cardiovascular mortality, myocardial infarction, stroke and HF (520).

6.4.2.2 Metformin intervention

In the current study, Ob-Met offspring showed a consistent decrease in HR throughout the life-course compared to Con and Ob offspring, which was associated with normalised SBP in early adulthood. It thus seems that the prenatal metformin intervention has prompted a negative chronotropic response to overcome the propensity to hypertension programmed by maternal obesity. Moreover, at 3 months

of age Ob-Met offspring showed normalised IVRT and DT suggesting improvement of diastolic function as well as hypertension programmed by maternal obesity in female offspring (Figure 6.14).

It is unclear through what mechanism the metformin intervention corrected SBP in female offspring. The finding of decreased HR suggests that metformin intervention may not have corrected the primary defect (e.g. TPR), although this cannot be conclusively determined at this stage. Gestational metformin was previously shown to correct hypertension in male rat offspring of fructose-fed dams when weaned onto HFHS, at least in part by down-regulating RAS components, uric acid, and oxidative stress in the kidney (380). This BP-lowering effect of prenatal metformin was likely AMPK-dependent as similar correction of programmed hypertension was seen in offspring prenatally exposed to other AMPK activators such as AICAR and resveratrol (521,522). Although renal morphology was perturbed in 3-week-old and 6-month-old male offspring in our model (University of Cambridge PhD thesis, Dr A. Pinnock) this remains to be assessed in females. Maternal obesity may contribute to programming of hypertension in offspring of obese dams by augmenting the neonatal leptin surge (163). Although not assessed, the metformin intervention might thus improve BP regulation by decreasing maternal fat mass in pregnancy and lactation (Chapter 3). Moreover, since metformin confers vasculo-protective effects on the adult as well as maternal circulation (245,247), the intervention might have beneficially affected developing vasculature *in utero* as well therefore potentially correcting the primary defect. Accordingly, late gestation metformin infusion into fetal sheep had beneficial effects on the pulmonary vasculature in a model of neonatal pulmonary hypertension (523). Alternatively, the decreased HR across life may reflect correction of an impaired baroreflex programmed by maternal obesity [as reported before in female rat offspring of HFD-fed dams (130)].

Intriguingly, the E/A ratio was decreased in Ob-Met compared to Con offspring, which was not seen in Ob females. Decreased E/A reflects impaired passive filling leading to overreliance on atrial systole and is a sign of impaired relaxation without drastically increased filling pressures. Therefore, the metformin intervention may have prevented an increase in filling pressures (as might be present in female Ob offspring) by correcting SBP, but offspring still show some diastolic dysfunction potentially directly resulting from exposure to maternal obesity *in utero* and/or in lactation. This would be consistent with Ob females showing pseudonormalisation of the E/A ratio at 3 months. Decreased E/A in Ob-Met could also reflect relatively better filling if E/A has started to increase in both Con and Ob offspring, although this would not necessarily be expected in young adult mice. Alternatively, Ob-Met offspring might have corrected some (IVRT and DT) but independently introduced other indices of diastolic dysfunction (E/A). Further experiments such as the Langendorff preparation and measurement of left atrial diameter (a marker of LAP) at 3 months of age will help determine which scenario is more likely.

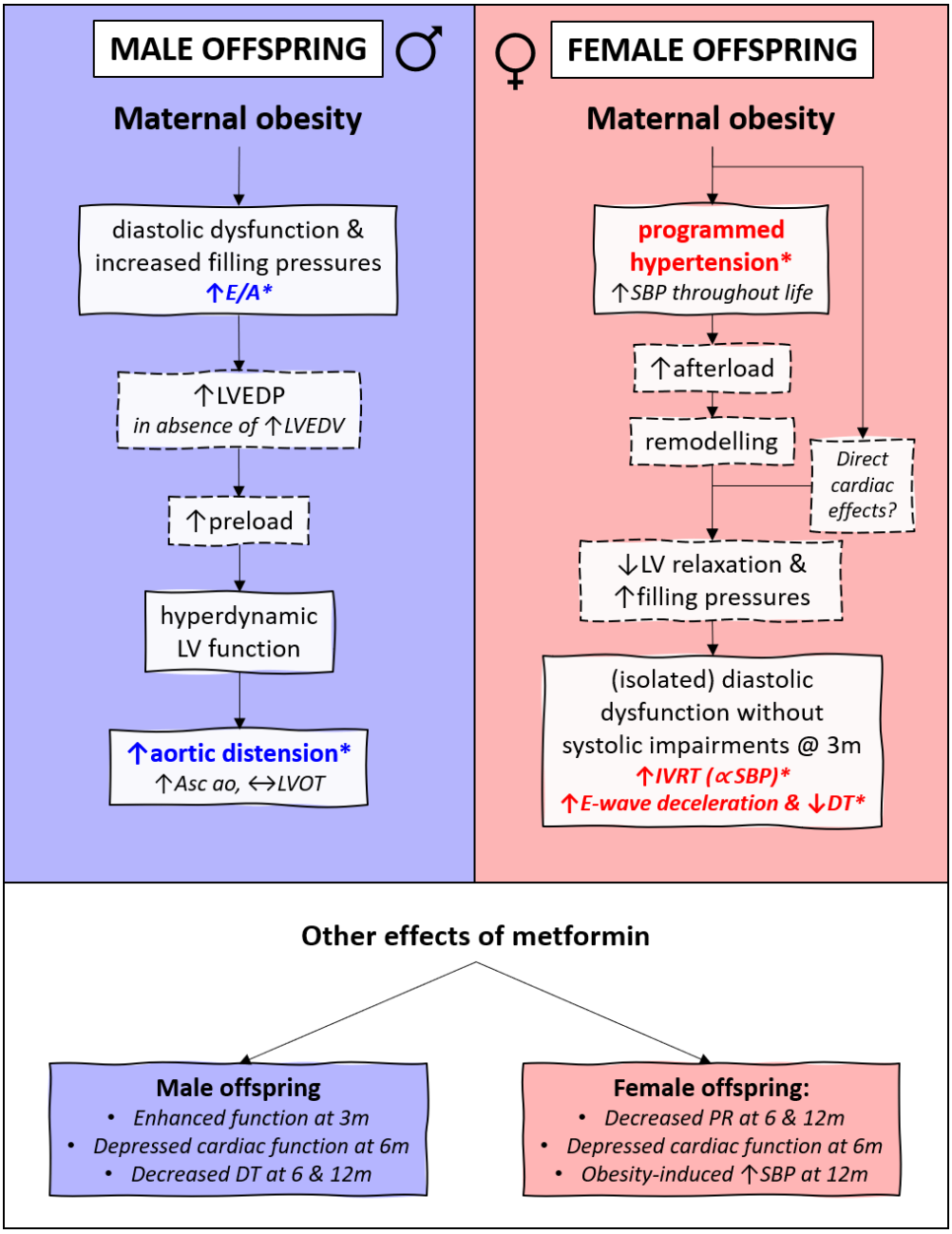

Figure 6.14: Hypothesised effect of maternal obesity and metformin intervention on cardiovascular health in offspring. *Solid lines: evidence presented in this study. Dotted lines: not directly assessed in this study.* ***Bold*:*** *parameters influenced by maternal metformin treatment. Asc ao = ascending aorta. DT = E-wave deceleration time. E/A = ratio between early and atrial filling velocities. IVRT = isovolumetric relaxation time. LV = left ventricle. LVEDP = LV end-diastolic pressure. LVEDV = LV end-diastolic volume. LVOT = LV outflow tract. PR = pulse rate. SBP = systolic blood pressure.*

