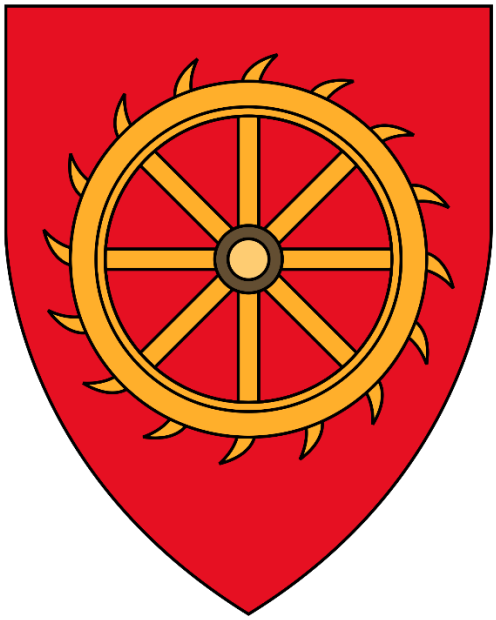
Maternal Obesity During Pregnancy: Cardiovascular Outcomes for Mother and Baby



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This thesis is submitted for the degree of Doctor of Philosophy

Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as specified in the text. I further state that no substantial part of my thesis has already been submitted, or is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution. It does not exceed the prescribed word limit of 60,000 words for the Clinical Medicine and Clinical Veterinary Medicine Degree Committee.

Signed [Signature redacted] Date 22.12.22

Phoebe Kate Wilsmore 22nd December 2022

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Summary

The prevalence of obesity has increased significantly over the last 30 years such that it is now more common worldwide to be overweight or obese than normal weight. As a result of this, increasing numbers of women are entering pregnancy overweight or obese. Obesity during pregnancy is associated with an increased risk of numerous negative obstetric outcomes including miscarriage, stillbirth, and neonatal mortality. Moreover, obesity is associated with an increased risk of women developing disorders of pregnancy such as gestational diabetes mellitus (GDM) and pre-eclampsia. These disorders are associated with the development of type 2 diabetes (T2D) and persistently high arterial blood pressure (hypertension) in the years after delivery. Hypertension is the number one risk factor for the development of cardiovascular disease (CVD), and CVD is the leading cause of death across the globe. Therefore, maternal obesity during pregnancy is associated with poor cardiometabolic health both during pregnancy and in later life.

Children of obese women are at an increased risk of both becoming obese themselves and developing hypertension and CVD. While some of this increased risk in the general population is attributable to a shared obesogenic postnatal environment, studies in both humans and animals reveal that it is also directly programmed by exposure to maternal obesity *in utero*. This forms part of the Developmental Origins of Health and Disease (DOHaD) hypothesis, which states that exposure to suboptimal environments during development programmes changes in long-term health. Together, this shows that maternal obesity during pregnancy is associated with poor cardiometabolic health in both mother and baby in the short and long-term, which will result in an increasing number of people suffering and dying from CVD. Therefore, it is paramount that interventions are developed in order to break the transgenerational cycle of obesity and cardiovascular dysfunction. Two interventions used in pregnancies complicated by GDM and that are being trialled in glucose-tolerant obese pregnancies are the application of an exercise intervention and the use of the glucose-lowering medication metformin. While evidence of improved maternal cardiometabolic function in response to these interventions has been found in human studies, longitudinal follow-up of children is limited.

The Ozanne group uses a well-established mouse model of maternal diet-induced obesity to investigate the effects of maternal obesity and glucose intolerance during pregnancy on the cardiometabolic health of both mother and baby. Using this model, the effects of maternal exercise and metformin intervention during pregnancy on the cardiometabolic health of both mother and offspring can be investigated.

Previous studies have found that maternal exercise intervention improves metabolic and cardiac function in young adult male offspring of obese dams but does not prevent the programming of hypertension. This is associated with improved metabolic health in the dam without a significant change in her adiposity. However, how obesity affects maternal cardiovascular function in pregnancy,

and whether this is affected by exercise, has not previously been investigated in this model. In Chapter 3 of this thesis, evidence of impaired cardiovascular adaptation to pregnancy was identified in obese dams, although this did not result in overt cardiac dysfunction in late gestation. Maternal obesity also resulted in fetuses being growth-restricted, which is well established to be associated with poor long-term cardiometabolic health. Neither the impaired cardiovascular adaptation nor fetal growth restriction were rescued by maternal exercise.

Changes in the autonomic control of the heart, including an increase in sympathetic activity and a decrease in parasympathetic activity, are a common feature of heart failure. Previous studies have suggested that maternal obesity programmes changes in autonomic control of the offspring heart. In Chapter 4 of this thesis, a new protocol was developed to investigate the response of the heart to sympathetic stimulation *in vivo*. This protocol is a powerful tool that can be adapted to investigate the effects of maternal obesity during pregnancy on cardiovascular function in male and female offspring.

Metformin administration during obese pregnancy has been found to improve maternal metabolic health in both human and animal studies. However, metformin exposure *in utero* has been found to programme increased adiposity and metabolic dysfunction in male and female offspring of obese dams. No studies have been published investigating the impact of metformin exposure during obese pregnancy on the blood pressure and vascular function of offspring beyond childhood in humans, or at all in animals. Hypertension and CVD are age-related disorders and so the effect of gestational metformin on blood pressure and vascular function must be investigated in aged offspring in order to draw clinically relevant conclusions about its effects. In Chapter 5 of this thesis, it was found that combined exposure to maternal obesity and metformin *in utero* programmed hypertension in aged offspring. This was associated with sex-specific changes in vascular reactivity. This suggests that maternal metformin administration during pregnancy is detrimental to long-term offspring cardiovascular health and highlights the importance of including both male and female offspring in studies of developmental programming.

Overall, this thesis investigated the effects of obesity on cardiovascular function in both pregnant mothers and their offspring and developed a new protocol for the investigation of cardiovascular function *in vivo*. It also assessed the effects of exercise intervention on maternal cardiovascular adaptation to pregnancy, and of metformin intervention on long-term cardiovascular health in offspring. Developing effective interventions in obese pregnancy is paramount for improving the cardiometabolic health of the population, both in reducing the incidence of CVD and T2D in overweight and obese mothers, and in preventing the programming of obesity and cardiovascular dysfunction in their offspring.

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Abbreviations and Acronyms

α1R	alpha-1-adrenoreceptor
AAC	area above the curve
ACh	acetylcholine
AChR	acetylcholine / cholinergic receptor
AET	aortic ejection time
AFE	Atwater free energy
AGEs	advanced glycation end products
AngII	angiotensin II
ANS	autonomic nervous systems
Apic-4	apical four-chamber view
ApoE	apolipoprotein E
ATP	adenosine triphosphate
AUC	area under the curve
AWERB	Animal Welfare and Ethical Review Board
β1R	beta-1-adrenoreceptor
BMI	body mass index
BW	body weight
CO	cardiac output
‘Con	control (lean) dams
Con	offspring of control (lean) dams
CVD	cardiovascular disease
DALI study	Vitamin D and Lifestyle Intervention in the prevention of GDM study
DBP	diastolic blood pressure
Deceler^{n}	mitral valve deceleration (rate / time)
DEXA	dual-energy X-ray absorptiometry
DOHaD	Developmental Origins of Health and Disease
E	embryonic / gestational day
ECG	electrocardiogram
EDHF	endothelium-derived hyperpolarising factor
EDV	end-diastolic volume (when referencing the heart)
EDV	end-diastolic velocity (when referencing uterine artery flow)
EF	ejection fraction
EMPOWaR	Efficacy of Metformin in Pregnant Obese Women, a Randomised Controlled Trial
eNOS	endothelial nitric oxide synthase
ESV	end-systolic volume
ET-1	endothelin 1
ETIP trial	Exercise Training and Weight Gain in Obese Pregnant Women trial
FFA	free fatty acid
FS	fractional shortening
FTO	fat mass and obesity-associated protein
GDF15	growth differentiation factor 15
GDM	gestational diabetes mellitus
GWAS	genome wide association study
GWG	gestational weight gain
HAPO study	Hyperglycaemia and Adverse Pregnancy Outcomes study
HFD	high-fat diet

HF-HS	high-fat, high-simple carbohydrate
HR	heart rate
Hyst. BW	hysterectomised body weight
IMPROVE	Improving Maternal and Progeny Obesity Via Exercise study
Inter^{n}	interaction
IP fat	intraperitoneal fat
IUGR	intra-uterine growth restriction
IV	intravenous
IVCT	isovolumetric contraction time
IVRT	isovolumetric relaxation time
IVS	intra-ventricular septum
K^{+} curve	potassium curve
Krebs	Krebs-Hensleit buffer
Lepr	leptin receptor gene
LGA	large for gestational age
LIMIT	Limiting Weight Gain in Overweight and Obese Women During Pregnancy to Improve Health Outcomes trial
L-NAME	L-NG-nitroarginine methyl ester, an eNOS inhibitor
LV	left ventricle / left ventricular
LVAW	Left ventricular anterior wall
LVEDP	left ventricular end-diastolic pressure
LVEnL	left ventricle endocardial length
LVID	left ventricle inner diameter
LVOT	left ventricle outflow tract
LVPW	left ventricular posterior wall
MAChR	muscarinic cholinergic receptors
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinases
MC4R	melanocortin 4 receptor
MiG trial	Metformin in GDM trial
MODY	maturity onset diabetes of the young
MOP trial	Metformin in Obese Non-diabetic Pregnant Women trial
MPI	myocardial performance index
MSFP	mean systemic filling pressure
MV	mitral valve
NA	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate
NFT	non-filling time
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NO	nitric oxide
NP	non-pregnant
NPY	neuropeptide Y
‘Ob	obese dams
Ob	offspring of obese dams
‘Ob-Ex	obese-exercised dams
Ob-Ex	offspring of obese-exercised dams
‘Ob-Met	obese-metformin dams
Ob-Met	offspring of obese-metformin dams

P2	second pregnancy
PA	pulmonary artery
PAT	pulmonary artery acceleration time
PBS	phosphate-buffered saline
PCOS	polycystic ovary syndrome
PE	phenylephrine
PET	pulmonary artery ejection time
PI	pulsatility index
PND	post-natal day
PNS	parasympathetic nervous system
POMC	pro-opiomelanocortin
Preg	pregnant
PregMet trial	Metformin in Pregnant PCOS Women trial
PreP2	pre-second pregnancy
PSAX	parasternal short-axis view
PSLAX	parasternal long-axis view
PSV	peak systolic velocity
PVN	paraventricular nuclei
PW Doppler	Pulsed Wave Doppler
RAGE	receptor for advanced glycation end products
RBC	red blood cell
RCT	randomised controlled trial
RI	resistive index
ROS	reactive oxygen species
RP fat	retroperitoneal fat
SBP	systolic blood pressure
SEM	standard error of the mean
SGA	small for gestational age
SNP	sodium nitroprusside
SNS	sympathetic nervous system
SV	stroke volume
SVR	systemic vascular resistance
T2D	type 2 diabetes
TDNMR	time-domain nuclear magnetic resonance
TGF-β	transforming growth factor beta
TOP study	Treatment of Obese Pregnant Women study
UK	United Kingdom
UPBEAT	UK Pregnancies Better Eating and Activity Trial
USA	United States of America
UtA	uterine artery
VCAM1	vascular adhesion molecule 1
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
VTI	velocity time integral
WAT	white adipose tissue
WHO	World Health Organisation

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Chapter 1: General Introduction

1.1 Obesity

1.1.1 The obesity epidemic

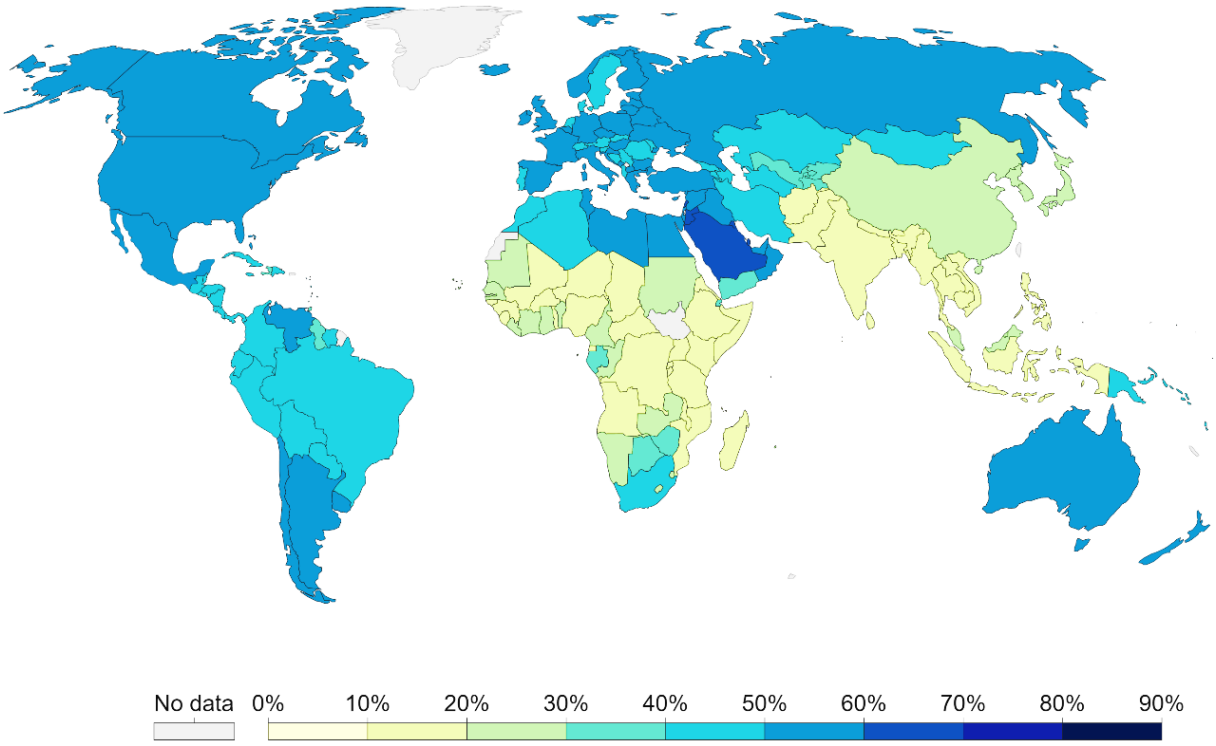


Figure 1.1: Percentage of adults who were overweight or obese worldwide in 2016. *Figure adapted from Our World in Data: https://ourworldindata.org/obesity* [1]

The rising prevalence of obesity and associated conditions is one of the largest global health challenges today. The most common way of categorising whether a person is a standard weight for their height versus under- or over- weight is to calculate their Body Mass Index (BMI). If someone has a BMI between 25 and 30 kg/m^2 they are classified as overweight and having a BMI greater than 30 kg/m^2 classifies an individual as obese. Between 1975 and 2016 the worldwide prevalence of obesity nearly tripled, with over 1.9 billion adults now being classified as overweight or obese[2]. This increase is observed in both the developed and developing world, with the majority of adults now being overweight or obese in more than half of all countries[1] (Figure 1.1). This has both direct and indirect health, social and economic impacts in the UK and across the globe. Almost two-thirds of adults in England were overweight or obese in 2018[3] and the NHS was estimated to have spent £6.1 billion on ill-health related to overweight or obesity in 2014[4]. This problem is currently only set to get worse; obesity in childhood is a strong predictor of adult obesity[5] and recent data from NHS Digital found that between 2006 and 2021 the prevalence of being overweight or obese has increased from 23% to 27.7% in 4-5 year olds, and from 35.2% to 40.9% in 10-11 year olds[6].

1.1 | Obesity

The socioeconomic impact of obesity worldwide is largely due to the fact that the excessive adiposity in obesity increases a person's risk of developing numerous health conditions, including several of the world's leading causes of death: heart disease, stroke, type 2 diabetes and numerous cancers (Table 1.1)[27]. For every 5 kg/m^2 increase in BMI above 25 kg/m^2, overall mortality increases by 29%[8].

Diabetes mellitus is a group of chronic metabolic disorders in which the body is not able to correctly regulate blood glucose levels due to impairments in insulin production and/or signalling, resulting in elevated blood glucose levels. If untreated, the elevated glucose levels result in tissue damage and even death. Throughout this thesis, diabetes mellitus is referred to simply as "diabetes". There are several different types of diabetes, including, type 1 and type 2 diabetes, gestational diabetes (GDM), and maturity onset diabetes of the young (MODY).

Obesity is a key contributor to the pathogenesis of type 2 diabetes (T2D) because the accumulation of excess adiposity results in the development of peripheral insulin resistance[9]. As peripheral insulin resistance increases, the beta cells in the Islets of Langerhans in the pancreas respond by increasing insulin secretion in order to maintain glucose homeostasis. In T2D, the pancreas is no longer able to compensate for the increased insulin resistance and so glycaemic control fails. In the UK, a person is considered diabetic if their fasting blood glucose level is greater than 7.0 mmol/L and/or their two-hour post-prandial blood glucose level is greater than 11.1 mmol/L. As many as one in five overweight and obese people in the UK are diabetic[3] and even in the absence of overt diabetes obesity results in metabolic dysfunction; in 2019 it was estimated that 7.5% of the world's population had impaired glucose tolerance[10], meaning that they are not able to properly respond to a glucose challenge and are at an increased risk of developing T2D.

	Overweight		Obese	
Co-morbidity	Male	Female	Male	Female
Type 2 Diabetes	2.4	3.92	6.74	12.41
Hypertension	1.28	1.65	1.84	2.42
Coronary Heart Disease	1.29	1.80	1.72	3.10
Stroke	1.23	1.15	1.51	1.49
Asthma	1.2	1.25	1.43	1.78
Colorectal Cancer	1.51	1.45	1.95	1.66
Pancreatic Cancer	1.28	1.24	2.29	1.60
Endometrial Cancer	n/a	1.53	n/a	3.22
Kidney Cancer	1.40	1.82	1.82	2.64

Table 1.1: Risk ratio of co-morbidities in adults who are obese or overweight compared to those who are a normal weight. *Data from Guh et al[7]*

Due to their increasing incidence across the world, both diabetes and obesity are recognised as epidemics by the World Health Organization (WHO), and diabetes is now the 9th biggest cause of death globally[11]. Approximately 537 million adults are living with diabetes worldwide, of which around 90% have T2D[12]. In the UK alone, over four millions adults have diagnosed T2D, although there are estimated to be a further 800,000 adults with T2D that have yet to be diagnosed and so are unaware that they have the condition[13], and treating diabetes and its complications accounts for more than 10% of NHS annual expenditure[14]. The increasing prevalence of obesity and diabetes is associated with an increasing prevalence of the metabolic syndrome, a syndrome loosely characterised by a combination of metabolic phenotypes that was first described in the 1920s[15]. While there is no set criteria for the metabolic syndrome, most definitions agree that it includes glucose intolerance, obesity, high blood pressure and dyslipidaemia, having abnormal lipid levels in the blood[16].