By 12 months of age, Ob-Met females had developed hypertension compared to controls, suggesting the anti-hypertensive effects of the intervention were no longer sufficient to maintain normal BP in these animals. As described in Chapter 5, at 12 months Ob-Met females show exaggerated adiposity compared to both Con and Ob offspring, as well as gWAT inflammation, increased HOMA-IR index and hyperinsulinaemia. Therefore, the metformin intervention rescues programmed hypertension in early life but induces obesity-associated hypertension upon ageing. This is supported by the significant

correlations between SBP and measures of adiposity (body weight, caloric intake and total WAT mass collected) at 12 months of age, which were not present at earlier timepoints. Several changes in Ob-Met females could underlie this obesity-induced hypertension phenotype. Obesity is associated with increased circulating blood volume (524) but since EDV or SV were unaltered in Ob-Met offspring volume overload is probably not responsible for hypertension in 12-month-old offspring. Insulin has also been implicated in the pathogenesis of obesity-induced hypertension via increased sympathetic nervous system activity and blunted insulin-stimulated vasodilation in obese individuals (525). Moreover, there were significant correlations between inflammatory adipokines (CRP, IL-6 and MCP1 and ICAM-1) and brachial artery endothelial function in the Framingham Heart Study suggesting inflammation may impair vascular function (526). This suggests potential causal roles for the hyperinsulinaemia and inflammatory WAT phenotype observed in 12-month-old Ob-Met offspring in the development of late-life hypertension. Lastly, DIO is associated with selective leptin resistance, leading to dampening of the appetite-suppressing effects of leptin while maintaining its ability to increase BP (527). Although not measured, hyperleptinaemia in obese Ob-Met females might thus drive the elevated SBP at 12 months. Metformin-related obesity and metabolic dysfunction in ageing female offspring might exacerbate vascular resistance independently to maternal obesity, leading to the highest TPR in Ob-Met females at 12 months of age and consequent hypertension. This is consistent with preliminary experiments from the laboratory indicating further impaired relaxation following parasympathetic stimulation (worse than Ob females) as well as exaggerated constriction upon sympathetic stimulation (not seen in Ob offspring) in femoral arteries from 12-month-old Ob-Met females (personal correspondence with P. Wilsmore, unpublished).

There were no significant differences in systolic function at 3 months or at 12 months of age. However, at 6 months of age a significant decrease in SV and CO was observed in Ob-Met compared to Con and Ob offspring. These changes are unlikely to reflect impaired systolic function following increased afterload since SBP is not elevated in Ob-Met offspring at this age. That being said, central BP has been shown to precede systemic BP changes and the presence of elevated central SBP can therefore not be definitively excluded (391). Alternatively, although not significantly different from Ob offspring, Ob-Met offspring already has the highest fat mass at 6 months of age. If it is assumed that adiposity increases TPR independently from maternal obesity, it could be argued that at 6 months TPR in Ob-Met offspring is beginning to exceed that of Ob offspring. As a result, the decreased SV and CO might reflect additional compensatory mechanisms to maintain SBP in the normal range. If so, one might expect to see similar changes at 12 months of age. However, since Ob-Met offspring began to diverge in body weight from 9 months of age, it is likely that by 12 months of age Ob-Met offspring had been exposed to several weeks of hypertension. This might have resulted in resetting of the baroreflex

response allowing for a higher SBP without triggering further compensatory responses such as decreased SV (528). However, the finding that the decrease in SV was related to a decrease in end-diastolic area and therefore reflects an impairment in filling rather than contraction complicates this theory. Instead, the decrease in SV or CO might be an indication that diastolic function is mildly impaired in 6-month-old Ob-Met offspring through unidentified mechanisms, related or unrelated to the start of obesity-induced hypertension in these offspring.

6.4.3 Sexual dimorphism

Maternal obesity had sex-specific effects on offspring, although similarities were also observed. Both sexes showed signs of diastolic dysfunction, but this was more severe in Ob males (restrictive pattern) than in Ob females (impaired relaxation only). Moreover, since in male offspring the E/A ratio already reflected a restrictive pattern at 3 months of age it can be inferred that diastolic dysfunction developed earlier in male than in female offspring. This is consistent with a recent report from a murine maternal HFHS model where female offspring showed later and less marked changes in diastolic function compared to male offspring, suggesting relative cardioprotection (502). Moreover, based on findings in the current study a different aetiology is expected between sexes: pressure overload in male offspring and hypertension in female offspring. However, we also cannot exclude that some LV stiffening effects programmed by maternal obesity are similar between sexes.

Sexual dimorphism in the programming of hypertension was previously seen in studies by Khan *et al.* who reported that female but not male rat offspring of HFD-fed dams had elevated SBP throughout life (80, 180 and 360 days)(90,117). Interestingly, both sexes showed blunted arterial endothelium-dependent relaxation response to acetylcholine suggesting vascular dysfunction was not the sole underlying cause. Instead, there may be sex-specific impairments in baroreflex function suggested by the observation that male offspring of HFD-fed dams were bradycardic and normotensive while female offspring had normal HR and elevated BP in the study (90,117). Similar finding were reported in mouse offspring of sucrose-fed dams with female-specific baroreflex resetting despite the presence of sympathetic dominance and arterial hypertension in both sexes (529). Although its involvement in the programming of female hypertension cannot be excluded, male offspring had normal SBP and HR in the current study and therefore it is unlikely that sexual dimorphism in baroreflex impairment is responsible for sex differences in BP.

There were also differences and similarities between male and female offspring exposed to prenatal metformin. Both offspring showed some correction of diastolic dysfunction, but this occurred early in females compared to later in life in males. This timing difference in diastolic improvement may reflect the sex-specific aetiology mentioned above, with female hypertension (and consequent diastolic

impairments) developing by 3 months of age compared to slower development of LV stiffening in male offspring of obese dams. Interestingly, diastolic improvement in the form of decreased IVRT was also observed in 4-year-old offspring from the MOP trial, suggesting similar early benefits as in female mouse offspring in the current study (391). Additionally, although absolute values were not different, the authors reported a decreased odds ratio for E/A to be clinically elevated in offspring of metformin- compared to placebo-treated obese mothers, suggesting protection against pathologically increased filling pressures as seen in our male offspring (391).

Both male and female Ob-Met offspring showed decreased SV and CO compared to Ob offspring at 6 months of age. As outlined in sections 6.4.1.2 and 6.4.2.2, these changes might be partly responsible for the corrected hyperdynamic function in male offspring and hypertension in female offspring. However, the similarity between these phenotypes poses the question whether similar intrauterine mechanisms underlie this phenotype of depressed cardiac function. Preliminary data from our laboratory indicates that metformin can be detected in the late gestation fetal heart (personal correspondence with Dr H. Blackmore and A. Hufnagel, manuscript in preparation), suggesting potential direct effects of metformin exposure on developing cardiomyocytes. Metformin is known to affect lipid metabolism in target cells (219), and indeed lipidomic analysis has shown alterations in lipid profile in Ob-Met fetal hearts (personal correspondence with Dr H. Blackmore, manuscript in preparation). Moreover, metformin influences cellular energetics by inhibiting complex I of the mitochondrial electron transport chain (leading to decreased ATP production) and activating AMPK (219). AMPK activation in adults is considered cardioprotective (530), and in fact downregulated cardiac AMPK signalling in offspring of overnourished mouse dams was proposed to underlie some of the programmed cardiac dysfunction (503). Paradoxical to the action of metformin in adult cells, prenatal exposure has been shown to decrease AMPK activation in CVD-related tissues like the kidney and perivascular adipose tissue in adult offspring (380,382), suggesting this could also be the case for the heart. AMPK activation should therefore be determined in both fetal and adult hearts of metformin-exposed offspring in our model. Whether through AMPK-dependent or independent mechanisms, metformin exposure *in utero* may have the potential to influence cellular energetics and cardiomyocyte function which if persistent could lead to the depressed LV systolic function seen at 6 months.

6.4.4 Strengths & Limitations

The major strength of this study is the longitudinal follow-up within the same animal from 12 weeks to 12 months of age, which allowed us to study how the effects of an obesogenic intrauterine environment and metformin intervention interacted with ageing. This is especially important since CVD is an age-related disease and parameters such as diastolic dysfunction can show biphasic

patterns. However, this strength is also a limitation: because the same animals were used at 3, 6 and 12 months of age, no tissues were collected at intermediate timepoints for molecular or histological analysis. This complicates the elucidation of the mechanism behind the diastolic dysfunction in 3-month-old females as well as the timing of cardiac stiffening in male offspring. Moreover, untangling mechanisms behind the early correction of SBP by the metformin intervention in female offspring is complicated by the obesity-induced hypertension that had developed by 12 months of age.