The COVID-19 pandemic further highlighted the secondary health effects of increased adiposity, with obese individuals who tested positive for SARS-CoV-2 being more likely to need to be hospitalised and even die from the disease[17]. As well as obesity affecting COVID-19 patients outcomes, recent studies suggest that the pandemic has led to an increase in the prevalence of childhood obesity in the UK[6].

1.1.2 Aetiology of obesity

Given its significant health and socioeconomic impact, it is important to establish how and why people become obese, and why obesity is becoming more prevalent worldwide. On a day-to-day basis, humans are able to regulate their body weight in spite of variations in how much energy they consume and expend. This is the concept of energy homeostasis. An imbalance of energy intake and energy expenditure over time determines whether a person loses, gains, or maintains their weight.

A major contributor to the ongoing obesity epidemic is how modern life impacts our ability to maintain energy homeostasis. It is widely accepted that modern life is more sedentary than previously, and that people tend to consume more calories due to changes in diet and feeding behaviours. Indeed, increasing obesity in developed countries is associated with urbanisation[18], providing evidence of the environmental influences on weight gain. A decrease in general activity in developed countries is largely due to the introduction of modern technologies such as cars, trains and video games making it less likely that people will move around, alongside a change in industry to mostly tertiary employment structures, such that many people have entirely sedentary office jobs. Moreover, modern diets contain more ultra-processed foods and are more calorie-dense than old diets[19], thus making it much easier and more common to consume more total calories in meals. In support of this, the Food and Agriculture Organization of the United Nations reported a worldwide increase in the average daily calorie kcal intake per person of 487 kcal between 1969 and 2015[20].

1.1 | Obesity

Although either a prolonged decrease in energy expenditure or increase in energy consumption can result in the development of overweight or obesity, studies suggest that it is increased average caloric intake that is primarily driving the obesity epidemic[21,22].

While the conceptual basis of the increase in global obesity may seem simple, a solution to the problem is not. Individual willpower and lifestyle alterations may be effective for some people, but at a population-scale this is not a feasible solution for reducing obesity. Most of the behavioural factors underlying why more people are overweight in the modern age are the result of social and economic frameworks that must be addressed at a cultural or political level if sustainable changes are to occur[23]. The role of excess sugar consumption in the increasing prevalence of obesity resulted in the UK government instating the Soft Drinks Industry Levy, commonly known as the "Sugar Tax", in April 2018[24]. This tax resulted in an increased rate of tax on drinks if they contained more than 8 g sugar per 100 mL, payable by manufacturers to HMRC, and rapidly resulted in most manufacturers reformulating their products to decrease their sugar content. In the first year after the implementation of the UK sugar tax, there was no change in the volume of soft drinks purchased but the sugar content of those drinks was decreased by 10%[25]. This presents clear evidence that changes to nationwide policy and regulations can impact public health without relying on changes to the behaviour of the general population.

Our changing lifestyle has clearly driven the increasing prevalence of obesity worldwide, but this does not fully explain the large variations in body weight between individuals in a similar modern environment. Twin and adoption studies have found that the heritability of BMI is fairly high: between 40% and 70%[26,27]. This suggests that there is a genetic component to whether someone is more or less likely to develop obesity. Some genetic mutations have been identified that cause severe obesity, notably those that disrupt leptin signalling[28–30] and loss-of-function mutations in the melanocortin 4 receptor (MC4R)[31]. These mutations are rare, with fewer than 100 patients with leptin receptor deficiency published worldwide[32], and MC4R mutations accounting for only 2-5% of cases of severe obesity[33]. In recent decades, numerous common variants have been identified by genome-wide association studies (GWAS). However, even the variant with the largest effect size on BMI, the FTO locus, still only explains 0.34% of inter-individual variation[34] and the cumulative proportion of BMI variability explained by GWAS-identified loci is still less than 5%[33].

Beyond genetic and lifestyle factors, more recently it has emerged that exposure to suboptimal environments between conception and the first two years of life can influence an individual's likelihood of becoming obese themselves. This has been termed the Developmental Origins of Health and Disease (see Section 1.3).

1.1.3 Age, sex, and obesity

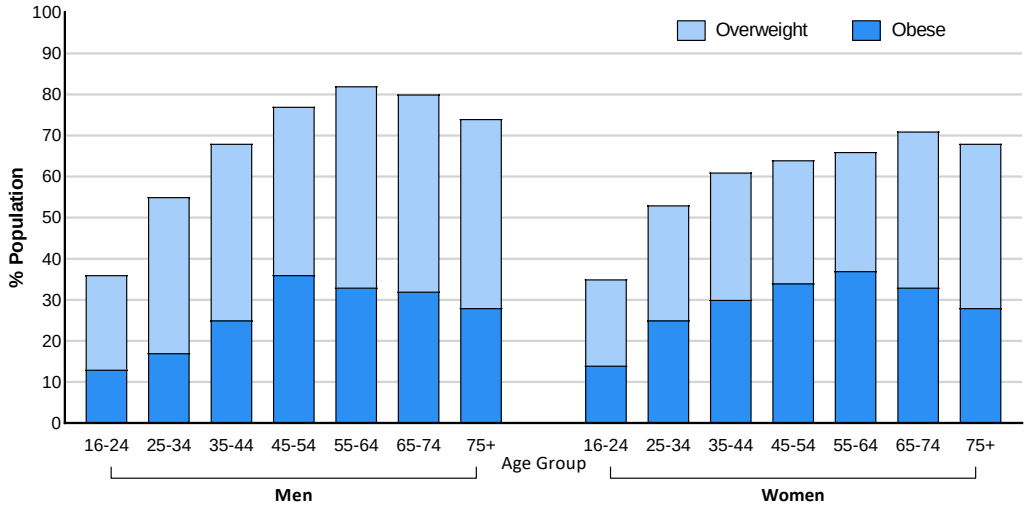


Figure 1.2: Prevalence of being overweight or obese by age and sex in the UK in 2018. *Data from NHS Digital, Health Survery for England 2018[35].*

While the obesity epidemic is not sex-specific, the prevalence of being overweight or obese is both age- and sex-dependent (Figure 1.2)[35]. In general people tend to gain increasing weight as they get older, and women tend to accumulate more fat than men at any given age. This is regardless of whether they are lean or obese, with women having a greater body fat percentage than men with a similar BMI[36]. Interestingly, in the UK obesity is more common in women than men but being overweight is more common in men[35].

Fat distribution also differs between men and women. Body fat can be characterised as either visceral, accumulating around the abdominal viscera, or subcutaneous. In both men and women the vast majority of body fat is subcutaneous, but, visceral fat accounts for a higher proportion of total body fat in men than in women[36,37]. This means that women accumulate more total body fat than men of the same age before reaching comparable amounts of visceral fat[38]. This is notable because a greater proportion of visceral fat is associated an increased risk of developing both the metabolic syndrome[39] and hypertension[40]. Visceral adipose tissue is more insulin resistant than subcutaneous adipose tissue[41,42] and increased visceral fat deposition is associated with increasing systemic insulin resistance. Indeed, this may underlie why men are more insulin resistant than women from late puberty through to adult life[43]. Moreover, men are diagnosed with T2D at a lower average BMI than women[44,45], which may contribute to the fact that middle-aged men are at a higher risk of developing T2D than age-matched women[46].

The mechanisms underlying the relationship between visceral adiposity and systemic insulin resistance remain unclear. A popular hypothesis is the Portal Theory, the basis of which was first proposed by Arner in 1997[47]. Venous blood from visceral fat drains into the portal vein, whereas that from subcutaneous fat drains into the vena cava. Therefore, the liver is directly exposed to free fatty acids (FFAs) and cytokines released from visceral fat, but not from subcutaneous fat. Moreover, visceral fat is more sensitive to catecholamine-induced lipolysis than subcutaneous fat[48], which promotes the release of FFAs. As visceral fat increases, lipolysis in this tissue will thus account for an increasing proportion of hepatic FFA delivery[36]. The Portal Theory proposes that increased delivery of FFAs and cytokines from visceral adipose tissue to the portal system results in the development of hepatic and subsequently systemic insulin resistance[49]. This is supported by experiments in dogs in which selective elevation of FFAs in the portal vein results in peripheral hyperinsulinaemia during a euglycaemic clamp[50]. However, this hypothesis has been challenged more recently as there is little supporting evidence in humans *in vivo*[51].

1.2 Cardiovascular disease

1.2.1 The world's biggest killer

Cardiovascular disease (CVD) is a general term describing conditions that affect the heart and vasculature. Two of the most common CVDs are coronary heart disease and stroke and these have been the two most common causes of death globally for the past 20 years (Figure 1.3)[11]. Coronary heart disease, also known as ischaemic heart disease, is the leading cause of death worldwide[52]. It is a condition in which blood flow to the coronary arteries is reduced or stopped entirely. A stroke is when blood supply to part of the brain is cut off. Together, CVDs took the lives of 17.9 million people worldwide in 2016, accounting for 31% of all global deaths[53]. In the UK alone, there are over 7.4 million people living with CVD and the annual healthcare cost of these diseases to the NHS is estimated at more than £9 billion[54].

1.2.2 Risk factors for cardiovascular disease

The number one risk factor for the development of CVD is persistently high arterial blood pressure, also known as hypertension, with more than half of heart attacks and strokes worldwide being associated with high blood pressure[55]. Consistent with this, the Global Burden of Disease Study has found high blood pressure to be the single largest contributing risk factor to all-cause deaths every year since 1990[56].

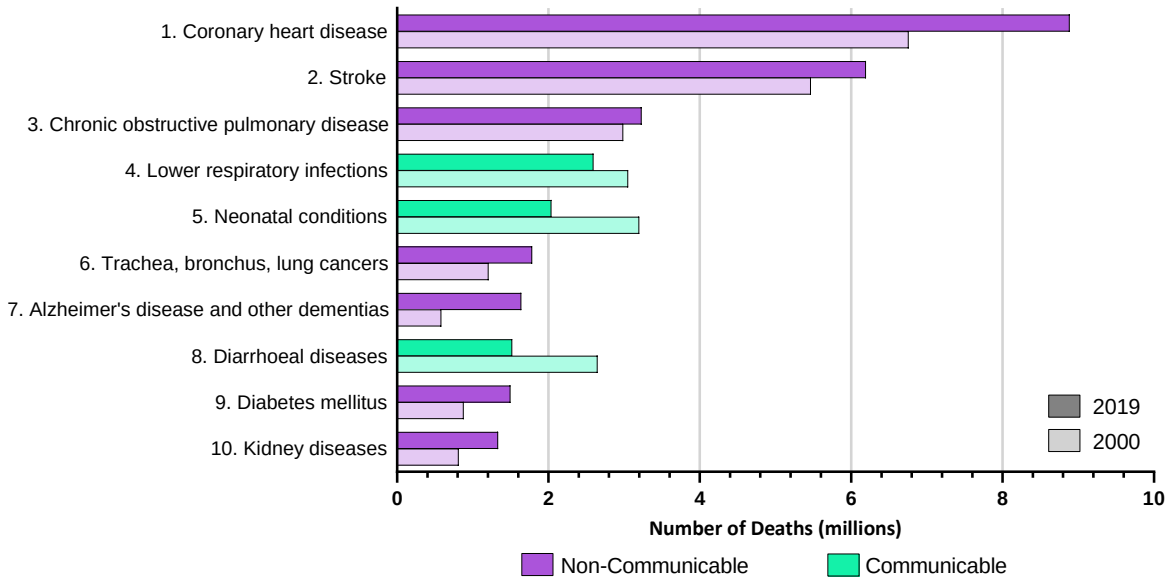


Figure 1.3: Leading causes of death in 2000 and 2019. *Data from WHO Global Health Estimates*[11]

Blood pressure is recorded as systolic blood pressure over diastolic blood pressure (SBP/DBP, measured in mmHg). SBP is the arterial blood pressure during systole, when the heart is contracting, and DBP is the arterial blood pressure during diastole, when the heart is relaxing between beats. The principles of blood pressure regulation are described in Section 3.1.1. In the UK hypertension is classified as 140/90mmHg or higher and a quarter of adults were hypertensive in 2015[57]. Obesity is associated with an increased risk of hypertension[7] and studies have found that a reduction in fat mass due to surgical removal[58] or after bariatric surgery[59] can reduce the need for previously-hypertensive people to take anti-hypertensive medications in order to maintain a healthy blood pressure. Numerous factors have been implicated in the pathogenesis of hypertension including increased sympathetic nervous system activity, changes in peripheral vascular reactivity, vascular remodelling and changes in sodium handling by the kidney[60].

Another major risk factor for CVD is diabetes. Diabetic adults are twice as likely to develop CVD as non-diabetic adults[46] and in the UK, one third of adults with diabetes die from CVD[61]. This association is in part due to hypertension and diabetes sharing many aspects of their pathophysiology, resulting in many people suffering from both conditions concomitantly. The San Antonio Heart study found that 50% of people with hypertension had impaired glucose tolerance or T2D, and 85% of people with T2D over the age of 50 had hypertension[62].

Other key risk factors for CVD include having a family history of CVD and being from certain ethnic backgrounds, both of which support a genetic component to CVD risk, as well as smoking, and dyslipidaemia[63]. Given the association between obesity and several of the key risk factors for CVD, it is unsurprising that while obesity does not cause CVD directly, it too is considered a major risk factor[63].

1.2 | Cardiovascular disease

The Framingham Heart Study is an ongoing study that began in the 1940s to identify risk factors of CVD. It found that the risk of heart failure increased with any increase in BMI above a healthy level, not just in cases of severe obesity[64]. Of the 4 million global deaths attributed to individuals being overweight or obese in 2015, more than 40% were due to CVD[65]. The rising rates of both obesity and diabetes worldwide are expected to be associated with an ever-increasing prevalence of CVD.

The risk of an individual developing CVD increases with age (Figure 1.4), although there are sex-dependent differences in prevalence, age of onset, and prognosis of CVD, with premenopausal women being relatively protected compared to age-matched men[66]. This is explored in more detail in Chapters 4 and 5. Moreover, while T2D is a risk factor for CVD in both women and men[46], diabetic women have a greater relative increase in CVD risk compared to diabetic men[67]. This is largely due to the fact that women tend to have a greater adiposity than men when they are diagnosed with T2D[44].

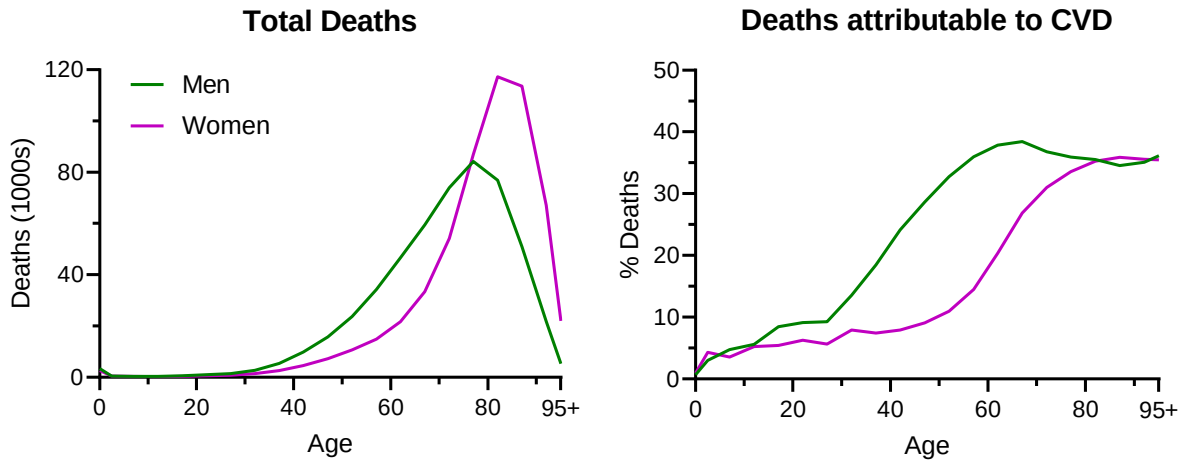


Figure 1.4: Deaths attributable to CVD in Finland between 1986 and 2009. *Data from Mikkola et al.*[68]

1.3 Developmental Origins of Health and Disease (DOHaD)

Given the rising global prevalence of obesity and diabetes, and the associations between these conditions and CVD which is already the single biggest cause of death worldwide, it is paramount that we understand the aetiology of these conditions in order to develop both effective preventative strategies and treatments. While the risk of developing these conditions has been found to be influenced by genetics and is clearly affected by current lifestyle, in recent decades it has become apparent that the conditions a fetus is exposed to during development also impact their long-term risk of obesity and cardiometabolic dysfunction.

1.3.1 The Thrifty Phenotype hypothesis

The first indication that exposure to a sub-optimal environment during early life impacts the long-term cardiovascular health of an individual came from a study by Barker et al. that found that differences in death rates among newborn babies in different parts of England and Wales paralleled those from coronary heart disease[69]. From these findings, Baker et al. inferred that there may be a common cause between the increased risk of neonatal mortality and adult mortality from CVD.

Further studies then expanded upon this and found that birth weight negatively correlated with death rate from coronary heart disease in a cohort of men and women from Hertfordshire[70,71], and also with body weight at one year of age in men only[70]. It was also found that systolic blood pressure both in childhood and adulthood was negatively correlated to birth weight[72]. Together, this suggested that a low birth weight results in an increased risk of cardiovascular dysfunction in adulthood. Shortly after these studies were published, the findings were recapitulated by studies in South India[73] and Helsinki[74]. In the original cohort of Hertfordshire men, it was also found that low birth weight and weight at one year of age correlated with impaired glucose tolerance or type 2 diabetes[75] and the metabolic syndrome[76] when individuals were in their sixties. Therefore, low birth weight was associated with an increased risk of cardiometabolic dysfunction in adulthood.