Historically, M-mode echocardiography was used to determine systolic function. However, this method calculates functional parameters from the internal diameter at the mid-cardiac point and therefore does not consider the shape of the LV. Therefore, in the current study systolic function was assessed using the B-mode LV Trace tool allowing more accurate determination of contractile capacity across the whole heart, which is a major strength of this study. Unfortunately, echocardiography cannot be used to measure ventricular and atrial pressures. Instead, this requires *in vivo* catheterisation which is invasive and not suitable for longitudinal studies.

PW Doppler flow dynamics (as used in this study to determine diastolic function) are influenced by age, preload, afterload and HR and consequently provide information on diastolic function *in situ* but not isolated cardiac effects (494). Furthermore, in order to fully understand the *in vivo* filling process PW Doppler should be combined with Tissue Doppler. This feature was not available in our animal facility at the time of this study. Because animals were exposed to repeated echocardiography, a limited selection of measurements was taken to avoid overexposure to anaesthesia throughout life. This prevented measurement of left atrial diameter as a marker of LAP which is best assessed in the 4-chamber-view (497), an image not taken in the current study due to time constraints. Similarly, RV dimensions are best assessed in a modified PSLAX view which was not included in our echocardiographic protocol for the same reason.

The gold standard for BP measurement in experimental conditions is radiotelemetry, which involves the surgical placement of a catheter directly into the aortic arch and allows for the measurement of undisturbed HR and BP over a prolonged period (510). Although radiotelemetry provides very accurate readings without interference of handling stress, this invasive technique requires surgery and is therefore not suitable for longitudinal assessment of BP (510,531,532). Tail cuff plethysmography is a non-invasive alternative that correlates well with invasive techniques in rodents (510,511,532), while also reflecting the way BP is measured in humans. However, the tail cuff technique does not generate accurate DBP measurements (533) and therefore DBP, pulse pressure and mean arterial pressure could not be estimated in this study.

6.5 Conclusion

Maternal obesity introduced diastolic dysfunction in offspring of both sexes. However, in male offspring this occurred later in life and was associated with hyperdynamic systolic function, while in female offspring this was seen early in life and secondary to elevated SBP which was maintained across the life-course. Exposure to maternal obesity therefore increases susceptibility to cardiovascular dysfunction in a sexually dimorphic manner. Although no overt HF was seen in the current study this predisposes offspring to CVD risk when exposed to a second hit such as postnatal overnutrition or further ageing. The metformin intervention corrected some of the programmed cardiovascular changes but also introduced independent cardiovascular alterations in a sex- and age-dependent manner, most clearly in females where obesity-induced hypertension was observed. The long-term effects of the intervention on offspring cardiovascular health are therefore not entirely beneficial and further studies are required to determine the full extent of the effects.

6.6 Key findings

- In male offspring, maternal obesity increased aortic width and impaired diastolic function that progressed with age, without changes in systolic function or blood pressure
- The metformin intervention corrected some of these changes in male offspring later in life (by 6 and 12 months of age, respectively) but introduced other independent alterations
- Maternal obesity programmed hypertension in female offspring, associated with diastolic impairment at 3 months of age
- The metformin intervention corrected these programmed changes in early life, but caused obesity-associated hypertension by 12 months of age

7 General discussion

The DOHaD hypothesis states that suboptimal environments during development confer long-term risks for exposed offspring. Prevalence of maternal obesity and/or GDM is rising at alarming rates (15,26,27) and is associated with obesity, metabolic and cardiovascular morbidity and even premature mortality in the offspring (69–75). Clinically relevant interventions are required to break the transgenerational cycle of obesity and associated comorbidities. Metformin is used to treat GDM in the UK and several other countries (30,228), and RCTs are investigating metformin as a candidate for implementation in pregnancies complicated by obesity or PCOS (233,240,346,534). However, metformin crosses the placenta and long-term follow-up of metformin-exposed offspring is sparse in both humans and animal models. Data regarding follow-up throughout the life-course, effects on female offspring and cardiovascular outcomes are especially lacking.

This thesis therefore aimed, using a mouse model, to determine both the short- and long-term effects of metformin exposure during obese pregnancy on adiposity and metabolic health in male and female offspring in early life (neonatal growth trajectory), young adulthood (8 weeks) and older age (12 months of age). Secondly, it aimed to investigate cardiovascular phenotypes in adult offspring at different time points across the life-course (3, 6 and 12 months of age).

7.1 Maternal and early offspring effects of prenatal metformin intervention

Feeding female mice a HFHS diet from preconception introduced obesity with increased fat mass before and during pregnancy as well as gestational and lactational hyperphagia. The metformin intervention decreased maternal fat mass in the pre-mating week and this difference was maintained until late pregnancy (fat mass) and at weaning (body weight), suggesting beneficial effects on maternal health consistent with human findings (261,278). In contrast, the metformin intervention did not impact on rate of litter losses, the decreased litter size or IUGR and catchup growth seen with obese pregnancy. Although this could indicate that the intervention is safe for offspring in the short-term [as commonly concluded from human studies (276,278,289)], the finding of increased gestation length poses the question whether fetal development is slowed by metformin. In combination with lactational hyperphagia following removal of metformin at the end of pregnancy the intervention may thus have introduced an IUGR with catchup growth phenotype independent of maternal obesity (Figure 7.1). Studies investigating fetal growth trajectory in metformin-treated pregnancies in humans and animal models are urgently needed to address this question given the known associations between these early growth patterns and long-term cardiometabolic health.

7.2 Effect of maternal obesity and metformin on offspring adipose tissue

Maternal obesity introduced hypertrophic adiposity in male and female offspring at 8 weeks of age and this increased adiposity compared to offspring of lean dams was maintained until 6 months of age. After 6 months, sex differences emerged: male Ob offspring were heavier and showed hypertrophic and hyperplastic adiposity with WAT inflammation and IR, whereas adiposity in female offspring was less prominent by 12 months of age (total WAT depot weight was increased only when expressed relative to body weight) and adipocyte hypertrophy had normalised [as previously seen in 4-month-old female but not male offspring of HFD-fed dams (535)]. Combined with the milder phenotype at 8 weeks of age, this suggests relative protection of female offspring against late-life obesity programmed by maternal overnutrition. At no point did maternal obesity cause metabolic abnormalities in the current study suggesting exposure to *in utero* overnutrition did not influence metabolic health at the ages investigated. However, the finding of increased adiposity remains striking as offspring were fed a healthy chow diet throughout life. Moreover, previous data from the lab showed that adiposity and IR were exacerbated in our model when offspring were weaned onto an obesogenic diet, suggesting that metabolic alterations might arise if offspring of obese dams are exposed to a 'second hit' postnatally (139). Similarly, metabolic abnormalities might be uncovered upon further ageing beyond 12 months (an alternative second hit) since the lifespan of mice is around 2 years of age (467).

The metformin intervention did not correct the adiposity phenotype programmed by maternal obesity, but instead led to exaggerated adiposity and adipose tissue dysfunction in both sexes in a time-dependent manner. In both sexes, offspring exposed to metformin during obese pregnancy showed WAT hypertrophy at 8 weeks of age prior to development of obesity. In males this was associated with adipocyte hyperplasia, WAT inflammation and local IR. Additionally, the intervention increased β-cell activity at this age. This might either be a direct pancreatic effect of early life metformin exposure, as has previously been reported with gestational or lactational metformin treatment (307,331,447), or a compensatory response to local IR (Figure 7.1). This protection did not persist as no changes in glucose tolerance or whole-body insulin homeostasis were observed at 12 months of age. However, as increased IR and circulating insulin with age are commonly seen in males (including in the current study), intervention effects may have been masked by a strong ageing effect in 12-month-old male offspring or alternatively require even further ageing to develop the phenotype. Glucose tolerance should therefore be tested at other time points across the life-course to further elucidate the effect of maternal metformin intervention on male offspring metabolic health. Interestingly, although Con and Ob offspring underwent age-related eWAT hyperplasia, adipocyte number failed to increase between 8 weeks and 12 months in Ob-Met offspring. Combined with the

finding of more extensive macrophage infiltration and increased liver weight [a finding that was previously reported in metformin-exposed offspring (301)], this leads to the hypothesis that WAT expansion capacity is restricted in Ob-Met males, which when combined with the early adiposity phenotype resulted in WAT dysfunction and ectopic lipid deposition in metabolic organs such as liver or muscle (Figure 7.1).