These findings led Barker and Hales to propose the Thrifty Phenotype hypothesis[77] which postulates that exposure to an undernourished environment *in utero*, which results in the birth of a small baby, programmes changes to metabolism in the fetal tissues to prepare the baby for a similarly undernourished environment *ex utero*. These changes include an increase in peripheral insulin resistance and decreased capacity for pancreatic insulin secretion. In a postnatal environment with limited glucose and nutrient availability, these changes would be beneficial as they would enable the glucose to be preferentially diverted to the brain, thus supporting proper development, and promote the efficient storage of nutrients when they were available by promoting fat storage. However, these changes become maladaptive if there is then a mismatch between the *in utero* and postnatal environments. When these babies are born into conditions of adequate or over-nutrition, the changes instead promote the development of obesity and cardiometabolic dysfunction.

In the Helsinki cohort, it was also found that the people that had the highest risk of CVD were those who were born small but were average or above-average weight by the time that they were seven years old[74]. This lead to the hypothesis that being born small but then growing rapidly after birth, a scenario termed postnatal catch-up growth or accelerated postnatal growth, was especially damaging to long-term cardiometabolic health and it is now well established that rapid postnatal growth is associated with childhood obesity[78] and that this can be independent of birth weight.

While these studies successfully related low birth weight to poor long-term cardiometabolic health, they could not necessarily infer how this linked to maternal nutrition. Studies of women who were pregnant during times of famine and their children provide more evidence of this.

The great Chinese famine occurred between 1959 and 1961 and was one of the deadliest famines in history, causing the deaths of more than 30 million people[79]. Children who were born during the famine were twice as likely to be hyperglycaemic as adults that those born before or after it, and had an increased risk of T2D[80], hypertension[81,82], and the metabolic syndrome[83].

The Dutch famine of 1944 – 1945 was a nationwide period of severe food-restriction during the German occupation of the Netherlands in World War II, with the harshest rationing lasting a little over five months. In line with other studies, adults exposed to the famine while they were in the womb had an increased risk of impaired glucose tolerance[84,85], and this reduction in glucose tolerance was greater than could be explained by the decrease in birth weight alone[84]. The acute time period of this famine means that it could be used to distinguish the effect of severe maternal undernutrition during early versus late gestation. As well as the impaired glucose tolerance, men and women who were exposed to the famine as fetuses in the first half of pregnancy had increased rates of obesity[86,87] and coronary heart disease[88], while these phenotypes were not as strong in those exposed only in late gestation.

The difference between early versus late exposure to famine during gestation is also seen in people who were pregnant during Holodomor, a famine in Soviet Ukraine between 1932 and 1933. People exposed to the famine during early gestation had the greatest risk of developing T2D as an adult[89]. Together these studies support the Thrifty Phenotype hypothesis.

1.3.2 Challenges beyond undernutrition

While the field of developmental programming first came about from studying the effect of maternal undernutrition, research has since expanded to look at a wider range of maternal health conditions that may result in a suboptimal environment for fetal development. Studies in humans include looking at the effect of maternal stress[90–92] and smoking[93] during pregnancy. Although long-term follow-up in these studies is lacking, current data suggest that exposure to these different suboptimal *in utero* environments also programme an increased risk of offspring obesity, glucose intolerance, and cardiovascular dysfunction.

While many of the original studies relating *in utero* development to long-term health found that poor outcomes negatively correlate with birth weight and thus that low birth weight is associated with poor cardiometabolic health, they studied populations of mostly lean mothers with a low prevalence of maternal obesity and GDM. The addition of studies in contemporary populations reveals that the relationship between birth weight and long-term outcomes is actually U-shaped[94]. Indeed, a study in Taiwan found that both low and high birth weight were associated with the development of T2D in adolescence[95], with the effect of being born small greater than that of being born large. Therefore, being born either small or large is associated with poor metabolic health in later life.

Studies from Poulsen at al. took these findings further, removing genetic variation from the equation by studying monozygotic twins in adulthood. In one study they showed that independent of genetics, larger birth weight babies had an increased fat mass throughout life[96]. Interestingly, in other studies of elderly monozygotic twins, they have found that the lower birth weight twin had increased insulin resistance and impaired insulin secretion[97], and in twins discordant for T2D, the diabetic twin was typically smaller at birth[98]. This suggests not only that being either large or small for gestational age influences later metabolic health independent of genetics, but also that obesity and impaired insulin signalling can be programmed independently of one another.

As the effects of a range of insults during fetal development and the first years of life have been further researched and identified as having long-term effects on health, the field of developmental programming has expanded beyond the original Thrifty Phenotype hypothesis to the broad definition of the Developmental Origins of Health and Disease (DOHaD). DOHaD describes how insults during critical developmental windows, both during gestation and infancy, impact on long-term health. Exposure to a suboptimal environment during this time programmes changes in tissue structure and function that can predispose an individual to disease in later life.

While these findings arise from human epidemiological studies, such studies have several limitations. Firstly, both T2D and CVD are age-related diseases. Much of our data investigating the effect of low birth weight and maternal undernutrition on long-term health and mortality is only possible due to well documented, population-wide historical challenges such as famines, and/or high-quality medical records being accessible. However, such data is not universally available to investigate the impact of other maternal conditions. Secondly, epidemiological studies require large numbers in order to enable us to reduce the influence of confounding factors such as postnatal environment and genetic variation. These limitations in human studies have led to the widespread use of animal models to address questions related to developmental programming, especially in relation to demonstrating causality of relationships and exploring underlying mechanisms. Animal models in DOHaD research is discussed in the context of maternal obesity in Section 1.5.3.

1.4 Obesity during pregnancy

Due to the worldwide increase in obesity, increasing numbers of women are overweight or obese. In the UK, this now accounts for over half of women of childbearing age[99]. Having a high BMI affects fertility; obese women have a lower chance of conception than non-obese women[100] and being overweight or obese results in it taking longer for a woman to fall pregnant[101]. Nevertheless, this still results in a high prevalence of maternal obesity during pregnancy. In fact, the prevalence of maternal obesity during pregnancy tracks with the number of obese people in the general population in England[102], and it is estimated that more than 38.9 million pregnant women were overweight or obese worldwide in 2014[103].

Once pregnant, obese women are more likely to miscarry than those with a healthy BMI[104], and they are approximately twice as likely to have a stillborn baby[105]. However, this is not only an effect of severe obesity, any increase in BMI above a healthy level increases the risk of stillbirth and neonatal death[106]. Obese women are also more likely to need a caesarean section and are at a greater risk of medical complications around birth regardless of method of delivery[107,108]. Once delivered, babies of obese women are at a higher risk of perinatal mortality and fetal abnormalities, particularly neural tube and structural cardiac defects[109], and the rate of neonatal unit admissions correlates with maternal BMI[110]. Moreover, babies born to overweight or obese mums are less likely to be born a healthy weight, being more likely to be born either macrosomic (above 4kg) or large for gestational age (LGA)[104,111,112] with increased subcutaneous fat[113] due to overnutrition, or small for gestational age (SGA)[104,112] due to intra-uterine growth restriction (IUGR), indicative of impaired fetal development. Excessive weight gain during pregnancy, known as gestational weight gain (GWG), in overweight and obese women further increases the risk of negative perinatal outcomes for both mother and baby[114,115]. It also creates a negative cycle as women with excessive GWG are likely to retain the increased weight after delivery[116] and thus enter subsequent pregnancies even heavier[117].

Obesity during pregnancy impairs maternal physiological adaptation to pregnancy, including important changes to both metabolic and cardiovascular function. This results in obese women being at an increased risk of developing two major pregnancy-related disorders: gestational diabetes mellitus (GDM)[118] and pre-eclampsia[119].

1.4.1 Obesity and gestational diabetes mellitus

More than one in six live births are affected by some form of diabetes during pregnancy[12], including both pre-existing diabetes and GDM. GDM is diabetes that is first diagnosed during pregnancy and is usually resolved after birth. It is the result of a failure of the maternal metabolism to adapt correctly to pregnancy. In normal pregnancy, maternal glucose handling changes significantly. This is necessary for healthy fetal development because glucose makes up a substantial fraction of fetal energy substrates and its transport across the placenta is entirely passive, using facilitative glucose transporters. This means that glucose delivery to the fetus is dependent on the concentration gradient between the maternal and fetal circulations. To raise glucose levels in the maternal circulation, the placenta secretes hormones, such as placental lactogen, progesterone and cortisol[120], and cytokines[121] that act on maternal tissues to increase insulin resistance[122] and stimulate hepatic gluconeogenesis[123]. To preserve maternal glycaemic control, there is a compensatory increase in maternal pancreatic insulin-secretory capacity via an increase in beta cell proliferation[124], and an increase in the glucose sensitivity of these cells[125]. Together, these changes ensure that the fetus receives sufficient glucose to develop, while the mother is able to remain glucose tolerant. The maternal insulin resistance also results in an increase in circulating maternal FFAs, another important fuel source for the growing fetus.

GDM occurs as a result of a failure of the increase in insulin secretion by the maternal pancreas to compensate for the pregnancy-induced increase in insulin resistance, resulting in a loss of glycaemic control. In both lean and obese women, developing GDM is associated with increased insulin resistance [126,127] compared to age-matched glucose tolerant pregnant women, and while women with GDM do exhibit increased insulin secretion as gestation progresses, it is not to the same extent as non-diabetic pregnant women [128]. Together, these result in hyperglycaemia in pregnancy.

The reported prevalence of GDM in different countries worldwide ranges from 1-30%[129]. While studies of GDM prevalence within multi-ethnic populations have shown that different ethnicities have different GDM-risk[130], there is variation in the screening strategies and diagnostic criteria used and so comparing prevalence between countries is difficult. Nevertheless, it is clear that GDM is a major problem worldwide, and that its incidence is increasing[131] due to the ongoing obesity epidemic. Having a high BMI is the most significant risk factor for GDM[131]; being overweight more than doubles a woman's risk of developing GDM, and for severely obese women the risk is more than eight times greater[118]. Indeed, using the diagnostic criteria recommended by the International Association of Diabetes and Pregnancy Study Groups[132], which has been adopted by WHO, it is estimated that up to a quarter of obese pregnant women could be diagnosed with GDM[133]. This is most likely due to pre-existing defects in glucose handling that impair maternal metabolic adaptation to pregnancy.

1.4 | Obesity during pregnancy

Obese and overweight women enter pregnancy with a higher baseline insulin resistance than lean women[9], and in non-diabetic pregnancy their relative increase in insulin resistance as gestation progresses is reduced compared to lean women[120]. This is in line with an increased risk of GDM, as increased insulin levels can be detected in early pregnancy in women who will later develop GDM[134]. However, the increase in glucose-sensitivity of insulin secreting cells is also reduced in obese pregnant women, even in the absence of diabetes[120]. Together, these suggest that overweight and obese women enter pregnancy with a lesser capacity to adapt to the metabolic challenges of pregnancy.

Women that develop GDM are more likely to be diagnosed with T2D in later life[135–137], this supports that women who develop GDM have underlying defects in their ability to maintain glucose homeostasis which are unmasked by the metabolic challenge of pregnancy. It may also suggest that developing GDM permanently dysregulates glucose-insulin signalling pathways in ways that increase the risk of developing T2D in the future. This presents an additional benefit to developing interventions to prevent GDM as they may also reduce secondary health risks to the mother.

Given the significant overlap in incidence of maternal obesity during pregnancy and GDM, it can be difficult to determine whether poor fetal outcomes are specifically due to their exposure to maternal hyperglycaemia or other features of an obese pregnancy. The Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) study was a large, multinational landmark study that included more than 25,000 women and that found that independent of maternal BMI, maternal hyperglycaemia positively correlated with the risk of numerous perinatal complications including: pre-eclampsia, pre-term delivery, and the need for delivery by caesarean section, as well as an increased incidence of LGA babies and increased admission of newborns to neonatal special care units[138].

1.4.2 Obesity and pre-eclampsia

Significant changes in maternal cardiovascular function occur during pregnancy in order to support the growth of the fetus and placenta. The normal adaptation of the maternal cardiovascular system to pregnancy is described in Section 3.1.2. Failure in this adaptation to pregnancy can result in impaired fetal growth and development, as well as maternal cardiovascular dysfunction. A key disease related to impaired maternal cardiovascular adaptation to pregnancy is pre-eclampsia.

Pre-eclampsia is a serious pregnancy complication that typically arises after 20 weeks of gestation and is most commonly defined as new-onset hypertension alongside proteinuria[139], the excretion of protein in the urine. It is estimated that 4.6% of pregnancies globally are affected by pre-eclampsia[140]. Maternal mortality from pre-eclampsia accounts for more than 60,000 global deaths each year[141], and tragically are mostly due to a lack of access to obstetric care in low and middle-income countries[142,143].

Risk factors for pre-eclampsia include diabetes and pre-existing high blood pressure[139], therefore it is unsurprising that obesity is associated with an increased risk of pre-eclampsia[119]. One systematic review of 52 studies found that, on average, the risk of developing pre-eclampsia was increased by 50% in women with a BMI ≥25kg/m^2 compared to those with a "normal" BMI[144], and Thadhani et al. found that being obese doubles someone's risk of developing the condition[119]. The association between obesity and increased risk of pre-eclampsia has been replicated in different populations[145]. This effect has also been found by studying individual women over time; while the risk of pre-eclampsia is highest in someone's first pregnancy and a previous normotensive pregnancy offers relative protection against pre-eclampsia[141], gaining weight between pregnancies is associated with an increased risk of pre-eclampsia in subsequent pregnancies[146,147].

In addition to pre-eclampsia, there are two other main hypertensive disorders of pregnancy: pre-existing hypertension and gestational hypertension, and together hypertensive disorders of pregnancy are estimated to affect 18 million pregnancies globally[148]. It is well established that obesity is associated with increased arterial blood pressure[149], and thus overweight and obese individuals are more likely to enter pregnancy with pre-existing hypertension. Gestational hypertension is new-onset hypertension in pregnancy without proteinuria, and being overweight doubles a woman's risk of developing gestational hypertension[119]. Therefore, the ongoing obesity epidemic can be expected to result in an increasing prevalence of hypertensive disorders of pregnancy. In line with this, the incidence of hypertensive disorders of pregnancy has increased by 10% in the past decade[148].

Women with a history of gestational hypertension or pre-eclampsia have an increased risk of developing hypertension[150] and CVD[151] after pregnancy compared to those who had a normotensive pregnancy, resulting in an even greater health burden. It is likely that this is at least in part due to the physiological stress of pregnancy unmasking pre-existing vulnerabilities or undiagnosed cardiovascular conditions that contribute both to their developing new-onset hypertension during pregnancy and the later hypertension and CVD. However, whether the complicated pregnancy also alters maternal cardiovascular function directly and subsequently increases the risk of developing hypertension and CVD after delivery is unknown[152]. If there is an element of new-onset hypertension during pregnancy being an independent risk factor for later hypertension and CVD, this further supports the need and value of developing effective intervention strategies during obese pregnancy to reduce the risk of these obesity-related complications.

1.4.3 Vascular damage in gestational diabetes

As introduced in Section 1.2.2 diabetes and hypertension often coexistence in patients and they share many aspects of their pathophysiology, particularly relating to vascular dysfunction and injury. Vascular damage is also a common feature of GDM and contributes to its pathogenesis. The hallmark characteristics of both T2D and GDM are chronic hyperglycaemia and insulin resistance. These factors promote vascular damage and endothelial dysfunction via several mechanisms, including the formation of advanced glycation end products (AGEs), increased oxidative stress, and promoting an inflammatory state.

Advanced glycation end products

AGEs are generated by glycation of proteins, lipids and nucleic acids[153] and chronic hyperglycaemia accelerates the formation of AGEs[154]. AGEs contribute to vascular damage and dysfunction via three key mechanisms. Firstly, AGEs accumulate inside vascular cells and can disrupt the structure and function of intracellular proteins[155]. Secondly, AGEs interact with vessel extracellular matrix components including collagen and elastin, increasing vessel stiffness[156]. Thirdly, AGEs bind to their cell surface receptor, RAGE, activating intracellular signalling pathways through transforming growth factor (TGF)-β and mitogen-activated protein kinases (MAPK)[153] as well as inducing expression of vascular adhesion molecule 1 (VCAM1) and vascular endothelial growth factor (VEGF). These pathways are associated with vascular fibrosis, calcification, and prothrombotic effects[157]. AGE-RAGE interactions also stimulate the production of reactive oxygen species (ROS) via activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidases[157] and can activate inflammatory genes[158].

Oxidative stress

An imbalance between the production of ROS and cellular antioxidant defence mechanisms leads to oxidative stress. ROS production in diabetes is increased due to numerous processes, including glucose-stimulated mitochondrial respiration[157] and increased expression and activity of NADPH oxidases[157,159], and vascular antioxidant capacity is reduced, such as due to dysfunctional endothelial nitric oxide synthase (eNOS)[159]. Oxidative stress causes vascular damage due to alterations to DNA, protein, lipids, and other macromolecules, resulting in fibrosis, cellular dysfunction, and inflammation.

Pro-inflammatory state

Obesity and diabetes are associated with a chronic state of systemic inflammation[160,161]. Within blood vessels, inflammation contributes to the development of endothelial dysfunction, thrombosis, vascular remodelling, and atherosclerosis[162].

1.5 Developmental programming by maternal obesity

1.5.1 Programming of obesity and metabolic dysfunction in humans

As described in Section 1.1.2, twin and adoption studies have found that the heritability of BMI is between 40% and 70%[26,27] and parental obesity is one of the strongest predictors of obesity in children[163] and young adults[164]. This may be expected given that there is a known genetic component to BMI (see Section 1.1.2) and that most children will share the same postnatal environment as their parents. Therefore, if the parent is living in an obesogenic environment, including consuming a high calorie diet, their children will experience the same environmental exposures. Interestingly, studies by Sorensen et al. suggest that the environment someone is raised in has a minimal effect on their BMI in adulthood as they found that an individual's BMI correlates strongly with that of their biological parents, and the extent of this is comparable whether they were raised by them or adopted[165].

While childhood BMI and subsequently obesity are associated with both maternal[166,167] and paternal[163] BMI, the association is stronger with maternal BMI[168–171]. This suggests that the mother's health may have a greater influence on future offspring metabolic health. Indeed, exposure to maternal obesity *in utero* has also been found to predispose offspring to the development of obesity independent of genetics and postnatal environment. The strongest evidence for this in humans comes from studies looking at the health of siblings who were born before or after their obese mother lost a significant amount of weight following bariatric surgery. Siblings born after their mother's weight loss had a lower incidence of macrosomia[172] progressing on to a lower incidence of obesity in young adulthood[172,173]. Moreover, they were more insulin sensitive, had a lower blood pressure and had an improved lipid profile compared to their older siblings[172]. This shows that differences in maternal BMI during early life result in differential programming of later cardiometabolic health, both in addition to and independent of genetic influences and postnatal environment.

Maternal obesity is associated with an increased risk of T2D in offspring[174,175], with an increased risk of developing glucose intolerance seen as early as 10 years of age[176]. This could be a secondary effect of programmed obesity. However, studies have found that maternal pre-pregnancy BMI is associated with childhood insulin resistance independent of current body size[177] and that fetuses of obese mums are more insulin resistant than those of lean mums while still in the womb[178]. This supports that maternal obesity programmes an increased risk of developing T2D both directly and indirectly via programming offspring obesity.

As discussed in Section 1.4.1, many obese pregnancies are also complicated with GDM. Exposure to GDM *in utero* is associated with an increased risk of diabetes[179] and the metabolic syndrome[180] in adult offspring. Once again, distinguishing the effects of maternal adiposity versus hyperglycaemia can be difficult. Song et al. found that GDM increases the risk of macrosomia independent of maternal obesity[181] and the HAPO-Follow-Up study found that independent of maternal BMI, maternal hyperglycaemia correlated with childhood adiposity[182] and GDM was associated with impaired glucose tolerance[183] in 10-14 year olds. Furthermore, as well as an increase in pregnant women developing GDM worldwide, as the prevalence of T2D increases more women are entering pregnancy diabetic. Dabelea et al. found that individuals born after their mothers were diagnosed with T2D had an increased risk of obesity and diabetes themselves compared to their older siblings who were not exposed to maternal diabetes *in utero*[184].

1.5.2 Programming of cardiovascular dysfunction in humans

In addition to increasing the risk of offspring being overweight or obese and developing T2D in adulthood, a high maternal BMI has been found to be associated with premature death of offspring[185]. This is in part mediated by an increased risk of offspring developing CVD[174] and increased hospital admissions for cardiovascular events[185]. Once again it is possible that this is a secondary effect due to the increased risk of obesity and T2D in adulthood and the association between these factors and CVD, rather than a direct effect of maternal obesity programming poor offspring cardiovascular health. Several studies that have found an increased risk of CVD in offspring of obese mothers have either not considered the current BMI of the offspring[174] or have found that any associations between maternal BMI and offspring cardiovascular risk are fully explained by their current BMI[186]. However, impairments in cardiac function have been identified in fetuses and newborns of obese mothers, suggesting that maternal obesity does directly influence cardiac development *in utero*.

Studies utilising echocardiography have reported changes in global strain in fetal hearts in early[187], mid[187,188] and late[187] gestation of obese pregnancies, which is indicative of subtle contractile dysfunction, as well as increases in intra-ventricular septum width in late gestation[187], revealing an effect on cardiac structure. Ece et al. also reported an increase in myocardial performance index (MPI) in late gestion fetuses of obese mothers[189], which is indicative of diastolic dysfunction, an impairment in the ability of the heart to relax and fill with blood between beats. These *in utero* findings have also been replicated after birth, with Guzzardi et al.[190] finding that babies of obese women had heavier and thicker left ventricles at birth. Nyrnes et al.[191] reported increases in intra-ventricular septum width and changes in cardiac strain in both newborn and 8-week-old babies of obese mothers.

Together, these studies show that while it does not result in overt cardiac dysfunction in fetuses or babies, exposure to maternal obesity during pregnancy results in changes in both cardiac structure and function. This may contribute to the increased risk of these children developing CVD in adulthood independent of their increased risk of obesity.

Given that the number one risk factor for CVD is hypertension, it is important to also consider the impact of maternal obesity on offspring blood pressure. Numerous studies have found that maternal pre-pregnancy BMI positively correlates with offspring SBP and DBP in childhood[192,193] and adolescence[194,195]. Indeed, a meta-analysis including offspring aged between 2 and 32 years old found that with every 5 kg/m^2 increase in maternal pre-pregnancy BMI, SBP increased by 1.9 mmHg and DBP by 0.5 mmHg[196]. While one study found that this association between maternal BMI and offspring blood pressure was lost when childhood BMI trajectory was taken into account[193], most report that the effect is reduced but does persist[194,196], with a few studies estimating that current BMI accounts for approximately half of the association between maternal BMI and offspring blood pressure[192,195].

As discussed in Section 1.4.2, obese women are twice as likely to develop pre-eclampsia as lean women[119]. Exposure to pre-eclampsia *in utero* is associated with increased offspring blood pressure immediately after birth[197], and increased SBP and DBP in childhood and adolescence[198]. Moreover, in the Helsinki Birth Cohort, maternal pre-eclampsia was associated with an increased risk of offspring having a stroke in later life[199]. Whether these associations are secondary effects of the IUGR seen in one third of pre-eclamptic pregnancies[200] or due to pre-eclampsia-specific programming is unclear[201]. However, one systematic review found that both SBP and DBP were increased in offspring of pre-eclamptic pregnancies compared to babies with similar birth weights from unaffected pregnancies[198], suggesting that this effect is independent of birth weight.

1.5.3 The use of animal models in DOHaD

As introduced in Section 1.3.2, animal models provide a valuable tool for DOHaD research. This is for several reasons. Firstly, they enable confounding factors such as genetics and postnatal environment to be tightly controlled and the species generally used have significantly shorter lifespans than humans, enabling the study of the progression of age-related disorders without research studies stretching over decades. Moreover, animal models enable us to interrogate underlying mechanisms in a more intensive way than human studies as animals can be euthanised and tissues or organs collected for further study, whereas clinically accessible human tissues are limited. Finally, using animal models enables us to employ different techniques to manipulate the conditions surrounding pregnancy, be it to elucidate causal mechanisms or investigate potential therapies, which is not feasible, or is unethical and/or immoral in human studies.

1.5 | Developmental programming by maternal obesity

Animal models to study maternal obesity during pregnancy have been established across multiple species and using numerous different techniques to induce obesity. By far the most common species used are rodents, although some researchers do work with larger animals such as sheep and non-human primates, including baboons and rhesus macaques. While non-human primates are the most similar to humans, they have a relatively long gestation period and lifespan, and are expensive to maintain[202]. The same challenges are present in sheep, albeit to a lesser extent. Sheep are a useful model as they typically have singleton pregnancies, like humans, and they have a similar temporal pattern of organ development as fetal humans. Moreover, their size facilitates complex maternal, fetal and placental physiology studies in non-anaesthetised animals by chronic instrumentation[203,204].

The vast majority of animal models for investigating developmental programming, including by maternal obesity, are established using rodents. Rodents have a short gestation period and lifespan, and they are comparatively cheap to maintain. Specific clonal strains of mice and rats can also be used, controlling for the effect of genetic variation. The use of mice specifically is also common due to the availability of numerous different transgenic lines, facilitating the development of highly specific models and the investigation of the effects of individual genes and how they interact with the environment, such as the diet the animal consumes. A key drawback of using mice in developmental programming research is that several maturational changes that occur during gestation in humans occur postnatally in mice.

There are many ways of inducing obesity and/or obesity-related disorders in an animal for scientific study. This includes numerous genetic models; different rodent strains with specific susceptibilities to cardiometabolic dysfunction have either arisen spontaneously or been developed by selective breeding, and specific transgenic mouse lines have been generated. Non-obese Diabetic mice and Bio Breeding rats can be used to investigate pre-pregnancy diabetes without obesity[205], and models for pre-eclampsia have been developed using ApoE-KO mice[206]. Some studies have used Agouti yellow mice as a model of obese, glucose-tolerant pregnancy[207] although others have shown that these animals are glucose intolerant before and hyperglycaemic during pregnancy[208,209]. Ob/ob and db/db mice are homologous for mutations in the genes for leptin and the leptin receptor (*Lepr*) respectively, resulting in severe obesity, but they are infertile. However, female mice heterozygous for the *Lepr*^{db} mutation are usually glucose-tolerant, but they develop glucose intolerance during pregnancy, making them a model for GDM[210]. Nevertheless, genetic models are unlikely to accurately recapitulate the complex physiological conditions of maternal obesity, and thus conclusions drawn using them are less likely to be translatable to humans. Moreover, secondary changes to the fetal genotype may have direct non-physiological effects that mask phenotypes programmed by maternal health.

Diabetes can be chemically induced at any time before or during pregnancy by injection with streptozotocin, an alkylating agent which is highly toxic to pancreatic beta cells[210]. While this effectively induces a diabetic phenotype, it reflects a loss of insulin secretion, like in type 1 diabetes, rather than the more clinically relevant dysregulation of glucose-insulin signalling involving both the pancreas and changes in peripheral insulin resistance which occurs in T2D and GDM.

Many research groups induce maternal obesity and metabolic dysfunction by altering maternal feeding. In some models, obesity can be induced by overfeeding of the same diet given to controls. This is most commonly done in sheep; ewes are fed a body-weight-matched food ration in line with the National Research Council's recommendations[211] and obesity is induced by giving a subgroup of sheep access to 50% more food for 60 days prior to mating[212]. Alternatively, different experimental diets can be given to animals in order to promote obesity. Such obesogenic diets include high fat diet (HFD) pellets, supplementation of pellets with sucrose water in order to increase sugar consumption, and a cafeteria-style diet, in which animals have a range of different palatable foods available to them, such as chocolate, marshmallows, peanuts and crisps[213,214]. Different composition HFD pellets exist, with usually between 40-60% of calories coming from fat[215,216], typically lard. While feeding these pellets can induce metabolic dysfunction, some studies have found that mice tend to eat less of them, resulting in comparable calorie consumption between HFD-fed and chow-fed animals[217]. This may be due to a combination of two factors. Firstly, HFD pellets may be less palatable than chow diets. Secondly, rodents are thought to be relatively good at modulating their food intake when fed a diet high in fat. This is something that we combat in our model of maternal diet-induced obesity (see Section 1.6) by also increasing the simple carbohydrate content of our diet in order to override satiety signals, promote increased calorie intake, and drive the development of obesity.

As discussed in Section 1.3.1, human epidemiological data suggests that exposure to suboptimal environments during different critical time windows programmes different changes in long-term health. This applies both to whether insults occur during early versus late gestation, and if they occur during fetal versus neonatal life. This is difficult to address in human studies and so once again, animal models are a useful tool. The cross-fostering of animals or timing of introduction and removal of experimental diets can be used to investigate the effect of maternal diet and obesity during these time windows. For example, switching dam diets at birth or newborns being cross-fostered between animals on different diets can be used to distinguish the effects of the *in utero* environment versus lactation[218–220]. Alternatively, the effect of exposure to maternal obesity can be distinguished from that to an altered metabolic environment *in utero* due to a maternal obesogenic diet in the absence of maternal obesity by only feeding animals a HFD during pregnancy[221,222], or by inducing maternal obesity by high-fat feeding but then feeding dams a control diet during pregnancy[223].

1.5.4 Evidence from animal models: obesity

In line with human data, multiple meta-analyses of animal studies find that offspring of obese mothers are heavier and have increased adiposity than offspring of lean mothers[224,225]. This is consistent across species. Importantly, studies in rodents have found a specific increase in visceral adiposity in offspring of obese pregnancy[226,227], which is well-established to be associated with metabolic dysfunction[39]. This outcome is consistently found when offspring are fed a healthy chow diet from weaning, and thus the effect is independent of post-weaning diet. Interestingly, White et al. found comparable body weights in 18-week-old rats between offspring of control dams that were fed a postnatal HFD and offspring of obese dams that were fed a postnatal control diet[228]. They suggested that this reveals that maternal high-fat feeding exerts an effect on adult offspring body weight of comparable magnitude as offspring post-weaning diet. However, this outcome was most likely because the HFD used was not very palatable; if a more palatable obesogenic diet was used, one would expect offspring high calorie feeding to have a greater effect on current body weight than a programmed effect by maternal obesity when offspring are fed a control diet postnatally.

The effect of maternal obesity on fetal growth is inconsistent between species, models and studies, with different models showing increased[229–231], decreased[232–234], or no change[235–237] in either fetal weight or birth weight. This is not necessarily at odds with human studies, as maternal obesity increases the incidence of both SGA and LGA or macrosomic babies, with similar increases at both ends of the birth weight spectrum[104]. Likely as a result of this variation, meta-analyses of animal studies typically conclude that maternal obesity does not consistently affect birth weight[224,225].

Studies in rodent models have found that maternal obesity programmes changes in offspring feeding behaviours resulting in hyperphagia[238]. This results in increased energy consumption and thus will contribute to the development of obesity. Changes in the feeding and reward circuits in the offspring hypothalamus that regulate appetite likely underlie these behavioural changes[239]. In line with this, Nguyen et al. found that mRNA levels of the orexigenic (appetite-stimulating) neuropeptide NPY were increased and those of the anorexigenic (appetite-suppressing) POMC were decreased in the hypothalami of offspring of obese rats[240]. Numerous studies have found differences in the hypothalamus with different timings of exposure to maternal obesity. Chang et al. found increased expression of orexigenic peptides in the paraventricular nuclei (PVN) of the hypothalamus of offspring of rats fed a HFD during pregnancy whether they remained with those dams for lactation or were cross-fostered onto control dams[241]. This suggests that these changes are established *in utero*.

Other studies have focussed on the importance of the *ex utero* developmental window of lactation. In rodents, there is a substantial increase in plasma leptin that occurs within the first two weeks of life, known as the neonatal leptin surge[242], which is critical to the normal development of hypothalamic feeding circuits[243]. Offspring of obese rats have been found to have an amplified and prolonged neonatal leptin surge[244], and this is associated with increased hypothalamic leptin resistance in one-month-old offspring. Hypothalamic leptin resistance is known to contribute to obesity[245,246], and offspring of obese dams remained leptin-resistant in this study and were also hyperphagic and heavier than offspring of lean dams by the time that they were three months old[244]. This relationship between an increased neonatal leptin surge and the development of hypothalamic leptin resistance, hyperphagia and increased adiposity has been replicated in mice[247]. These studies infer that there are different stages in hypothalamic development that span both gestation and the suckling period, and that exposure to maternal obesity during any of these stages can alter offspring neurobiology, which may predispose them to hyperphagia and thus obesity postnatally.

As well as programming changes in appetite, exposure to maternal obesity *in utero* may alter food preference. One study in rats found that offspring whose mothers were fed an obesogenic cafeteria-style diet during pregnancy and lactation not only ate more of that same diet after weaning than offspring of control dams, but would also preferentially select fatty, sugary and salty foods[214]. This presents another way in which maternal obesity may programme offspring feeding behaviours, resulting in an increased risk of obesity.

1.5.5 Evidence from animal models: metabolic dysfunction

Maternal obesity is associated with increased offspring insulin and glucose levels regardless of species[224], which is indicative of differences in insulin sensitivity and impaired glucose homeostasis, thus recapitulating human studies. The impaired glucose homeostasis has been attributed to programmed changes in both insulin resistance and beta cell function.

<u>Programmed changes in insulin resistance</u>

Using animal models enables easier interrogation of tissue-specific insulin resistance due to the availability of tissues for experimentation. Altered expression of insulin signalling proteins has been identified in adipose tissue from offspring of obese dams in mice[248]. This suggests that as well as programming an increase in total and visceral fat mass, maternal obesity may programme increased insulin resistance of that adipose tissue; both of which will result in an increase in peripheral insulin resistance. Two other key tissues in determining peripheral insulin resistance are the liver and skeletal muscle.

1.5 | Developmental programming by maternal obesity

Changes in expression levels of proteins involved in hepatic insulin signalling have been reported in adult mice[249], rats[250] and sheep[251] exposed to maternal obesity *in utero*. Moreover, maternal obesity is associated with liver steatosis, excess lipid accumulation, in adult mice[226]. Increased hepatic lipid accumulation and dysregulated metabolism pathways have also been found in fetal non-human primates of obese pregnancy[237]. Lipid accumulation in the liver is a common manifestation of the metabolic syndrome[252], as it suggests that the body's ability to store excess energy in adipocytes is dysfunctional due to increased peripheral insulin resistance.

Molecular indices of insulin resistance have also been identified in skeletal muscle of fetal[236] and adult[253] offspring of obese sheep. In these same studies, changes in protein expression consistent with upregulated adipogenesis were found in fetal skeletal muscle, resulting in increased intramuscular adipocytes and triglycerides in both fetuses[236] and adult offspring[253] of obese ewes. This is of note as increased intramuscular triglyceride is associated with insulin resistance[254,255]. Alterations in insulin-signalling proteins have also been found in skeletal muscle in offspring of obese mice[256].

Programmed changes in beta-cell function and insulin secretion

Several studies have investigated the impact of maternal obesity on pancreatic endocrine function. Decreased beta cell mass has been found in late gestation fetal sheep[257]. This suggests that exposure to maternal obesity *in utero* results in impaired development of the pancreas. In spite of being born with reduced beta-cell mass, rodent studies have found that offspring of obese dams have an increase in pancreatic mass and islet mass at weaning[258259] and in adulthood[260], due to an increase in both alpha and beta-cell masses[259,260]. This is consistent with a compensatory increase in endocrine cell number in order to increase insulin-secreting capacity in response to increased peripheral insulin resistance. However, isolated islets from adult offspring of obese dams have decreased glucose-stimulated insulin secretion *ex vivo*[227] compared to controls. This suggests increasing pancreatic endocrine cell number may not be sufficient to compensate for programmed deficits in insulin secretion. Indeed, rats exposed to maternal obesity *in utero* but fed a control diet throughout life have both peripheral insulin resistance and pancreatic beta-cell dysfunction in late adulthood[227], representing an eventual failure in pancreatic endocrine compensation for increased peripheral insulin resistance.

To distinguish the effects of maternal obesity from maternal diet on offspring metabolism, one study in mice induced obesity in dams by 12 weeks of HFD feeding and then returned them to control diet for 8 weeks before mating[223]. Interestingly, they found that both fetuses and newborn offspring of obese dams fed a control diet had increased beta-cell mass compared to non-obese controls, with corresponding hyperinsulinaemia. This suggests that pre-pregnancy obesity and maternal high-fat diet during pregnancy may have opposing effects on fetal pancreatic development.