In contrast, in female metformin-exposed offspring hypertrophic adiposity in young adulthood was associated with an altered WAT response to fasting, alternative macrophage activation in gWAT and an improvement in whole-body insulin sensitivity. It thus seems that the metformin intervention introduces compensatory mechanisms to maintain glucose homeostasis in both sexes but does so in a different manner, which has been reported before in models of metformin intervention (307,447). The mechanisms by which the metformin intervention improved insulin sensitivity remain unknown, although protection against pro-inflammatory changes may play a role. As for male offspring, this protection was not long-lived as after 6 months of age it was female Ob-Met offspring that diverged from Con and Ob offspring in both body weight and fat mass, showing hyperplastic (but not hypertrophic) adiposity at 12 months of age. This was associated with both peripheral (gWAT) and systemic (HOMA-IR) insulin resistance, compensatory increases in β-cell output and consequent hyperinsulinaemia (Figure 7.1). Moreover, WAT inflammation was detected in Ob-Met female offspring only. Due to the feed-forward loop of pro-inflammatory changes in WAT described in Chapter 4, it is likely that the WAT inflammation contributed to the development of IR and that hyperinsulinaemia in turn exacerbated the pro-inflammatory signature of the WAT. Notably, liver weight also tended to be increased in female Ob-Met offspring suggesting that perhaps the WAT expansion limit had also been reached in these offspring. The increased macrophage infiltration in both male and female Ob-Met offspring could thus either reflect an intrinsic programming effect of intrauterine metformin or be a signal of exhausted WAT expansion capacity.

7.3 Effect of maternal metformin intervention on offspring cardiovascular function

Maternal obesity was associated with cardiovascular alterations in both male and female offspring. In males, Ob offspring had diastolic dysfunction leading to pressure overload and hyperdynamic systolic function indicated by a widening of the ascending aorta. This was likely an intracardiac effect of impaired relaxation because SBP was not altered in these offspring. The finding of cardiac effects in the absence of hypertension shows that these two outcomes are programmed by maternal obesity through different mechanisms, as was previously suggested by the exercise intervention in our maternal obesity model (128). In contrast, female offspring of obese dams were hypertensive through-

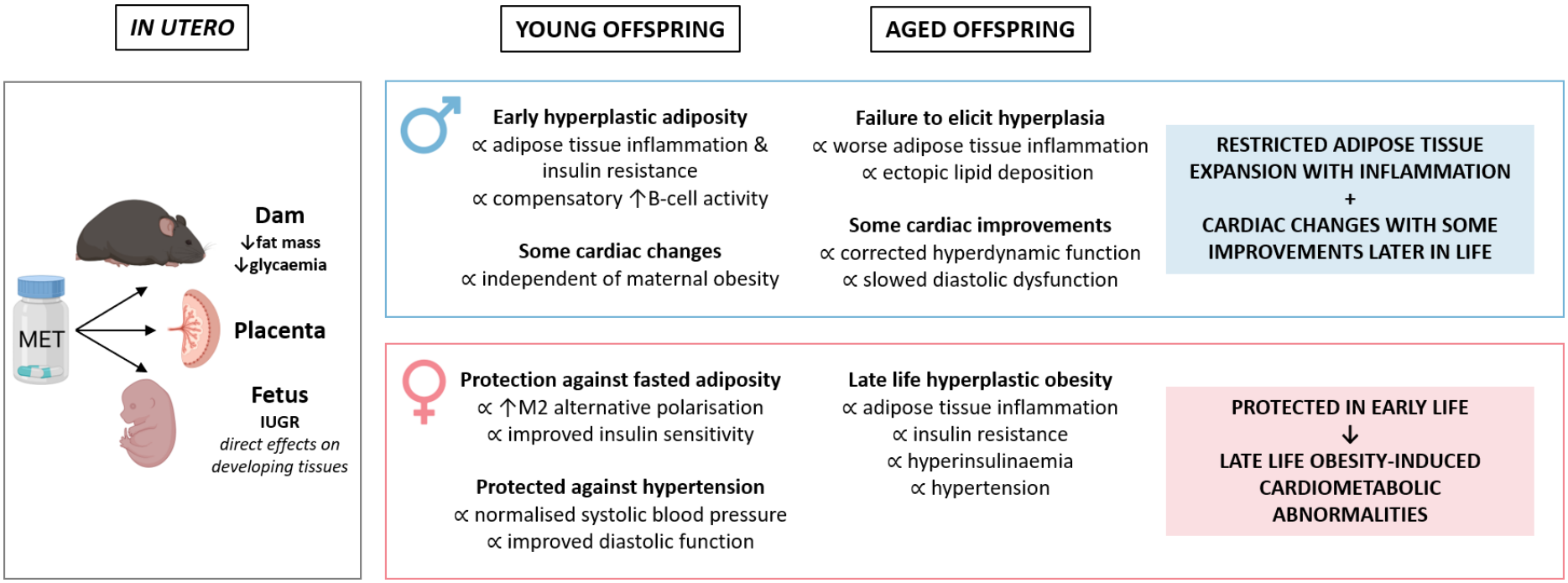

Figure 7.1: Summary of findings with regards to the metformin intervention in this thesis.
Image created with BioRender.com.

out life, which led to diastolic dysfunction at 3 months. Even though no systolic impairments were observed in these offspring, this predisposition to altered cardiovascular function can still have a large impact on offspring health. Although HFpEF has a better prognosis than HFrEF, mortality is still increased compared to the general population (499) and even pre-clinical diastolic alterations increase the risk of HF and cardiovascular mortality (498). Furthermore, in programming contexts a second hit is often required for more severe dysfunction to emerge. In our model, weaning offspring onto an obesogenic diet confers additional hypertension risk that is additive to the effects of maternal obesity (129). Changes in risk factors such as obesity and IR were also exacerbated by the combined exposure to maternal and postnatal obesogenic diet (139).

The metformin intervention corrected some of the changes that were observed with maternal obesity but exaggerated others. In male offspring, although diastolic dysfunction was not completely prevented, prenatal metformin halted the progression of diastolic dysfunction with age as evidenced by a plateauing E/A ratio between 6 and 12 months leading to normalisation of the aortic distension seen in Ob offspring (Figure 7.1). However, the intervention also introduced enhanced LV function at 3 months followed by cardiac depression at 6 months, and DT was decreased. Therefore, the metformin intervention introduced some cardiovascular alterations independently of maternal obesity that should be taken into account when assessing the benefit versus risk of the intervention. In female offspring, the metformin intervention corrected hypertension seen in 3- and 6-month-old offspring of obese dams. Since hypertension is a major predictor of HF (491) this is considered a beneficial effect. However, by 12 months of age female metformin-exposed offspring had developed obesity-induced hypertension. This illustrates the need to look both short- and long-term and also to investigate cardiometabolic physiology at the systemic level, as the initial cardioprotection of the intervention was completely reversed secondary to the adipose tissue effects.

7.4 The importance of long-term follow-up

Non-communicable diseases develop with age, and ageing exacerbates the effect of developmental programming by insults that include maternal obesity. In a developmental context it is therefore important to look beyond initial maternal and/or neonatal outcomes to determine the efficacy of an intervention. The exercise intervention studied in our laboratory, for instance, does not affect maternal body weight or correct IUGR in offspring, but has long-term beneficial effects on cardiac function in male offspring (128). Contrastingly, this thesis has shown that although metformin intervention may be beneficial for the mother [decreased adiposity in our model, and decreased GWG,

macrosomia and PE risk in humans (259,278,284)], this does not mean there are no adverse outcomes in offspring later in life as this thesis has shown (Figure 7.1).