1.5.6 Evidence from animal models: fetal cardiac dysfunction

As well as effects on offspring obesity and glucose tolerance, exposure to maternal obesity *in utero* results in programmed changes in offspring cardiovascular structure and function in animal models. As in humans, changes are present before birth and therefore cannot solely be explained as secondary effects of the programmed changes in adiposity and metabolism. Studies in sheep have consistently found an increase in fetal heart weight in offspring of obese ewes from mid gestation[261–264], which is associated with increased wall thickness at both mid[261,262] and late gestation[265]. This is in line with the human data discussed in Section 1.5.2. The increased heart weight and wall thickness is associated with cardiomyocyte hypertrophy[261,262,265], an increase in cardiomyocyte cell size. Fetal cardiac hypertrophy is not inherently pathological. Indeed, it is a fundamental process in the growth and development of the fetal heart[266]. However, increased cardiomyocyte hypertrophy may indicate pathological remodelling of the fetal heart in response to a suboptimal *in utero* environment, and these changes may impact heart structure and function after birth[267]. In addition to cardiomyocyte hypertrophy, evidence of impaired contraction has been found in isolated cardiomyocytes from fetal sheep of obese ewes, which was associated with impaired calcium handling[265]. Moreover, fetuses of obese ewes had a disorganised cardiac structure, with irregular arrangement of cardiac muscle fibres[261,262,265] and lipid droplet accumulation[261], as well as an increase in cardiac fibrosis compared to controls[268]. These studies reveal changes in the electrophysiology and gross morphometry of the fetal heart that are indicative of pathological hypertrophy[269,270]. Together, these studies suggest that exposure to maternal obesity *in utero* results in pathological remodelling of the fetal heart which may persist postnatally and result in an increased susceptibility to cardiac dysfunction independent of adult offspring adiposity and metabolism.

Several of the cardiac phenotypes programmed by maternal obesity identified in sheep have been recapitulated in rodent models, including an increase in fetal heart weight[271,272]. Disorganised arrangement of cardiac muscle fibres has been found in fetal and newborn rats[271] as well as alongside increased cardiac fibrosis in one week old mice[273]. Increased lipid deposition in the heart has also been found in newborn rats exposed to maternal obesity *in utero*[215]. Findings of cardiac hypertrophy have also been recapitulated in minipigs, with newborn offspring of HFD-fed sows having increased left ventricular (LV) mass and cardiomyocyte size compared to controls. These changes were associated with changes in myocardial metabolism, including increased glucose uptake and increased glycogen accumulation, resulting in myocardial insulin resistance[190]. This is of particular note as myocardial insulin resistance may contribute to the development of cardiac hypertrophy[274], and changes in cardiac metabolism are a hallmark of pathological cardiac remodelling[269].

1.5.7 Evidence from animal models: adult cardiac dysfunction

Changes in the structure of the adult heart have been identified in offspring of obese mice that are consistent with those seen in fetal and neonatal rodents. Data from the mouse model of maternal diet-induced obesity used in this thesis are discussed in Section 1.6.2. Vaughan et al. have identified cardiomyocyte hypertrophy in 3-month-old male offspring of obese dams using a similar model[275]. In postnatal life, hypertrophy is a mechanism to increase the pumping capacity of the heart and is typically a compensatory response to an increase in strain in the walls of the heart. As in fetal life, cardiac hypertrophy postnatally is not inherently pathological. Physiological hypertrophy of the heart is critical to normal growth during childhood, increasing cardiovascular fitness with exercise training, and cardiac adaptation to pregnancy. It is associated with increased cardiac muscle mass with a corresponding expansion of the cardiac capillary network, and is not associated with structural or functional abnormalities[270]. However, pathological hypertrophy is associated with structural changes including fibrosis and reduced capillary density[270], metabolic reprogramming, reactivation of fetal gene expression, and impaired calcium handling[269]. Pathological hypertrophy leads to cardiac dysfunction and is associated with heart failure, the inability of the heart to pump sufficient blood around the body to meet the metabolic demands of the body. The aforementioned cardiac hypertrophy in 3-month-old male offspring of obese dams was associated with fetal gene re-expression[275], consistent with pathological hypertrophy. Echocardiography also revealed indices of diastolic, but not systolic, dysfunction in these male offspring at both three and six months[275], and up to two years of age[272]. These studies reveal that exposure to maternal obesity *in utero* alters offspring cardiac structure, and that this results in diastolic dysfunction even if the offspring are fed a healthy chow diet throughout life.

In addition to, and likely as a consequence of, the changes in heart structure and impaired diastolic function programmed in fetal life, studies have found that exposure to maternal obesity *in utero* results in an increased susceptibility to systolic dysfunction, which may be unmasked if offspring are subjected to a postnatal challenge. Offspring of obese dams fed a postnatal high-fat diet themselves have increased cardiac hypertrophy compared to those of control dams in both mice[276] and sheep[277], in line with what is seen in chow-fed offspring. Turdi et al.[276] used echocardiography to investigate *in vivo* cardiac function in adult mice. In this study, 20-week-old offspring of obese dams fed a control diet post-weaning had no impairment in fractional shortening (FS), a measure of contractile function, while those fed a postnatal HFD had a decrease in FS regardless of maternal diet. However, the effect was more pronounced in offspring of obese dams, suggesting that maternal obesity worsens the susceptibility of the offspring heart to developing systolic dysfunction in response to a postnatal HFD.

1.5.8 Evidence from animal models: hypertension and vascular dysfunction

Numerous animal studies have found that offspring of obese mothers have higher arterial blood pressure than those of lean mothers. Increases in SBP have been reported in male offspring in rodent models of maternal diet-induced obesity or HFD feeding during pregnancy at four[278] as well as six[220] months and up to as much as one[279,280] and two[280] years of age. Many studies only include male offspring and so there is less data from females, although studies have also reported elevated SBP in female offspring of obese dams at both 6 months[219,281] and one year[281] of age. Indeed, meta-analyses of animal studies conclude that exposure to maternal obesity *in utero* increases SBP independent of current age[224], and that this effect is consistent across species. Some studies have also reported an increase in DBP in both male[220] and female[219,281] offspring of obese dams. However, DBP data is more limited than SBP data because measuring DBP accurately in rodents requires the use of radiotelemetry, which is invasive and more expensive than non-invasive tail cuff plethysmography, what most studies use. As introduced in Section 1.2.2, numerous factors have been implicated in the pathogenesis of hypertension including increased sympathetic nervous system activity and changes in peripheral vascular reactivity[60], both of which have been identified in offspring of obese mothers.

While it is more expensive and invasive than tail cuff plethysmography, using radiotelemetry to measure arterial blood pressure has many strengths. As well as allowing for accurate measurement of DBP, it also means that arterial blood pressure data can be recorded in a free moving animal without restraint throughout a 24-hour period. Radiotelemetry can thus offer a greater insight into blood pressure homeostasis. Samuelsson et al.[282] found that both male and female offspring of rats fed a HFD for five weeks before and then throughout pregnancy and lactation had increased arterial blood pressure compared to controls during the night, when rats are more active. Spectral analyses of heart rate variability in the radiotelemetry data found increased low-frequency oscillations in the traces from offspring of obese dams. This is associated with increased sympathetic nervous system activity[283], and thus provides potential insight into one of the mechanisms underlying the programming of hypertension by maternal obesity during pregnancy.

Studies using a particular rat model in which dams are fed a HFD from ten days before pregnancy until the end of lactation have found that maternal high-fat feeding results in increased offspring SBP at multiple ages and have used wire myography to investigate whether changes in vascular reactivity might contribute to this. In this model, increased SBP is associated with impaired endothelium-dependent relaxation of isolated mesenteric[219,280,281] and femoral[284,285] arteries, and segments of thoracic aorta[286]. This combination of increased SBP and impairment in endothelium-dependent femoral artery vasodilation has also been found in offspring of mice fed a

HFD for 4 weeks before mating and throughout gestation and lactation[278]. A different study in 16-week-old offspring of rats fed a HFD for 12 weeks before mating found no effect of maternal obesity on offspring endothelium-dependent relaxation of isolated mesenteric arteries[287], however, they did not measure blood pressure in this study and so it is unknown whether these offspring were hypertensive. Many studies have reported no difference in vascular reactivity to sympathetic vasoconstrictors in offspring of obese dams[278,280,285–287], although one study did find an increased reactivity to noradrenaline in isolated femoral arteries from 2-month-old offspring of obese rats[284]. These data suggest that changes in vascular reactivity may contribute to the increased risk of hypertension in offspring of obese mothers.

1.5.9 Evidence from animal models: sex differences in cardiovascular function

While several of these programmed cardiovascular phenotypes have been found in both male and female offspring of obese mothers at various ages, there is also evidence of sex-specific effects of maternal obesity or high-fat feeding on offspring cardiovascular structure and function. For example, Xue et al. found that feeding rats a high-fat diet only between mating and delivery resulted in male-specific pathological cardiac hypertrophy[222]. This suggests that male offspring may be more susceptible to negative cardiac effects of exposure to maternal high-fat feeding. In support of this, an increased susceptibility to cardiac damage following ischaemia-reperfusion has been specifically found in male offspring of obese dams *in vivo* in mice[288] and *ex vivo* in rats[222].

While few studies have published blood pressure data in both male and female offspring, those that have found that maternal obesity has both age- and sex-dependent effects on the programming of offspring hypertension. In the model described previously in which rats are fed a high-fat diet from ten days before pregnancy, no effect of maternal high-fat feeding on arterial blood pressure, as measured by radiotelemetry, was identified in male offspring at three or six months or one year of age, although they did have a decreased heart rate at six months of age[219]. Female offspring of HFD-fed dams, on the other hand, had increased SBP and DBP at both six months and one year of age, with no difference in heart rate[281]. A different study in which female rats were fed the same obesogenic diet that we use in the Ozanne group from five weeks before pregnancy onwards found that while arterial blood pressure was elevated in both male and female one-, three- and six-month-old offspring, this was associated with decreased heart rate in female offspring only at one month and in male offspring at three months[282]. They found no differences in heart rate in six-month-old hypertensive offspring. However, studies on the impact of exposure to maternal obesity *in utero* on cardiovascular structure and function that include both male and female offspring are limited, thus further research into sex-specific programming effects at different ages is required.

1.6 Model of maternal diet-induced obesity during pregnancy

The mouse model of maternal diet-induced obesity used by the Ozanne group was first published by Samuelsson et al. in 2008[217]. Female mice are fed a highly palatable high-fat, high-simple carbohydrate (HF-HS) obesogenic diet consisting of 45% HFD pellets supplemented with sweetened condensed milk from when they are weaned onwards, including throughout both gestation and lactation. After six weeks of being fed this diet, mice are heavier than controls, hyperleptinaemic and hyperinsulinaemic[289], resulting in them entering pregnancy with an increased body weight and adiposity[249,290,291]. These characteristics persist through pregnancy[217]. However, obese dams are not glucose intolerant compared to controls before pregnancy (personal correspondence with Dr J. Schoonejans, unpublished), and they become glucose intolerant by late gestation[292,293]. This glucose intolerance resolves after birth[294]. Together, these characteristics make this a reasonable mouse model of maternal diet-induced obesity during pregnancy with a GDM-like phenotype. Dams fed the HF-HS diet remain obese[295] as well as hyperinsulinaemic and hyperleptinaemic until weaning, by which point they also develop hypercholesterolaemia[217,296].

1.6.1 Programming of body weight, adiposity, and metabolism

Across different studies using this model, offspring of obese dams have had a similar birth weight[290] to offspring of control dams, or been growth restricted in late gestation[293] or at birth[297]. When offspring are weaned onto a control diet, it has been consistently found that there is no difference in body weight[297–299] between offspring from obese or control dams from weaning until at least 8 weeks of age. In the majority of studies, there has also been no difference in offspring adiposity at 8 weeks of age, whether measured by DEXA[290,296] or fat pad weight[291,295,300], although other studies using TDNMR have found increased total fat mass in offspring of obese dams[298,301,302] at this age. When fed a control diet postnatally, offspring of obese dams begin to diverge and become heavier and fatter than offspring of controls at around 12 weeks of age in males and 20 weeks of age in females[217]. While they do not have consistently increased adiposity compared to controls, 8-week-old offspring of obese dams show signs of adipocyte hypertrophy[297,300] and males exhibit adipocyte hyperplasia, an increased number of adipocytes in their white adipose tissue[297]. This suggests that despite no difference in body weight or adiposity, offspring of obese dams have dysfunctional adipose tissue at this age.

As well as no difference in body weight or adiposity, 8-week-old offspring of obese dams are not glucose intolerant[296,299], nor do they have differences in fed[299] or fasted glucose, fasted triglycerides, FFAs or cholesterol[291,301] compared to controls. However, while they have no differences in fed insulin[299], they do have elevated fasted insulin[290,296]. Elevated fasting insulin levels without glucose intolerance suggests that the pancreas is having to secrete more insulin to maintain glycaemic control,

which is indicative of peripheral insulin resistance. Indeed, hyperinsulinaemic-euglycaemic clamps reveal that 8-week-old offspring of obese dams exhibit whole-body insulin resistance[303], and markers of impaired insulin resistance in offspring of obese dams have also been identified at 8 weeks in adipose tissue[296] and at 12 weeks in liver[249]. Therefore, in our model of maternal diet-induced obesity, offspring of obese dams show both tissue-specific and whole-body insulin resistance at 8 weeks of age, prior to the development of obesity.

There is no difference in beta-cell mass or pancreatic insulin content in 8-week-old offspring of obese dams but some sex-specific effects of maternal obesity on insulin secretion have been identified[299]. A decrease in insulin granule density in beta cells was found in both male and female offspring, however this was only associated with a decrease in granule docking in males. Meanwhile, isolated islets from female offspring of obese dams only had increased basal insulin secretion, as well as in response to glucose and amino acid stimulation. This suggests that in our model of maternal diet-induced obesity during glucose-intolerant pregnancy, in spite of 8-week-old offspring not yet being obese or glucose intolerant, there are programmed changes in both insulin sensitivity and insulin secretion that may contribute to later metabolic dysfunction.

1.6.2 Programming of cardiovascular structure and function

As well as programmed changes in metabolic function, changes in cardiovascular function have been identified in this model. Despite no difference in body weight, 8-week-old male offspring of obese dams consistently have an increased heart weight[290,295,298,301], which is associated with cardiomyocyte hypertrophy[290,295,301] and increased cardiac wall thickness[295,298]. This is consistent with the studies in fetal and newborn sheep, rodents, and minipigs discussed in Section 1.5.6, and adult mice discussed in Section 1.5.7. The hypertrophy observed in 8-week-old offspring of obese dams in our model is associated with an increase in cardiac expression of fetal genes[290,298,301], which is indicative of pathological hypertrophy. These changes are associated with increased insulin signalling in the heart[290], which likely contributes to the cardiac hypertrophy.

Changes in *in vivo* cardiac function have also been identified in male offspring at this age using echocardiography, with exposure to maternal obesity *in utero* resulting in a decrease in ejection fraction[298,301], which is indicative of systolic dysfunction. This suggests greater cardiac dysfunction in our model than in the studies discussed in Section 1.5.7, as systolic dysfunction has been found in the absence of postnatal high-fat feeding. This is further supported by *ex vivo* experiments in 12-week-old male offspring, as isolated hearts on a Langendorff preparation display signs of systolic dysfunction[295]. Utilising the Langendorff preparation, evidence of cardiac sympathetic dominance was also identified[295]. Cardiac sympathetic dominance is a marker of heart failure[304].

This is consistent with the data from Samuelsson et al. discussed in Section 1.5.8[282], in which differences in heart rate variability indicated increased sympathetic nervous system activity in the hearts of offspring of obese rats[283]. However, evidence of cardiac sympathetic dominance has not previously been investigated *in vivo* in our model of maternal diet-induced obesity.

When investigating the programming of hypertension by exposure to maternal obesity, previous studies using tail cuff plethysmography found an increase in evening SBP in 8-week-old offspring of obese dams compared to controls[298,301]. Moreover, studies in 12-week-old offspring using the same model also found increases in SBP of offspring of obese dams during the night using radiotelemetry[217]. This increase in SBP is associated with both impaired endothelium-dependent relaxation and increased reactivity to sympathetic vasoconstrictors in mesenteric arteries at this age[217].

To date, all *in vivo* and *ex vivo* functional experiments assessing cardiovascular function by the Ozanne group in this model have only been performed in male offspring, and so there may also be sex-specific effects that are yet to be identified. Moreover, we have not completed any effective studies of either diastolic cardiac function or vascular reactivity in offspring of obese dams in our model. Indeed, while studies of the effect of maternal obesity on offspring cardiovascular function have included investigations into changes in arterial blood pressure, vascular reactivity, and cardiac structure and function, no studies identified in the literature or previously undertaken by the Ozanne group have looked at all three of these factors in a single study. This is incredibly important as these three factors are inherently linked to one another.

Chronic hypertension results in increases in left ventricular pressure and subsequently increased left ventricular wall stress. This stimulates myocardial hypertrophy that acts to normalise wall stress, but that can result in impairments in cardiac diastolic function. If the hypertension is not corrected, the hypertrophy eventually fails to compensate for the increased wall stress and subsequently the heart dilates, and systolic function is impaired. This results in heart failure and illustrates the interrelated nature of blood pressure homeostasis and cardiac function. In the Framingham heart study, 91% of people who developed heart failure first developed hypertension[305]. As described in Section 1.2.2, the pathogenesis of hypertension is associated with numerous factors including changes in peripheral vascular reactivity[60]. Therefore, studying arterial blood pressure, vascular reactivity, and cardiac function in a single study is extremely powerful in the characterisation cardiovascular health.

1.6.3 Combined exposure to maternal obesity and a postnatal obesogenic diet

Feeding of our experimental HF-HS diet postnatally results in increases in fasting insulin, leptin and cholesterol independently of maternal diet and combining this with exposure to maternal HF-HS diet *in utero* results in more severe hyperinsulinaemia and hyperleptinaemia[298].

This supports that exposure to maternal obesity predisposes offspring to an increased risk of metabolic dysfunction, especially if challenged with a hypercaloric postnatal environment. Moreover, exposure to a postnatal HF-HS diet resulted in comparable *in vivo* systolic dysfunction with or without maternal obesity, whereas the effects on offspring blood pressure were additive. That is, exposure to either maternal or postnatal HFD resulted in elevated arterial blood pressure in 8-week-old offspring compared to offspring of lean dams fed a healthy chow diet from weaning, and those exposed to both maternal and postnatal HFD had even higher SBP. This suggests that the programming of cardiac dysfunction and hypertension are independent of one another. Offspring with markers of systolic dysfunction only had concurrent cardiac re-expression of fetal genes if they were exposed to maternal obesity *in utero* and not if they only received the HF-HS diet postnatally. This suggests that the programming of cardiac dysfunction by maternal obesity is via independent mechanisms to those linking it to postnatal diet.