The data presented in this thesis also highlight the importance of investigating intervention effects at different ages throughout life. Specifically, the metformin intervention improved insulin sensitivity in 8-week-old female offspring but the opposite was observed in 12-month-old offspring. Similarly, male offspring showed signs of compensatory β-cell activity in young adulthood which had disappeared with age. This mimicked the resolution of glucose intolerance in male offspring of obese dams in our model between 6 months (470) and 12 months of age. Moreover, the development of obesity-induced hypertension in female Ob-Met offspring as well as the more drastic diastolic dysfunction in Ob males and late-life correction thereof by the metformin intervention heavily depended on long-term follow-up. Lastly, without investigating WAT cellularity at two ages it would not have been possible to assess the temporal relationship between hypertrophy and hyperplasia.

Long-term follow-up in the current study also aided the identification of sex differences. At 8 weeks of age, female Ob and Ob-Met offspring showed less drastic adiposity than their male counterparts without signs of WAT inflammation, but it was unclear whether the intervention had delayed or prevented the development of obesity. Our longitudinal data showed that the increase in adiposity appeared later in female compared to male offspring as fat mass differences had emerged in both sexes by 12 weeks of age. Moreover, further ageing revealed that maternal obesity affected body weight in male offspring only, and although the metformin intervention effect on male and female offspring adiposity was similar between 3 and 6 months of age, sex-specific effects on WAT cellularity and insulin homeostasis developed later in life. If offspring follow-up had ceased at 6 months of age (a common study endpoint), the adverse effects of the metformin intervention on female obesity, SBP and metabolic health and the impaired WAT hyperplasia in aged males would have been missed.

7.5 Translational importance

With any animal model the question is how the findings translate to the human situation. The experimental model used in this thesis is clinically relevant with respect to the induction of maternal obesity (a preconception HFHS diet mimicking the obesogenic Western world), GDM (dams are glucose intolerant in gestation only) and metformin treatment (clinically relevant dose and dosing period). Moreover, parallels exist between the data presented in this thesis and outcomes of human trials, suggesting translational impact in our experimental model. Several trials in GDM and PCOS pregnancies have shown measures of increased adiposity in young metformin-exposed offspring

compared to insulin and placebo groups respectively (294,297,299,335,388,390), and IUGR-like growth trajectories have been reported before (257,271,294,297,335). These similarities in early outcomes might indicate that our longitudinal data is predictive of the human situation as well.

Human cohort studies have not investigated the inflammatory signature of adipose tissue in exposed offspring due to obvious ethical constraints. Serum CRP levels were not different in cord blood from metformin-exposed offspring in the EMPOWaR and MiG trials (232,274), nor in a subgroup of 4-year-old offspring from the MOP trial who did not show adiposity at that age (391). However, despite the lack of difference in cord blood CRP (232), increased levels of serum ferritin were found in the metformin arm of the 9-year-old New Zealand subgroup in the MiG trial (388). Indeed, serum ferritin is a marker of inflammation that is increased in patients with (components of) the metabolic syndrome (536). Moreover, ferritin can be produced by adipocytes. Since offspring also showed visceral adiposity at this age this does not exclude the possibility that WAT inflammation might be present.

Data on cardiovascular outcomes in human offspring is sparse. Initial follow-up of the PregMet pilot trial suggested increased SBP in 8-year-old offspring from metformin-treated PCOS mothers, but this study was small and confounded by a higher BMI in the metformin arm (299). Hence, direct comparison between their findings and ours is difficult. In contrast, the recently reported putative improvement in echocardiographic diastolic function indices in 4-year-old offspring from metformin-treated obese mothers could reflect the normalisation of diastolic dysfunction seen in offspring in this study, particularly in females where the correction was seen early in life. If the cardiovascular findings in the current study can be translated to humans, it would be interesting to see if female offspring from this trial also develop adiposity and obesity-induced hypertension later in life.

7.6 Clinical impact

Metformin treatment is widely used in the developed world (228) and increasingly popular in the developing world, hence the intervention will impact many pregnancies world-wide. Moreover, since trials generally show beneficial effects on early outcomes in mother and neonates (reviewed in Chapter 3) and women with GDM tend to prefer metformin to insulin (260), this number is likely to increase in the future. Moreover, metformin is being investigated for multiple other indications including PCOS (276,313), obesity (233,274,346) and pregnancies complicated by preeclampsia (537). The potential clinical impact of the data in this thesis is therefore large.

The metformin intervention in this study led to increased adiposity and WAT dysfunction in male and female offspring and introduced obesity-associated metabolic abnormalities and hypertension in aged

female offspring, warranting caution for use especially in pregnancies with female fetuses. However, metabolic health was unaffected in aged male offspring and the intervention was associated with short-term benefits in insulin homeostasis. Moreover, results from the longitudinal cardiovascular study suggests attenuation of some diastolic defects with maternal metformin treatment (although the obesity phenotype may worsen cardiovascular function later in life). Additionally, maternal fat mass was decreased therefore preventing excessive interpregnancy weight gain [associated with adverse pregnancy outcomes itself (33)] and leaving the mother leaner before her next pregnancy. Therefore, the relative benefits and adverse effects need to be weighed against one another. First of all, it is vital that these outcomes are addressed in human trials, as we cannot exclude that species-specific differences exist in vulnerability of developing organ systems to metformin. Moreover, the intervention might have different effects depending on clinical indication (GDM, T2DM, PCOS or glucose tolerant obesity), as illustrated by the heterogeneity in offspring outcomes using animal models that slightly differ in experimental design (301–304,307,359,392). Similarly, the timing of administration (from the second trimester in GDM compared to before or at conception in T2DM and PCOS, see section 1.7.1) is likely to matter. The wide range of offspring outcomes in literature (with both beneficial and adverse effects) warrant further investigation into long-term effects.

Although the data is this thesis suggest caution with respect to metformin treatment in pregnancies in the developed world, advice for the developing world [where maternal obesity and GDM are also increasing (538–540)] may be different. In low income countries, insulin may not be a suitable treatment for GDM: it is expensive, requires refrigerated storage and careful transport of fragile vials as well as more frequent monitoring and training of pregnant women for safe insulin injections (27). Moreover, poor glucose meter availability increases the risk of maternal hypoglycaemic episodes with insulin (270). Data on metformin-treated pregnancy in the developing world is sparse (28,541), and studies are often underfunded preventing access to sophisticated measures of offspring outcomes (542). It should be noted that compared to those complicated by obesity or PCOS, diabetic pregnancies always require treatment. In parts of the developing world where access to insulin is limited (only 10% of pharmacies stock insulin) and/or subject to financial barriers (>60% of households in low-income countries cannot afford insulin compared to <30% for metformin), oral hypoglycaemic agents are the only alternative to untreated GDM (543). In these cases, the (more long-term) metabolic programming effects by metformin need to be balanced with the embryotoxic effects of hyperglycaemia in pregnancy (544).

Data from this thesis found several changes that were more detrimental in offspring of metformin-treated compared to untreated obese pregnancy. The finding of offspring adiposity is concerning as

both childhood and adult obesity are already an increasing problem in the developing world (540) which will only be augmented with future generations due to the programming effects of both maternal and paternal obesity (165,545). On the other hand, improvement of short-term maternal and neonatal outcomes with metformin may be a priority in developing areas where maternal and infant mortality is high (488). Metformin may have beneficial effects on placental vascular function with the potential to prevent IUGR in pregnancies complicated by preeclampsia. Preeclampsia remains the main cause of maternal mortality worldwide, with the majority of maternal deaths occurring in the developing world (546). Moreover, preeclampsia is the main contributor to IUGR in developing countries (547). Studies in Iberian sows using an IUGR-model suggest metformin treatment may improve organ development and increase developmental competence of exposed fetal and neonatal offspring (362,363), suggesting potential for enhanced survival in metformin-treated preeclampsia. Indeed, RCTs looking into metformin for preeclampsia treatment (with the primary outcome of extended gestation time) in non-Western countries are currently underway (537). However, the potential IUGR with metformin use [as shown in animal models (301) and suggested by human trials (294)] in the developed world is concerning for offspring survival in low-income countries. Indeed, in an RCT investigating T2DM pregnancies in Pakistan, an increased risk of IUGR-related C-sections were observed in the metformin only arm, and metformin use (with or without supplementary insulin) increased risk of SGA across the study (336).

7.7 Follow-up questions & future directions

7.7.1 How does the metformin intervention affect fetal growth and adipogenesis?

As described in Chapter 3, the effect of metformin on birth weight is inconclusive in animal models, and in GDM-complicated pregnancies the increased risk of macrosomia complicates the distinction between IUGR and normalisation of birth weight. The only study investigating fetal body composition to date is the EMPOWaR trial in glucose tolerant obese women that found no differences in fetal subcutaneous adiposity, liver weight or hepatic fat deposition at 28 or 36 weeks (389), but no data is available for GDM or PCOS pregnancies. Therefore, it is vital to determine the intrauterine growth trajectory of metformin-exposed fetuses in both hyperglycaemic and glucose tolerant pregnancies. At different gestational ages, parameters such as placental and fetal body weight, crown-to-rump length, abdominal circumference and head circumference could be measured to provide an indication of fetal growth across ages. However, this would require the generation of multiple fetal cohorts. Alternatively, micro-ultrasound measurements of clinically relevant parameters including crown-to-

rump length, biparietal diameter, abdominal diameter and placental thickness could be performed in the same dam across gestation (548).

It would also be informative to study the potential effect of metformin on developing adipocytes. Since it is possible to isolate and culture pre-adipocytes from neonatal mice (549), expression of adipogenic markers could be measured in preadipocytes collected from neonatal offspring from control, obese and obese metformin-treated dams. Expression of genes characteristic of a 'starvation-like' transcription programme (439) as well as AMPK activation could be measured to assess whether metformin has had a direct effect. Alternatively, isolated preadipocytes could be cultured in presence of metformin to assess potential effects on differentiation or adipogenesis. Adipogenesis markers could also be measured in adipocytes from weanlings (535) to test the hypothesis that adipogenesis is enhanced in the early postnatal period following lactational hyperphagia after cessation of metformin treatment. Moreover, estimated adipocyte number could be determined at this age.

7.7.2 Does metformin promote ectopic lipid deposition in offspring?

As described in Chapter 5 and section 7.2, the metformin intervention is hypothesised to restrict visceral WAT expandability in male (and potentially female) offspring. In order to test this hypothesis, the presence of ectopic lipid deposition in liver and muscle tissues at 12 months of age could be investigated using molecular (Folch assay) or histological (lipid droplet quantification using HALO) methods. If ectopic lipid deposition is increased this is a strong indicator of restricted WAT expansion. Expression of lipogenic enzymes would also help to determine whether lipid storage is increased. Additionally, insulin signalling proteins and markers of oxidative stress or apoptosis can be measured in these tissues since these are common sequelae of lipotoxicity. The former could also be measured in WAT as lipolysis suppression is inadequate in insulin resistant adipocytes, consequently increasing flux to other organs (399).

7.7.3 What underlies the diastolic dysfunction in offspring of obese dams?

Diastolic dysfunction was observed in both male and female offspring of obese dams. However, in females this was accompanied by elevated SBP which was not seen in male offspring, suggesting different aetiology. Impaired relaxation can be caused by structural remodelling leading to cardiac fibrosis or LV hypertrophy (494). Using our model it was previously shown that in 8-week-old male offspring maternal obesity introduced systolic dysfunction, cardiac fibrosis and LV hypertrophy which was likely independent of hypertension (128,129,132,133). Therefore, a primary cardiac defect may

underlie the diastolic dysfunction in male offspring in the current study. It would be informative to measure collagen deposition (Western Blots and Picrosirius Red stain) and hypertrophy (stereology and wheat-germ agglutinin stain for cardiomyocyte size, and qPCR for expression of genes associated with pathological hypertrophy) in male hearts at the ages studied in this report to see if structural defects accompany the development of diastolic dysfunction. Moreover, measurement of left atrial diameter (echocardiography or histology) and/or pressure (isolated Langendorff preparation or *in vivo* cardiac catheterisation) could be performed to conclusive determine the presence of pressure overload. This will also help distinguish between normal and pseudonormal filling patterns. LV hypertrophy can also result from hypertension and consequently impair diastolic function. Previously no LV hypertrophy was seen in 8-week-old female offspring of obese dams despite hypertension (University of Cambridge PhD thesis, Dr Jessica Beeson). Cardiac hypertrophy should therefore be determined in 12-week-old female offspring to determine whether the diastolic dysfunction at this age may result from hypertension-induced hypertrophy. Moreover, wire myography could be performed on vessels from these animals to determine whether hypertension in female offspring results from altered vascular function. Lastly, diastolic dysfunction may also be related to impaired active relaxation responses in cardiomyocytes (494). Molecular markers of calcium recycling such as SERCA2 could be measured in ventricular tissue at ages where diastolic dysfunction was observed in male (6 and 12 months) and female (3 months) offspring in the current study.

7.7.4 What happens to these offspring upon further ageing?

Obesity, T2DM and CVD are age-related diseases. Although offspring was followed up until 12 months of age, the lifespan of mice is around 2 years and therefore this equates to middle age (467). Further ageing might thus be required for overt cardiovascular and metabolic dysfunction such as HF and T2DM to develop, acting as a 'second hit'. While ageing to 12 months was sufficient to introduce hypertension and hyperinsulinaemia in female offspring of obese metformin-treated dams, male offspring did not show alterations in BP or glucose homeostasis. Moreover, glucose tolerance during an ipGTT was unchanged in either sex. Follow-up of these offspring to 18 or 24 months of age is required to conclusively determine the long-term impact of maternal obesity and metformin intervention on offspring cardiometabolic health. Moreover, this will also allow us to address whether offspring of obese dams exhibit accelerated ageing by determining the lifespan, and to elucidate whether this is affected by the metformin intervention.

7.8 Concluding remarks

This thesis aimed to determine the longitudinal effects of maternal obesity and metformin intervention on the adiposity, metabolic and cardiovascular outcomes in male and female offspring. Maternal obesity introduces adiposity in exposed offspring which was more severe in males. Moreover, offspring developed diastolic dysfunction, which occurred early in life and was associated with hypertension in female offspring compared to later in life and independently of blood pressure in male offspring. The metformin intervention did not correct the adiposity phenotype but introduced exaggerated adiposity and WAT dysfunction by young adulthood in males (8 weeks) and later in life in females (12 months of age). In female offspring this was accompanied by obesity-induced hypertension, insulin resistance and hyperinsulinaemia. Concerns are therefore warranted for the use of metformin in pregnancies complicated by maternal obesity and/or GDM, especially if pregnant with a female fetus. In conclusion, this thesis reports sex- and age-specific effects of maternal obesity and prenatal metformin intervention, highlighting the need for short- and long-term follow-up of both male and female offspring exposed to early life insults and prenatal interventions.

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Appendix

A. Metformin dosing calculations

Metformin was dissolved in 1ml water and then stirred into 75g condensed milk. Therefore, the 'dose' calculated below refers to the amount of metformin that is required to enter the 75g milk pot to generate a daily intake of 300mg/kg, as calculated from recent body weight and milk intake (estimated intake of 2g/d was used to calculate the first dose). Therefore, in the example below, 317mg metformin needed to be dissolved in 1ml water for dosing.

$$Metformin\ dose\ (mg) = \frac{body\ weight\ (kg) \times 300mg \cdot kg^{-1} \cdot d^{-1} \times 75g\ milk\ pot}{milk\ intake\ (g \cdot d^{-1})}$$

Body weight and milk intake of female								
Day	**Weighing Date**	**ID**	**BW (kg)**	**Days**	**Milk start**	**Milk end**	**Milk intake (g)**	**Milk intake (g/d)**
Monday	29/01/2018	18M6	0.0348	3	161.4	154	7.4	2.5
Amending the dose to 300mg/kg/day								
Day	**Dosing Date**	**ID**	**BW (kg)**	**Milk intake (g/d)**	**Dose (mg/kg/d)**	**Milk pot (g)**	**mg MET in 75g milk**	
Tuesday	30/01/2018	18M6	0.0348	2.5	300	75	**317**	