1.6.4 The effect of ageing on programmed phenotypes

As introduced in Section 1.6.1, while offspring of obese dams fed a regular chow diet in this model are not heavier or consistently fatter than those of control dams at 8 weeks of age[290,291,295–301], these phenotypes develop as the offspring age[217,306]. The adipocyte hypertrophy and hyperplasia present in 8-week-old male offspring of obese dams in the absence of increased adiposity[297,300] also progresses as the offspring age and develop obesity themselves. By one year of age, male offspring of obese dams are not only heavier and fatter than controls, their white adipose tissue is also dysfunctional with increased expression of inflammatory genes[306]. Similarly, offspring exposed to obesity *in utero* are glucose tolerant at 8 weeks of age but have increased peripheral insulin resistance. However, by six months of age, this has progressed such that they are glucose intolerant[303]. Therefore, programmed differences in body composition and metabolism become greater with age.

Whether the same worsening of programmed phenotype with advancing age occurs with offspring cardiovascular health in this model has not yet been investigated to the same extent. Given that CVD is an age-associated disease and exposure to maternal obesity *in utero* is associated with an increased risk of CVD, it is paramount that we understand how programmed changes in cardiovascular function progress with age. The only published study investigating cardiovascular function in this model beyond three months of age found that SBP remained elevated in both male and female offspring of obese dams at six months of age when measured by radiotelemetry[217]. Moreover, while they found altered vascular reactivity in mesenteric arteries at three months of age, these differences were no longer present at six months of age[217].

1.7 Interventions in obese and diabetic pregnancy

The knowledge that the conditions that a fetus is exposed to *in utero* have a powerful impact on long-term cardiometabolic health means that pregnancy presents a valuable opportunity to intervene and improve the health of the population by reducing the risk of someone developing cardiometabolic disease as an adult before they are even born. Such interventions in obese and diabetic pregnancy aim to correct or compensate for the negative programming effects of maternal adiposity and the dysregulated metabolic milieu. There are two broad types of interventions: lifestyle and pharmacological. Most interventions currently being assessed were proposed or developed as treatments for GDM. However, given that GDM is associated with obesity and that even in the absence of overt maternal diabetes, obesity programmes poor maternal, neonatal and offspring outcomes, several of interventions are now also being investigated for use in glucose tolerant obese pregnancy.

1.7.1 Lifestyle interventions

In clinical practice, lifestyle intervention is the first line treatment for GDM. Typically, this involves healthcare professionals offering advice, recommendations, and guidance on improving the quality of someone's diet and increasing the amount of physical activity that they do. Such lifestyle interventions result in successful glycaemic control in as many as two thirds of GDM-affected pregnancies[307]. To date, numerous randomised controlled trials (RCTs) have been carried out to investigate the efficacy of different lifestyle interventions during overweight or obese pregnancy on maternal health and neonatal outcomes. However, there is a great deal of variation in how these RCTs are set up, and what effects they have found. The key ways that trials differ include: the inclusion criteria around maternal BMI, the timing and duration of the intervention, how the intervention is delivered, the type of exercise used, and whether it includes both exercise and diet changes or only one of them. Several RCTs investigating the effect of maternal exercise intervention during overweight or obese pregnancy and their findings are discussed below. In all cases participants were randomly assigned to either the intervention group or a control group when they were recruited, with control groups receiving standard antenatal care, and so results are relative to an internal control.

<u>Trials investigating the effect of exercise intervention alone</u>

The Treatment of Obese Pregnant Women (TOP) study[308] included 389 women in Copenhagen, Denmark, and included both an exercise-only intervention group, and a combined exercise and dietary intervention group, which is discussed in the next section. Women were recruited between 11 and 14 weeks of gestation and the average pre-pregnancy BMI was 34kg/m^2. For the exercise intervention, women were given a pedometer and encouraged to reach a minimum of 11,000 steps per day.

Progress was monitored by recording daily step counts for seven consecutive days every four weeks, and women received a text message at the beginning of the measurement period encouraging them to reach this goal. This intervention was thus very cost effective and simple to administer by health care professionals, and it did result in a decrease in the average GWG of 1.5 kg. However, in spite of the effect on GWG there were no significant effects on the incidence of GDM or hypertension, and no effect on birth weight or other maternal and neonatal outcomes.

The Vitamin D and Lifestyle Intervention in the prevention of GDM (DALI) studies[309] were much larger; they were conducted across nine European countries and included almost 900 women, although this also included groups who received a healthy eating intervention only, as well as interventions that involved Vitamin D administration – the results of which will not be discussed here. Women with a BMI ≥ 29 kg/m^2 were recruited on average at 15 weeks of gestation, and those in the exercise intervention group then devised a plan for increasing physical activity for the rest of their pregnancy with a lifestyle coach. Progress was evaluated and achievable goals discussed at a minimum of three further face-to-face sessions, plus optional telephone "booster calls". This exercise intervention thus involved more contact with health care professionals than was used in the TOP study and was focused more on the psychological aspect of changing behaviour through coaching rather than a simple step-count goal. However, this intervention did not impact GWG or maternal or neonatal outcomes[310]. Nevertheless, they did report an improvement in maternal aerobic fitness, which could improve maternal health going forward.

The Improving Maternal and Progeny Obesity Via Exercise (IMPROVE) study set out to study a more structured exercise intervention, albeit without an increase in healthcare professional time and input. It included 75 women in Auckland, New Zealand[311] recruited at 20 weeks of gestation with an average BMI of 33 kg/m^2. The intervention group received a written programme of between three and five 30-minute sessions of moderate-intensity cycling to be completed at home each week. There was no effect of the exercise intervention on any perinatal outcomes for either mother or baby, including both GDM incidence and birth weight. However, on average women in the intervention group only completed one third of the exercise sessions. Sensitivity analyses then found that greater compliance to the exercise protocol was associated with reduced BMI and fat mass in the postpartum period, suggesting that increased compliance with the same intervention may have yielded more effects. While there were no overall differences in either GWG or postpartum adiposity, as in the DALI studies women in the intervention group demonstrated improved aerobic fitness. An RCT using an almost identical intervention strategy was carried out by the same group a little under ten years prior in non-obese pregnant women[312]. This study included 84 women with an average BMI of 25.5 kg/m^2.

In this study, the average compliance in the exercise group across the intervention period was 75%, suggesting that motivating leaner women to increase their physical activity during pregnancy is easier than obese women.

Another RCT using stationary bike cycling was carried out by Wang et al. with vastly different results[313]. This study included 300 pregnant women with a pre-pregnancy BMI between 24 and 28 kg/m^2 in Peking, China. Women were recruited at an average of 10 weeks of gestation, and participants in the exercise group then attended supervised cycling sessions at least three times a week until late gestation. Each session was a minimum of 30 minutes long and consisted of interval cycling: alternating high intensity sprinting or working against resistance with low-to-moderate intensity cycling. The exercise intervention decreased average GWG by more than 2 kg and also resulted in a 45% reduction in GDM incidence. Moreover, babies born to women in the exercise group had a lower birth weight than controls. However, there was still no effect on the incidence of hypertensive disorders of pregnancy. Compliance was much higher in this trial: with 90% of participants in the exercise group more than 80% compliant with the exercise program. Therefore, while both this trial and IMPROVE utilised stationary cycling as an exercise intervention, this trial included leaner women who started the intervention earlier in pregnancy, and who completed more of the exercise sessions which were likely each more intensive than those in IMPROVE.

An RCT in Campinas, Brazil, of 80 women[314] found differences in the effect of an exercise intervention during pregnancy between overweight and obese mothers. The exercise intervention consisted of one 40-minute supervised session of light-to-moderate intensity exercise per week, plus guidance on completing five further sessions at home, starting at approximately 18 weeks of gestation. When stratified by pre-pregnancy BMI, the exercise intervention resulted in reduced GWG in overweight women, but not in obese women. This suggests that reducing GWG may be a more achievable goal for overweight women than obese women. No other differences in perinatal outcomes were reported.

The Exercise Training and Weight Gain in Obese Pregnant Women (ETIP) trial[315] applied a similar structure of intervention as the Wang. et al. trial but using different types of exercise. It included 91 women in Tronheim, Norway, with an average BMI of 34kg/m^2 who were recruited between 12 and 18 weeks of gestation. The exercise intervention consisted of three one-hour supervised sessions per week, plus the recommendation to carry out a further exercise session at home at least once per week. Supervised sessions included both resistance (i.e. bodyweight training) and endurance (i.e. jogging on a treadmill) training. In this trial, half of the women in the exercise group adhered to the full program. This exercise intervention did not affect GWG but did both reduce the incidence of GDM and reduce SBP in late pregnancy. This suggests that interventions can improve maternal health without affecting body weight. No effects on neonatal outcomes were found[316].

1.7 | Interventions in obese and diabetic pregnancy

Trials investigating the effect of combined diet and exercise intervention

The TOP study also included a combined exercise and dietary intervention[308]. Women in the combined group received the same pedometer and advice to do 11,000 daily steps as those in the exercise-only group and achieved similar step numbers in each measurement week. In addition, they received fortnightly contact with a dietitian, alternating face-to-face and via telephone, which included weight measurements and advice on how to improve and adjust their diet. Therefore, this represented an increase in the cost of delivering the intervention compared to the exercise-only arm. As in the exercise-only group, this combined intervention resulted in decreased GWG compared to controls, and the authors state that this GWG reduction was non-significantly greater than that seen in the exercise-only group. Once again there were no other effects on maternal or neonatal outcomes.

The DALI studies also included a combined healthy eating and physical activity intervention group[309]. In this group, guidance on how to improve diet quality was delivered by the lifestyle coach alongside the physical activity recommendations. Unlike those who only received the exercise intervention, women who received this combined intervention did have reduced GWG compared to controls[310], although there remained no other effects on maternal or neonatal outcomes. This suggests that a combined diet and exercise intervention may be more effective than exercise alone at reducing GWG.

The Limiting Weight Gain in Overweight and Obese Women During Pregnancy to Improve Health Outcomes (LIMIT) trial[317] included more than 2000 women in South Australia. Women with an average BMI of 31 kg/m^2 were recruited at approximately 14 weeks of gestation and randomly allocated to receive either standard care or dietary and lifestyle advice. In the dietary and lifestyle advice group, women met with a research technician at the beginning of the trial and were given individualised meal plans and nutritional advice and encouraged to set achievable goals for improving the quality of their diet and the amount of physical activity that they did. They then had follow-up conversations intended to reinforce this information either in person or over the phone every 2-4 weeks until the end of the third trimester. This trial was therefore fairly similar to the DALI combined diet and lifestyle study, albeit much larger. However, in this trial no effect of the intervention was found on GWG, as well as other maternal and neonatal outcomes.

The UK Pregnancies Better Eating and Activity Trial (UPBEAT)[133] included over 1400 women with an average BMI of 36 kg/m^2 who were recruited between 15-19 weeks of gestation and randomly allocated to standard care or a behavioural intervention. Similar to LIMIT, the behavioural intervention consisted of individualised guidance on diet and physical activity delivered by a health trainer. However, in UPBEAT the intervention group received one-hour sessions every week for nine weeks with the health trainer, in which they reviewed their progress and set ongoing, achievable goals.

UPBEAT therefore had a similar method of delivery of intervention as LIMIT, but with a greater amount of contact between participants and health trainers. Women in the intervention group had reduced GWG than women given standard care. This was associated with an improved quality of diet and an increased amount of physical activity. There was no effect on birth weight or incidence of either GDM or pre-eclampsia.

A study in Leuven, Belgium[318], further set out to investigate the impact of active face-to-face contact versus a more passive approach in a dietary and lifestyle intervention. A total of 122 women with an average pre-pregnancy BMI of 33.5kg/m^2 and an average gestational age at recruitment of 10 weeks were included. The passive intervention group were given a brochure that provided advice on improving diet quality and increasing physical activity during pregnancy during their first antenatal visit, and no further contact outside of routine prenatal care. The active intervention group were given the same brochure, and also attended three one-hour group sessions with a trained nutritionist over the remainder of their pregnancy, with the aim of these sessions being to reinforce the recommendations of the brochure. There was no effect of either intervention on GWG, nor on other outcomes including GDM, maternal hypertension or birth weight. This study also published findings on how the interventions affected feeding behaviours in these women. Women in both intervention groups consumed fewer calories than controls. This was associated with a decrease in saturated fat intake, an increase in protein content, and an increase in vegetable consumption. This suggests that these women did engage in a healthier lifestyle as a result of the intervention, although this did not impact on the outcomes measured. Moreover, no significant difference in the dietary habits between the active and passive intervention groups were reported, suggesting that ongoing contact with a dietician does not inherently improve the outcomes of a dietary intervention. In this trial, neither intervention strategy resulted in a change in physical activity.

Overall, data from human trials on the effect of exercise intervention during obese pregnancy on maternal and neonatal outcomes offer conflicting results. Studies that have used animal models to investigate the impact of exercise intervention during obese pregnancy are described in Chapter 3. In the Ozanne group, we have developed an exercise intervention using our mouse model of maternal diet-induced obesity in which dams are run on a treadmill for 20 minutes a day for one week prior to mating and throughout pregnancy. Using this model, we have demonstrated an improvement in cardiometabolic function in male offspring of obese mothers[292,301]. When considering how the intervention alters maternal health and thus what may underlie the improved offspring health, we have found that the exercise intervention does not impact maternal weight or adiposity but does improve metabolic function[292]. This is in line with what was found in the ETIP trial, where GDM incidence was reduced with an exercise intervention even without an effect on GWG.

Given the importance of maternal cardiovascular function in obese pregnancy on maternal and fetal outcomes, and that several RCTs in humans have found that an exercise intervention improves maternal cardiorespiratory fitness, it is important that we characterise the maternal cardiovascular function in our model and the impact of this exercise intervention on it.

1.7.2 Pharmacological interventions

Lifestyle interventions fail to restore glycaemic control in approximately one third of women with GDM[307]. Traditionally, the standard pharmacological treatment for GDM if lifestyle interventions fail is insulin. When administered correctly, insulin treatment is highly effective at controlling maternal glycaemia[319] and reduces the risk of macrosomia[320,321]. Insulin does not cross the placenta, and thus it is able to treat the maternal metabolic dysfunction without having any direct effects on the developing fetus, although it is possible that it exerts effects on the placenta. However, insulin treatment requires injections which many people find uncomfortable and requires more health care professional time to support the individual and optimise their insulin treatment. Insulin is also expensive and must be kept refrigerated, making it less suitable for use in developing countries. Moreover, even when well trained in the process, women using insulin treatment frequently experience mild hypoglycaemic episodes[129]. Therefore, oral diabetic medications are increasingly attractive alternatives to insulin as they are cheaper, and easier to administer and store.

One such class of medications are sulphonylureas, which bind to the sulphonylurea receptor 1 subunit of ATP-sensitive potassium channels in pancreatic beta cells. This binding causes closure of the channel, causing the beta cell membrane to depolarise, resulting in the opening of voltage-gated calcium channels on the cell membrane and the influx of calcium into the beta cell thus stimulating insulin release by calcium-dependent exocytosis. Therefore, sulphonylureas act by increasing insulin secretion. Glyburide, also known as glibenclamide, is a sulphonylurea that is commonly prescribed to treat GDM in the USA[322]. The first study comparing the use of insulin and glyburide in GDM treatment found them to be equally effective at controlling maternal glycaemia and found no difference in fetal outcomes, including the incidence of macrosomia and likelihood of being admitted to neonatal intensive care[323]. This suggested that glyburide was a good alternative to insulin in the treatment of GDM. However, more recent studies and meta-analyses have found that compared to insulin, maternal glyburide treatment for GDM is associated with increased birth weight and an increased risk of macrosomia[324–327], which is associated with an increase in neonatal fat mass[324].

Unlike insulin, glyburide can cross the placenta and is found in the cord blood at up to 70% of the concentration in the maternal serum[328]. This likely underlies to increased fetal growth, as glyburide in the fetal circulation would be expected to stimulate fetal pancreatic insulin secretion, and insulin is a major growth factor *in utero*. Also linked to this, maternal glyburide treatment has been found to be associated with neonatal hypoglycaemia[326]. This likely underlies the increased risk of neonatal intensive care admission in babies of women treated with glyburide compared to insulin reported by some studies[326,327]. Therefore, glyburide may not be an appropriate treatment for women with GDM due to the short-term impact it has on neonatal health, let alone whether it may correct or worsen any longer-term impacts of exposure to maternal diabetes *in utero*. In many countries, including the UK, the first line pharmacological treatment for diabetes during pregnancy is the synthetic biguanide drug metformin (1,1-dimethylbiguanide hydrochloride).

1.7.3 Metformin intervention

The use of biguanide drugs originates from *Galega officinalis*, a traditional herbal medicine high in guanidine used since the 17th century to treat diabetic-like symptoms[329]. Synthetic guanidine derivatives, including metformin, were first synthesised in the 1920s[330] and metformin was first found to lower blood glucose in rabbits[331] and dogs[332] in 1929. However, there was little follow-up on the potential use of metformin as an antidiabetic drug until it was first reported as a highly effective treatment for diabetes in humans by Jean Sterne in 1957[333]. Today, metformin is the most commonly prescribed glucose-lowering medicine in the world. While the specific mechanism of action of metformin remains unclear and debated, it is known to increase intestinal[334], adipose, and skeletal muscle[335] glucose uptake, and decrease hepatic glucose production[336]. This results in a decrease in blood glucose and an increase in insulin sensitivity without increasing insulin secretion, thus reducing the risk of hypoglycaemia compared with insulin treatment. Metformin also increases circulating levels of the peptide hormone GDF15[337] in non-pregnant individuals, which is associated with reduced food intake and sustained decreases in body weight[338].

The first study of the use of metformin during pregnancy to treat women with pre-existing or gestational diabetes was published in 1979, and suggested it was safe for use during pregnancy[339]. NICE guidelines in the UK recommend that women that are already taking metformin for pre-existing T2D may either continue taking metformin or switch to insulin when they become pregnant[340], and health guidelines from several countries including the UK[340], Canada, and New Zealand[341] now endorse metformin for the treatment of GDM. Indeed, the Metformin in GDM (MiG) trial found that metformin is generally preferred to insulin by pregnant women, likely due to it not requiring injections[342].

1.7 | Interventions in obese and diabetic pregnancy

A meta-analysis from our group concluded that metformin administration reduces GWG compared with other pharmacological antidiabetic treatments[341], and others have reported reduced weight gain compared to insulin-treated mothers[325]. Metformin may also reduce the risk of pregnant women developing gestational hypertension[325] and pre-eclampsia[341], and could be an effective treatment to prolong gestation in women with pre-term pre-eclampsia[343]. Together, these findings suggest that metformin administration may have numerous positive effects on the cardiometabolic health of pregnant diabetic women.