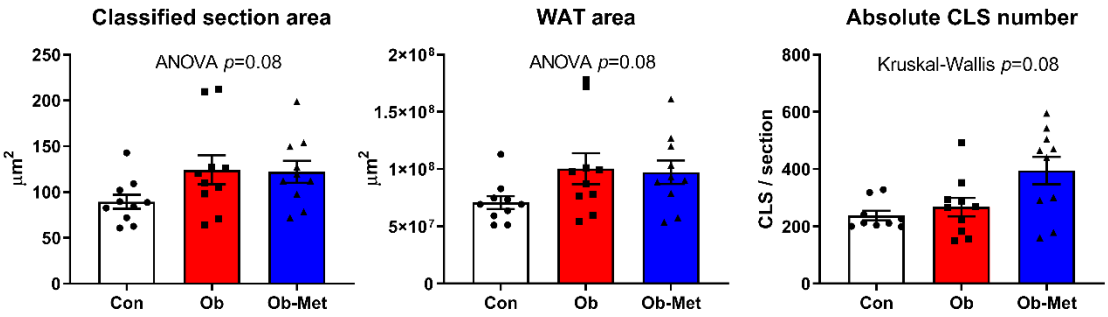
In order to estimate the amount of metformin ingested, the formulae below were used. In the example below, the dam had ingested 10.0mg metformin per day in the first four days of gestation, leading to an estimated amount of 257mg/kg/d over this period.

$$Metformin\ ingested\ (mg \cdot kg^{-1}) = \frac{Metformin\ dose\ (mg)}{75g\ milk\ pot} \times milk\ intake\ (g \cdot d^{-1})$$

$$Metformin\ ingested\ (mg \cdot kg^{-1} \cdot d^{-1}) = \frac{\frac{Metformin\ dose\ (mg)}{75g\ milk\ pot} \times milk\ intake\ (g \cdot d^{-1})}{body\ weight\ (kg)}$$

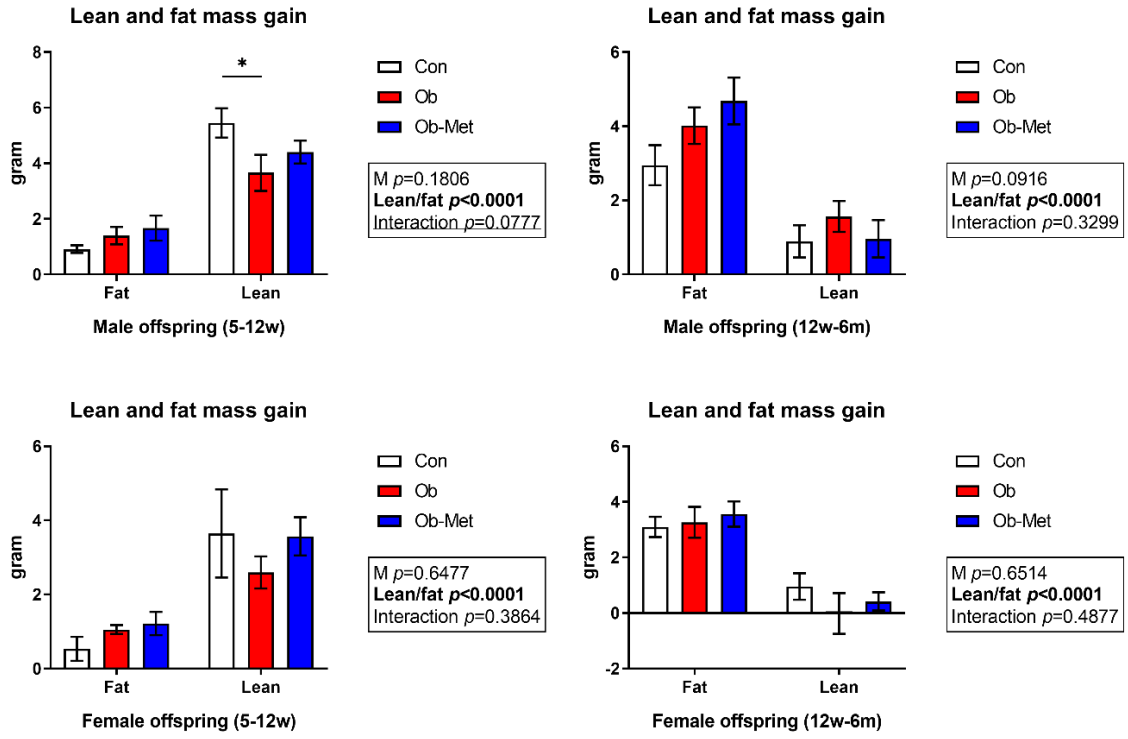
Weigh Date	**Dosing period**	**18M16**	**day**	**BW (kg)**	**Days**	**Milk (start)**	**Milk (end)**	**Intake (g)**	**Intake (g/d)**	**Dose (mg/75g)**	**Metformin ingested (mg/d)**	**Metformin ingested (mg/kg/d)**
12-Mar		Monday (start)	0	0.0373								
16-Mar	d0-4	Friday	4	0.0379	4	160.1	142.4	17.7	4.425	380	**22.4**	**592**
19-Mar	d4-7	Monday (mate)	7	0.0376	3	160.2	154.4	5.8	1.9	247	**6.4**	**169**
23-Mar		**Plug on Friday**	E1	0.0377								
27-Mar	E1-5	Tuesday	E5	0.0390	4	161.1	153.2	7.9	2.0	380	**10.0**	**257**
30-Mar	E5-8	Friday	E8	0.0396	3	161.1	152.7	8.4	2.8	380	**14.2**	**358**
03-Apr	E8-12	Tuesday	E12	0.0431	4	160.4	154.5	5.9	1.5	318	**6.3**	**145**
07-Apr	E12-15	Friday	E15	0.0479	3	159.8	155.4	4.4	1.5	380	**7.4**	**155**
10-Apr	E15-19	**Removed on Tues**	E19	0.0522	4	160.3	155.9	4.4	1.1	420	**6.2**	**118**

B. Section size and absolute crown number in 8-week-old male fed offspring



Appendix B.1: Section size and absolute crown number in 8-week-old male fed offspring
The p-values reflect outcomes of one-way ANOVA or Kruskal-Wallis test for non-parametric data. CLS = crown-like structures. WAT = white adipose tissue.

C. Lean and fat mass gain until 6 months of age



Appendix C.1: Lean and fat mass gain until 6 months of age
*Top: male offspring. Bottom: female offspring. Box: results from two-way ANOVA the effect of lean or fat mass (A), the maternal environment (M) and the interaction between them. *p<0.05 with Tukey's multiple comparison. Numbers are n=9 Con, n=10 Ob, n=9 Ob-Met for male offspring and n=9 Con, n=8 Ob, n=10 Ob-Met for female offspring.*