As well as women with diabetes, many women with polycystic ovary syndrome (PCOS) take metformin before and during pregnancy. Hyperinsulinaemia and insulin resistance are key features of PCOS; while the majority of women with PCOS are obese, this hyperinsulinaemic insulin resistance is also present in those who are lean[344,345] and is believed to contribute to the disease pathology by increasing circulating androgens and suppressing ovulation. Nestler and Jakubowicz first demonstrated that metformin was effective at decreasing insulin resistance and circulating testosterone in obese women with PCOS in 1996[346], and the same in non-obese women shortly afterwards[347]. As a result of this, metformin-treatment improves fertility in women with PCOS by increasing ovulation[348]. Moreover, women with PCOS are three times more likely to miscarry in early pregnancy than healthy women, and metformin administration during the first trimester reduces the risk of spontaneous miscarriage in women with PCOS who have previously had a miscarriage without metformin treatment[349]. As well as increasing fertility and reducing the risk of miscarriage, and in line with findings in diabetic women, metformin treatment during pregnancy has been found to reduce GWG in women with PCOS[350,351], which may decrease the risk of these women developing GDM[351].

The Efficacy of Metformin in Pregnant Obese Women, a Randomised Controlled Trial (EMPOWaR) and Metformin in Obese Non-diabetic Pregnant Women (MOP) studies were established to investigate the effect of metformin treatment in obese pregnancy without maternal diabetes. They explored the effect of metformin administration from week 12-18 of gestation until delivery compared to placebo in morbidly obese (BMI >35 kg/m^2, MOP) or obese (BMI >30 kg/m^2, EMPOWaR) women. The MOP study found that treatment with metformin reduced GWG but did not affect GDM incidence[352,353], while EMPOWaR found no effect on GWG and a trend for reduced GDM[354]. The MOP study found that metformin treatment reduced pre-eclampsia incidence[352,353] whereas EMPOWaR did not. However, EMPOWaR did find that metformin-treatment reduced inflammatory markers[354], which may decrease the risk of pre-eclampsia. Neither study found an effect of metformin exposure *in utero* on birth weight, although this may be due to low compliance with the metformin dosing.

Together, this means that an increasing number of babies are being exposed to metformin *in utero*. While numerous positive effects of gestational metformin treatment on maternal health have been reported, metformin freely crosses the placenta and is found at comparable levels in the maternal and fetal circulations[355]. This means that metformin is able to act directly on the developing fetal tissues, independent of maternal glycaemia. Concerns that metformin may directly impact fetal development and a lack of evidence on long-term offspring health led to the American Diabetic Association publishing a letter in 2006 urging caution about metformin use in pregnancy[356].

1.7.4 Effects of exposure to metformin *in utero* on offspring health

A meta-analysis from our group showed that, in contrast to glyburide, gestational metformin treatment is associated with decreased birth weight and a reduced incidence of macrosomia compared to insulin treatment[324]. This occurs without an increased risk of SGA neonates[357]. This may suggest that metformin is effective at reducing the negative effect that maternal diabetes has on increasing birth weight. However, two-year-old children of metformin-treated mothers are heavier than those of mothers treated with insulin, and by mid-childhood they have an increased BMI[357]. This suggests that prenatal metformin exposure may be programming a phenotype of IUGR with postnatal catch-up growth, resulting in an increased risk of childhood adiposity[78]. As discussed earlier, this would suggest that children exposed to metformin *in utero* may in fact be at an increased risk of obesity and cardiometabolic disease in later life. Long-term data in humans is lacking, with only a couple of studies publishing offspring weight and body composition beyond birth, and follow-up of children from the severely limited number of RCTs not extending beyond 10 years old.

Given the association between maternal obesity and diabetes and offspring cardiovascular dysfunction, it is important to investigate cardiovascular function in children exposed to metformin *in utero* to determine if metformin improves or worsens the programmed effects of exposure to maternal obesity and diabetes. The first study to report on offspring cardiovascular outcomes after maternal metformin treatment was a pilot study from the Metformin in Pregnant PCOS Women (PregMet) trial in Norway. This study found a trend for an increase in SBP in 8-year-old metformin-exposed children, without any difference in body weight or BMI[358]. This suggests that fetal exposure to metformin may programme a hypertensive phenotype. However, a larger follow-up study in 5- to 10-year-old children from the same trial did not find any effect of gestational metformin on blood pressure, although metformin-exposed children did have an increased BMI[359]. This follow-up study also found an increase in heart rate with metformin-exposure if the mother had a pre-pregnancy BMI greater than 30 kg/m^2, and sex-specific analyses revealed an increase in heart rate in sons of metformin-treated women only when not considering maternal BMI[359].

1.7 | Interventions in obese and diabetic pregnancy

The only published cardiovascular follow-up of children exposed to metformin in the context of GDM comes from the MiG trial. This study found no effect of metformin-exposure on blood pressure in 2-year-old children[360]. They also found no difference in total fat mass, although metformin-exposed children had increased sub-scapular skin folds, indicating an increased amount of subcutaneous fat[361]. The authors therefore propose that prenatal metformin-exposure may programme a more favourable pattern of fat distribution in children of diabetic mothers, which would potentially be protective against future metabolic dysfunction[39] and hypertension[40].

By far the most in-depth cardiovascular phenotyping of children exposed to metformin *in utero* comes from follow-up of the MOP trial[353] of maternal metformin-treatment versus placebo in morbidly obese, non-diabetic pregnant women as this study used echocardiography to investigate cardiac function. The average age of children at follow up was 3.9 years. Researchers found no difference in either SBP or DBP as measured using a standard inflatable cuff on the upper arm, which measures the blood pressure in the brachial artery. However, they then used the brachial blood pressure waveform to mathematically derive a value of aortic SBP and found that this was reduced by prenatal metformin. Moreover, pulse pressure, the difference between DBP and SBP, was reduced with *in utero* exposure to metformin. Pulse pressure increases with advancing age, and this is associated with increased central artery stiffness[362]. Therefore, reduced pulse pressure may indicate a beneficial effect of metformin on offspring vascular health. Echocardiography did not find any differences in LV mass or LV wall width, suggesting there was no difference in cardiac hypertrophy. The only measure of systolic function reported was fractional shortening, which was not different between groups. A change was found in one measure of diastolic function, isovolumetric relaxation time, which was indicative of improved diastolic function in children exposed to metformin *in utero*[363]. No differences were found in the other indices of diastolic dysfunction analysed and thus caution must be exercised when drawing conclusions on diastolic function in these individuals, as guidance from the American Society of Echocardiography and the European Association of Cardiovascular Imaging states that no single index of diastolic dysfunction should be used in isolation[364].

More recently, follow-up of children of an average age of 5.78 years was published from EMPOWaR[365], which also looks at the effect of metformin intervention in obese, non-diabetic pregnancy. As in the MOP trial follow-up, this study found no effect of gestational metformin on blood pressure. They also report a mathematically derived value for “central” SBP and DBP, and unlike the MOP follow-up, these were also not different between groups.

Together, follow-up data from human trials is limited and offers conflicting results of the effect of prenatal exposure to metformin on cardiovascular function, and no studies have investigated possible sex-specific differences. Regardless, the offspring in these studies are still very young and longitudinal follow-up is required to properly assess the effect of *in utero* metformin on cardiometabolic health. Findings of the effects of *in utero* exposure to metformin from studies using animal models are discussed in detail in Chapter 5, although published data almost exclusively considers the impact on offspring metabolic health, rather than cardiovascular function. Given the association between exposure to maternal obesity and/or diabetes during pregnancy and poor cardiovascular outcomes in offspring, it is paramount that we investigate how maternal metformin treatment on top of maternal obesity impacts long-term cardiovascular health.

1.8 Thesis aims

This thesis addresses how obesity during pregnancy impacts maternal and offspring cardiovascular health and explores two different intervention strategies applied to the mother during pregnancy: metformin administration and an exercise intervention. It has three specific aims with results for each presented across three results chapters. The specific aims are:

1) To establish the impact of obesity on maternal cardiovascular adaptation to pregnancy and determine whether an exercise intervention can offset any negative effects.

This will be achieved using our established mouse model of maternal diet-induced obesity with or without treadmill running. *In vivo* tail cuff plethysmography and late-gestation echocardiography and uterine artery ultrasound will be used to investigate maternal cardiovascular adaptation to pregnancy, while *ex vivo* wire myography will provide insight into vascular function in pregnant dams.

2) To identify sex-specific differences in the programming of offspring cardiac and vascular function by maternal obesity.

This will be achieved using *in vivo* tail cuff plethysmography and echocardiography, and *ex vivo* wire myography to characterise the cardiovascular phenotype of both male and female offspring of obese dams at 8 weeks of age. A new protocol will be developed in order to investigate whether maternal obesity programmes changes in offspring cardiac sympathetic reactivity *in vivo* using stimulated echocardiography.

3) To determine whether exposure to maternal obesity *in utero* results in sex-specific differences in blood pressure and vascular function with advancing age, and the consequences of metformin intervention in obese pregnancy on these factors.

This will be achieved using a cohort of 12-month-old offspring generated using our established mouse model of maternal diet-induced obesity with or without gestational metformin treatment. Programming of blood pressure and changes in vascular structure and function will be assessed by *in vivo* tail cuff plethysmography, *ex vivo* wire myography, and histological analyses of aortas.

Chapter 2: General Methods

2.1 Animal model

All animal studies were conducted according to the UK Home Office Animals (Scientific Procedures) Act 1986 and following ethical review by the University of Cambridge Animal Welfare and Ethical Review Board (AWERB). Wild-type C57Bl/6J mice were generated from in-house breeding colonies. At weaning, females were randomly assigned to either a highly palatable high fat - high simple carbohydrate (HF-HS) obesogenic diet or a control diet, both fed *ad libitum*. The obesogenic diet consisted of 45% HFD pellets (45% denoting the percentage calories derived from fat) (Special Dietary Services) supplemented with *ad libitum* access to sweetened condensed milk (Nestle). Micronutrient mineral mix (AIN93G, Special Dietary Services) was dissolved in the milk to ensure mice fed the obesogenic diet did not become mineral-deficient. The control diet used was RM1 laboratory chow (Special Dietary Services). Macronutrient composition of all diets is shown in Table 2.1.

	RM1	RM3	45% HFD	Condensed Milk
AFE (kcal/g)	3.52	3.61	4.54	3.25
Composition (%w/w)				
Fat	2.71 (soya oil)	4.2 (soya oil)	22.6 (soya oil and lard)	8.0
Protein	14.38	22.45	23.0	7.3
Starch	44.97	33.88	28.34	0
Simple Sugars	4.05	4.37	10.49	56

Table 2.1: Energy content and macronutrient composition of diet components. *HFD = High fat diet. AFE = Atwater Free Energy. %w/w = percentage wet weight.*

At six weeks of age, all mice were mated for a first pregnancy (P1). The purpose of this first pregnancy was to ensure that dams were proven breeders. Data were not collected from dams during P1 and litters were not used for further in experiments in this thesis but were made available to others in line with The Three Rs principle. Mice were paired with wild-type stock males (supplied by Charles River) for up to four days, during which time all females were maintained on their assigned pellet diet, although condensed milk was removed from those on the HF-HS obesogenic diet. When not mating, stock males were maintained on RM3 laboratory chow (Special Dietary Services) (Table 2.1). Mating was confirmed by the presence of a copulatory plug. Upon noting a plug, mice were separated from the male and housed in pairs with other females who had plugged within two days of each other. If females did not plug within four days, they were separated from the male and returned to their home cage for at least three days before being re-paired with a new male. Females were allowed up to three attempts to plug. Pregnant females were allowed to litter naturally, and first litters were weaned at post-natal day 21 (PND21), at which point females were considered “pre-second pregnancy dams” (PreP2 dams). Subsequent action depended on the dam’s experimental cohort and are described in each chapter.

2.2 Non-invasive blood pressure measurement

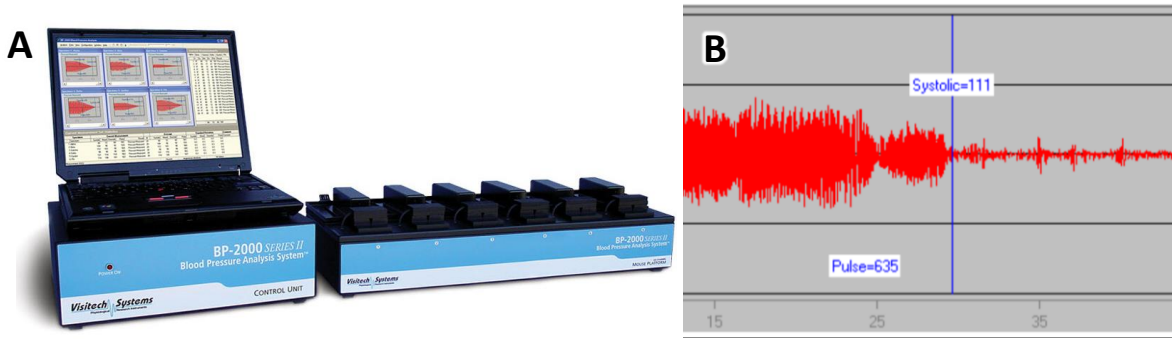


Figure 2.1: Visitech BP-200 tail-cuff plethysmography system. *System set-up (A) and representative blood flow trace showing point where systolic blood pressure is measured (B).*

Blood pressure measurements were recorded across all cohorts and specifics of each are described in the Methods sections of the relevant chapter. All blood pressure measurements were made in the conscious animal using a BP-200 tail-cuff plethysmography system (Visitech) (Figure 2.1(A)).

The system works as follows: non-anaesthetised animals are placed on a heated metal platform and restrained by placing a dark, magnetic cover over them, from which their tail protrudes. An inflatable cuff is placed around the base of the mouse's tail and the tail placed between a light source and a photodetector before being secured using surgical tape. Blood flow is measured by transmission photoplethysmography, which uses light scattering to measure vessel dilation and therefore blood flow. Mice are left to acclimatise for a few minutes before measurement cycling is initiated. In a given cycle, heart rate is measured over 30 seconds prior to cuff inflation. Cuffs are then inflated over 15-30 seconds to a maximum pressure of 180mmHg before deflating. SBP is recorded as the cuff pressure at which blood flow through the tail is completely occluded, as shown in Figure 2.1(B). Any data from the first five cycles in a run were discarded, and a minimum of 16 further cycles run. In all cases, animals were subjected to at least two days training before experimental measurements were recorded.

Mice are typically more mobile and their recorded SBP is elevated on the first day of training, as they are stressed when they are not yet accustomed to the machine. They calm down considerably and are mostly still by the second day. On the one occasion that a mouse remained clearly stressed on the second day of training, they were removed from the machine and not subjected to further blood pressure measurements. All sessions were performed at the same time of day to control for the natural circadian rhythm of blood pressure. At the end of each week, blood pressure measurements were exported from the Visitech BP-200 machine and recorded in Excel. Data from an animal were only included if at least ten successful SBP and HR measurements were obtained with an intra-animal coefficient of variation of less than 15%.

2.3 Wire myography

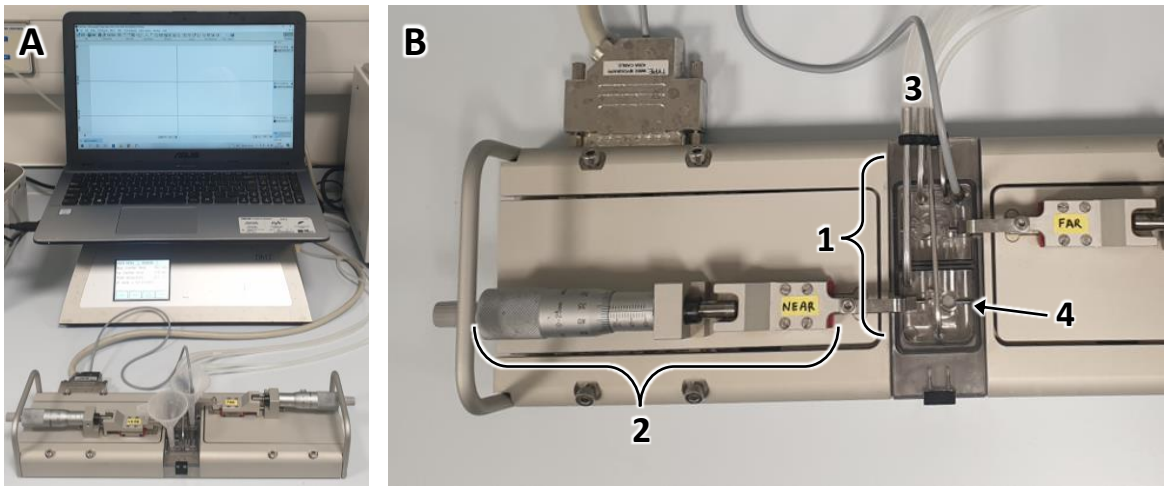


Figure 2.2: 420A Dual Wire Myograph system*. (A) System set-up including chamber unit, interface unit and laptop with LabChart. (B) Close-up of chamber unit showing 1. two chambers, 2. one micrometer, 3. suction outputs and gas inputs, 4. transducer pin without a jaw attached.*

Wire myography was performed on excised femoral arteries in all experimental cohorts, with uterine artery myography also being performed in the E19 cohort (see Section 3.2.6). Wire myography was performed using a 420A Dual Wire Myograph system from Danish Myo Technology (DMT, Denmark) (Figure 2.2). The procedure for excising and mounting femoral arteries on the system is described below, the equivalent procedure for uterine arteries is described in Chapter 3 (Section 3.2.6). The normalisation protocol described in Section 2.3.2 was applied to all vessels. Following normalisation and the potassium curve, different protocols were used in each cohort to generate dose-response curves to different vasomotive agents and these are described in each chapter.

2.3.1 Femoral artery excision and mounting

The femoral artery is a continuation of the external iliac artery that supplies oxygenated blood to the lower limbs. In order to excise the femoral artery, the euthanised mouse was first pinned in the supine position with its legs extended and the skin between the knee and groin removed. The femoral vein and nerve that run alongside the artery were separated from the artery by blunt dissection, and then any connective and adipose tissue around the area was gently removed.