D. Correlations of inflammatory gene expression with serology/fat weight in 12-month-old male offspring

Males		*F4/80*	*Cd11c*	*Mip1a*	*Tnf*	*iNos*	*Ccr2*	*Mcp1*	*Il1b*	*Il6*	*Hif1a*
Chol	a	**0.331**	**0.091**	**0.225**	**0.379**	0.316	0.629	**0.191**	**1.035**	-0.524	0.792
	r^2	**0.616**	**0.661**	**0.446**	**0.668**	0.033	0.187	**0.537**	**0.264**	0.073	0.126
	p	**<0.0001**	**<0.0001**	**0.0003**	**<0.0001**	0.374	0.024	**<0.0001**	**0.007**	0.183	0.082
TG	a	-0.015	-0.004	0.015	-0.010	-0.046	-0.048	-0.004	-0.157	0.214	-0.157
	r^2	0.021	0.024	0.037	0.008	0.017	0.022	0.004	0.087	0.178	0.082
	p	0.467	0.430	0.337	0.652	0.506	0.445	0.759	0.135	0.028	0.147
FFA	a	0.029	0.008	0.039	0.046	0.130	0.128	0.026	0.007	0.140	0.110
	r^2	0.052	0.053	0.163	0.105	0.077	0.092	0.105	0.000	0.057	0.025
	p	0.236	0.229	0.033	0.080	0.145	0.103	0.086	0.953	0.221	0.421
Glucose	a	0.096	0.024	0.090	0.155	**0.949**	0.338	0.061	0.539	-0.510	1.130
	r^2	0.026	0.022	0.035	0.063	**0.220**	0.034	0.026	0.035	0.034	0.137
	p	0.393	0.436	0.329	0.1731	**0.009**	0.321	0.391	0.332	0.336	0.048
Insulin	a	**36.70**	**10.40**	**28.07**	**41.40**	50.00	**94.900**	**25.85**	**142.40**	-93.32	103.30
	r^2	**0.487**	**0.542**	**0.475**	**0.559**	0.082	**0.324**	**0.606**	**0.298**	0.140	0.141
	p	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.1480	**0.002**	**<0.0001**	**0.003**	0.054	0.059
IR	a	**1.813**	**0.498**	**1.435**	**2.129**	3.607	**5.037**	**1.253**	**6.659**	-4.831	**6.351**
	r^2	**0.533**	**0.556**	**0.551**	**0.666**	0.204	**0.411**	**0.639**	**0.306**	0.177	**0.255**
	p	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.018	**0.0002**	**<0.0001**	**0.003**	0.029	**0.009**
%B	a	17.86	4.824	9.125	16.84	8.354	**64.35**	11.04	56.21	-73.78	65.83
	r^2	0.223	0.208	0.094	0.173	0.004	**0.297**	0.186	0.087	0.167	0.107
	p	0.015	0.019	0.136	0.031	0.752	**0.003**	0.028	0.144	0.038	0.111
Epi fat*	a	**1.378**	**0.777**	**0.724**	**1.296**	1.083	**2.490**	**1.108**	1.067	-1.115	2.063
	r^2	**0.518**	**0.589**	**0.314**	**0.570**	0.085	**0.567**	**0.570**	0.087	0.151	0.170
	p	**<0.0001**	**<0.0001**	**0.002**	**<0.0001**	0.119	**<0.0001**	**<0.0001**	0.119	0.038	0.026

Appendix D.1: Correlations of inflammatory gene expression with serology/fat weight in 12-month-old male offspring
*The slope (a), r^2 and p-values reflect results from linear regression analysis. Bold = significant correlation p<0.01. Grey = strong correlation $r^2 > 0.4$. *linear regression for epi fat weight performed using log-transformed gene expression data.*

E. Correlations of inflammatory gene expression with serology/fat weight in 12-month-old female offspring

Females		***F4/80***	***Cd11c***	***Mip1a***	***Tnf***	***iNos***	***Ccr2***	***Mcp1***	***Il1b***	***Il6***	***Hif1a***
Chol	a	0.043	-0.010	-0.028	-0.010	-0.100	0.264	-0.005	-0.103	0.071	0.210
	r^2	0.004	0.003	0.016	0.000	0.022	0.092	0.001	0.035	0.008	0.061
	p	0.720	0.750	0.486	0.911	0.410	0.086	0.893	0.309	0.619	0.159
TG	a	0.035	0.013	0.045	0.002	0.185	0.091	0.035	-0.046	-0.106	-0.244
	r^2	0.006	0.011	0.096	0.000	0.170	0.023	0.058	0.015	0.038	0.180
	p	0.670	0.555	0.080	0.972	0.015	0.387	0.169	0.495	0.265	0.011
FFA	a	-0.295	-0.042	-0.019	0.027	0.026	-0.303	-0.057	-0.104	0.237	0.128
	r^2	0.135	0.054	0.008	0.002	0.001	0.086	0.053	0.024	0.063	0.017
	p	0.030	0.195	0.630	0.808	0.835	0.091	0.189	0.390	0.146	0.460
Glucose	a	0.668	0.129	-0.034	0.277	0.095	0.790	0.138	-0.007	-0.400	0.417
	r^2	0.184	0.111	0.005	0.057	0.004	0.155	0.093	0.000	0.045	0.046
	p	0.013	0.067	0.706	0.188	0.738	0.026	0.090	0.977	0.233	0.233
Insulin	a	**80.84**	**28.89**	9.225	**65.20**	60.01	92.46	**28.25**	25.01	-59.95	-53.62
	r^2	**0.256**	**0.485**	0.033	**0.267**	0.148	0.191	**0.329**	0.035	0.097	0.068
	p	**0.003**	**<0.0001**	0.334	**0.003**	0.033	0.014	**0.001**	0.323	0.082	0.150
HOMA IR	a	**4.129**	**1.401**	0.393	**3.119**	2.811	**4.579**	**1.385**	1.050	-2.873	-2.038
	r^2	**0.317**	**0.543**	0.029	**0.293**	0.153	**0.223**	**0.378**	0.029	0.106	0.046
	p	**0.001**	**<0.0001**	0.371	**0.002**	0.029	**0.007**	**0.0002**	0.368	0.069	0.236
HOMA %B	a	40.28	**18.77**	8.348	45.40	42.79	50.67	17.08	21.75	-40.30	-65.96
	r^2	0.076	**0.247**	0.032	0.156	0.089	0.068	0.144	0.032	0.052	0.122
	p	0.128	**0.005**	0.348	0.028	0.102	0.156	0.035	0.346	0.208	0.050
Gonadal fat	a	**0.631**	-	0.180	-	-	-	-	0.074	-0.481	-0.653
	r^2	**0.208**	-	0.197	-	-	-	-	0.004	0.088	0.146
	p	**0.006**	-	0.010	-	-	-	-	0.724	0.084	0.024
Gonadal fat*	a	-	**1.378**	-	**1.454**	**1.784**	**1.790**	**1.224**	-	-	-
	r^2	-	**0.635**	-	**0.308**	**0.208**	**0.239**	**0.527**	-	-	-
	p	-	**<0.0001**	-	**0.001**	**0.007**	**0.003**	**<0.0001**	-	-	-

Appendix E.1: Correlations of inflammatory gene expression with serology/fat weight in 12-month-old female offspring
*The slope (a), r^2 and p-values reflect results from linear regression analysis. Bold = significant correlation p<0.01. Grey = strong correlation $r^2 > 0.4$. *linear regression for gonadal fat weight performed using log-transformed gene expression data.*

F. Additional cardiovascular parameters in female offspring

	Age	Con (n=7-12)	Ob (n=7-12)	Ob-Met (n=7-12)	A	M	A*M	post-hoc
E-wave (mm/s)	**3m**	665 ± 37	613 ± 25	574 ± 22	*ns*	*ns*	*ns*	
	6m	652 ± 24	631 ± 28	654 ± 28				
	12m	632 ± 22	631 ± 24	602 ± 21				
A-wave (mm/s)	**3m**	539 ± 21	492 ± 16	564 ± 39	*ns*	*ns*	*ns*	
	6m	524 ± 13	525 ± 27	507 ± 25				
	12m	521 ± 19	497 ± 23	460 ± 30				
Ascending aorta (diastole)	**3m**	1.23 ± 0.02	1.19 ± 0.03	1.22 ± 0.02	***<0.0001***	*ns*	*ns*	***3m vs 6m**** ***3m vs 12m***** ***6m vs 12m****
	6m	1.27 ± 0.02	1.26 ± 0.04	1.32 ± 0.03				
	12m	1.34 ± 0.03	1.36 ± 0.05	1.42 ± 0.05				
Ascending aorta (systole)	**3m**	1.40 ± 0.02	1.36 ± 0.02	1.35 ± 0.02	***0.0047***	*ns*	*ns*	***6m vs 12m****
	6m	1.43 ± 0.03	1.40 ± 0.03	1.40 ± 0.03				
	12m	1.42 ± 0.03	1.48 ± 0.03	1.48 ± 0.03				

Appendix F.1: Additional cardiovascular parameters in female offspring

*Right-hand columns reflect results from repeated measures mixed effects model testing the effect of age (A), the maternal environment (M) and the interaction between them (A*M) with Tukey's multiple comparison test. ns = not significant, **p<0.01, ***p<0.001, ****p<0.0001.*