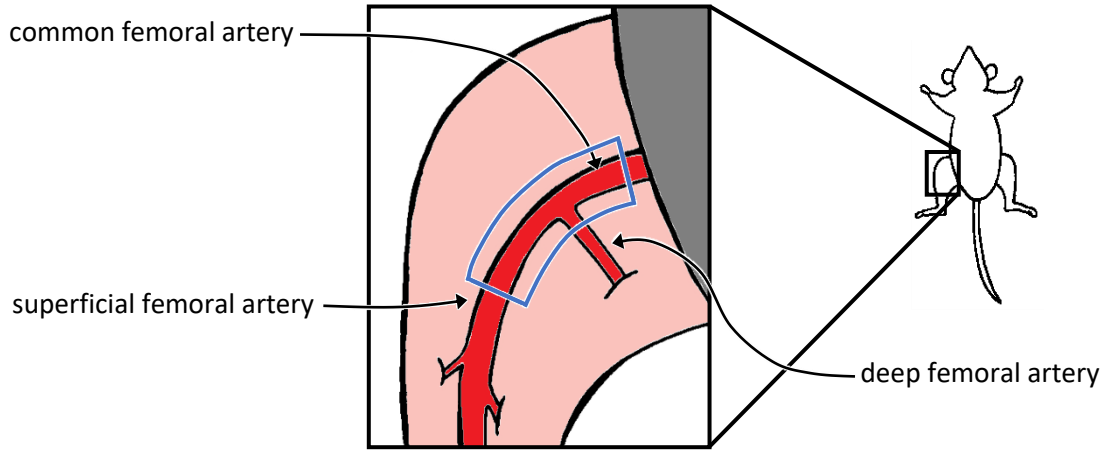


Figure 2.3: Diagram illustrating segment of femoral artery excised for wire myography.

Taking care not to clamp the artery itself with forceps at any time, it was then excised and placed in physiological Krebs-Hensleit buffer (Krebs) (mM: NaCl 118.5, NaHCO3 25.0, KCl 4.7, MgSO4·7H20 1.2, KH2PO4 1.2, CaCl2 2.5, glucose 2.8). The segment excised (Figure 2.3) included the branchpoint of the deep and superficial femoral artery, so that this piece of vessel could be held by the forceps during dissection and mounting without risk of damage to the main section of the femoral artery. After any remaining adipose tissue was removed, a segment approximately 2 mm in length was mounted on two 40 µm diameter stainless steel wires in the myograph chamber according to the protocol illustrated in Figure 2.4. The myograph chamber was filled with Krebs kept at 37°C and aerated with a gas mixture of 95% O_2 and 5% CO_2. One jaw of the myograph was connected to a micrometre screw and the other to a force transducer which was connected to a data acquisition system (LabChart 8, ADInstruments), such that the magnitude of any force exerted on the wire by the vessel could be recorded. When only femoral artery myography was being performed, both femoral arteries were excised and mounted across the two chambers of the myograph.

The first wire is secured to one jaw and the vessel segment threaded on to the wire (A). The wire is gently pulled taut and the other end of the wire secured to the jaw (B). The second wire is then threaded through the vessel (C), taking care not to damage the endothelium. The jaws are then closed (D) and the second wire secured (E). Finally, the jaws are moved apart so that the wires are barely not touching, and no negative force is recorded on the force transducer.

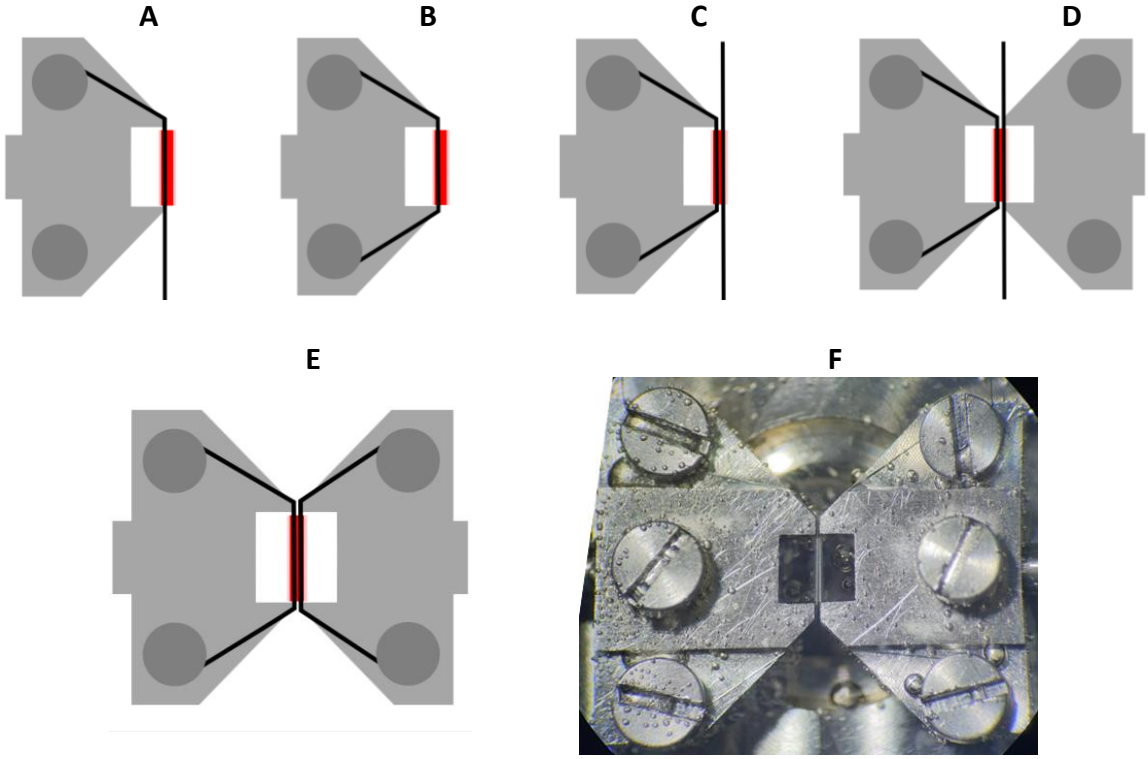


Figure 2.4: Mounting a vessel on the myograph. *Basic protocol for mounting any arterial segment on the 420A wire myograph (A-E), and a photograph of a mounted femoral artery segment (F).*

2.3.2 Vessel normalisation and potassium curve

After mounting, vessels were left to rest for a stabilisation period of at least 20 minutes before being subjected to a normalisation protocol consisting of alternating exposure to a modified Krebs buffer containing 64 mM K^+ (in exchange for Na^+ to maintain osmolality), which depolarises the vascular smooth muscle cells (VSMCs) such that they contract, resulting in vessel constriction, and the jaws being moved apart. This process was repeated until the magnitude of contraction elicited by exposure to 64 mM K^+ plateaued. This occurred when the wires were typically 250 – 300 µm apart for femoral arteries, and 175 – 225 µm apart for uterine arteries. The distance between the two wires was increased by no more than 25 µm at a time so as to avoid damaging the vessel. A potassium curve (K^+ Curve) (Figure 2.5(A)) was then run, in which vessels were exposed to different isosmotic Krebs solutions of increasing K^+ concentration (16-125 mM). Any vessels that did not generate a maximal contractile force of 1 mN or more in response to K^+ were rejected.

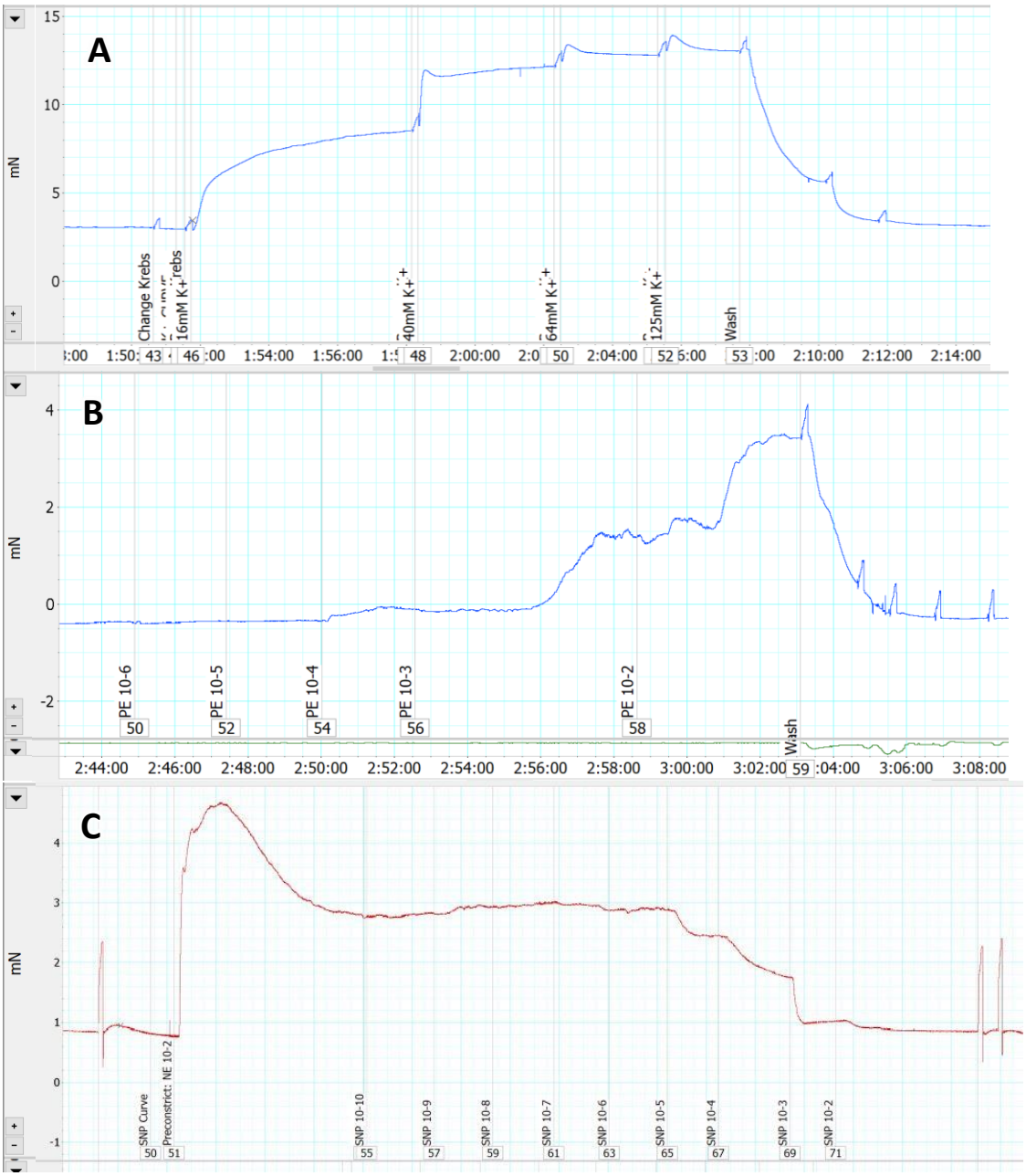


Figure 2.5: Representative images of LabChart traces during myography. *A K^+ curve (A), vasoconstrictor (phenylephrine) curve (B) and vasodilator (sodium nitroprusside) curve (C).*

Cumulative concentration dose-response curves to different vasomotive agents were then performed, the specifics of which are discussed in the relevant chapters. As well as a K^+ Curve, Figure 2.5 shows representative traces from LabChart of a vasoconstrictor curve, in this case the selective alpha-1-adrenoreceptor agonist phenylephrine (PE), and a vasodilator curve, in this case the NO-donor sodium nitroprusside (SNP). When running a vasodilator curve, vessels were first pre-constricted by exposure to the non-specific adrenoreceptor agonist noradrenaline (NA). Vessels were washed three times with Krebs at the end of any curve in order to wash away any residual agent in the chamber, and then allowed to rest for at least 15 minutes between each treatment.

Once all dose-response curves had been run, vessels were allowed to rest for a further 20 minutes and then exposed to isosmotic Krebs solutions containing first 64 mM K^+ and then 125 mM K^+. This was done in order to check that the contractile function of the vessel had not deteriorated significantly over the course of the experiment.

2.3.3 Myography data analysis

Once all myography data was collected for a given cohort, all files were blinded and randomised. Vessel-response data were extracted from blinded LabChart files manually and entered into Excel. For each curve, raw tension as measured on LabChart for every drug dose was recorded at the point where the response plateaued. These measurements were then expressed relative to the baseline vessel tension for that curve.

$$\textbf{Curve baseline} = \frac{(\textbf{Resting tension before curve}) + (\textbf{Resting tension after curve})}{\textbf{2}}$$

Response magnitude for each dose during a vasoconstrictor curve was expressed both as tension change from baseline, in mN, and relative magnitude of this change compared to the maximum response from the matched vessel's K^+ Curve (%K^+).

For analysis of vasodilator curves, the magnitude of contraction elicited by the noradrenergic pre-constriction was first calculated to give a value of 0% relaxation or 100% contraction.

$$\textbf{Pre} - \textbf{constriction} = (\textbf{NA response plateau}) - (\textbf{Curve baseline})$$

Response magnitude for each dose during a vasodilator curve was then expressed relative to the pre-constriction contraction.

$$\%\ \textbf{Relaxation} = \textbf{100 x}\ \frac{(\textbf{NA response plateau}) - (\textbf{Dose plateau})}{\textbf{Pre} - \textbf{constriction}}$$

Curves were then analysed using Prism 9.0 (GraphPad). To analyse dose-response curves to vasomotive agents, curves were first graphed to examine the highest dose of each vasomotive agent to which there was no significant response in any group, and individual curves were then adjusted to use the vessel tension at this dose as the baseline measurement. Curves were analysed using mixed-effects models and a post hoc Tukey's multiple comparisons test was performed if there was a significant effect of group. In offspring cohorts, statistical analyses of dose-response curves were conducted separately in male and female offspring. If there was a significant response to the lowest dose of vasomotive agent applied, curves could not be adjusted to a consistent baseline. The greatest response amplitude to any dose of a given vasodilator was extracted in order to give a measure of maximal percentage relaxation.

Chapter 3: Maternal Adaptation to Pregnancy

3.1 Introduction

3.1.1 Blood pressure regulation

In physiological studies, researchers often consider mean arterial pressure (MAP), rather than SBP and DBP separately.

$$\mathbf{MAP} = \mathbf{DBP} + \frac{\mathbf{1}}{\mathbf{3}}(\mathbf{SBP} - \mathbf{DBP})$$

According to Darcy's Law, flow is proportional to the pressure difference between two points, and inversely proportional to the resistance between them. When applied to the circulatory system, this means that the blood flow through the systemic circulation depends on the difference between MAP and right atrial pressure, and the total resistance of the systemic vasculature. Given that under normal conditions right atrial pressure is close to 0mmHg and therefore negligible, and that blood flow through the systemic circulation is equivalent to cardiac output, MAP can be said to be determined by cardiac output (CO) and systemic vascular resistance (SVR).

$$\mathbf{MAP} = \mathbf{CO\ x\ SVR}$$

Therefore, changes in CO or SVR will result in changes to MAP.

Determinants of systemic vascular resistance

SVR is the sum of the resistance of the systemic circulation, which is primarily a function of arteriolar resistance. The variable with the greatest effect on vascular resistance is vessel diameter. Therefore, widespread changes in vessel diameter, also known as vasomotion, of arterioles changes SVR. Vasomotion consists of vasoconstriction, a decrease in vessel diameter which increases vascular resistance, or vasodilation[1], an increase in vessel diameter which decreases vascular resistance. Both arteries and arterioles consist of three layers: the tunica externa, tunica media and tunica intima (Figure 3.1). Contraction of the vascular smooth muscle cells (VSMCs) of the tunica media results in vasoconstriction, and relaxation in vasodilation. The contraction of arteriolar VSMCs and therefore SVR is centrally regulated by the sympathetic nervous system (SNS). The SNS stimulates vasoconstriction via noradrenaline (NA), which is released from sympathetic vasoconstrictor nerves and acts on alpha-1-adrenoreceptors (α_1Rs) on VSMCs to stimulate contraction (alpha-adrenergic vasoconstriction). Peripheral arterioles are subject to resting sympathetic tone; tonic activity of the vasoconstrictor nerves that innervate them, causing them to be slightly constricted at rest. This means that vasoconstriction is induced by increasing noradrenergic stimulation of VSMCs and vasodilatation by inhibiting it.

[1] Throughout this thesis, an increase in vessel diameter will be referred to as vasodilation. However, it is noted that many consider vasodilatation to be a more accurate term.

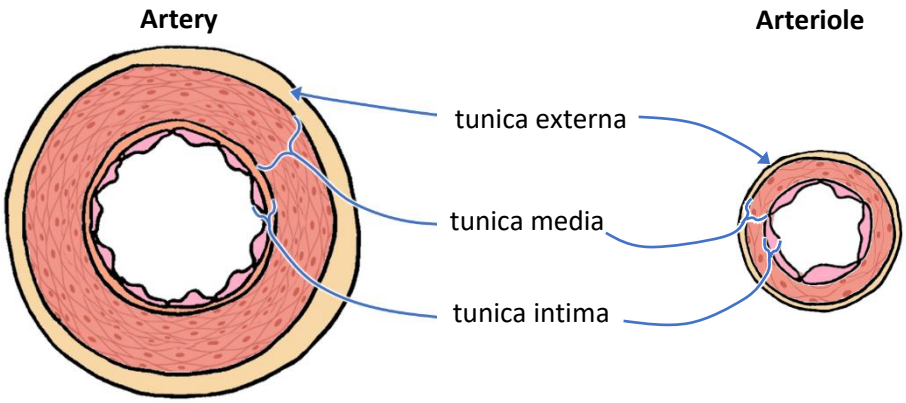


Figure 3.1: Structure of an artery and an arteriole. *Illustration adapted from BioRender.com.*

Parasympathetic nerves release acetylcholine (ACh) which causes endothelium-dependent vasodilation by binding to M_3 muscarinic cholinergic receptors (mAChRs) on the vascular endothelium, activating both nitric oxide (NO)-dependent and NO-independent pathways. The NO-dependent pathway consists of stimulating endothelial NO synthase (eNOS) to produce NO. NO is an endogenous vasodilator which then diffuses from the endothelial cells into the VSMCs, causing them to relax. NO-independent pathways of ACh-mediated relaxation involve other secondary paracrine signals, including prostaglandins and endothelial-derived hyperpolarising factors (EDHFs). However, the majority of blood vessels in the systemic circulation are not innervated by the parasympathetic nervous system (PNS). Therefore, changes in parasympathetic vasodilator nerve activity do not play a role in the regulation of SVR in the same way that those of sympathetic vasoconstrictor nerve activity do. Nevertheless, arteriolar endothelial cells play a major role in the regulation of blood pressure via the release of local vasomotive factors including NO and prostaglandins. Experimentally decreasing plasma NO levels by eNOS-inhibitor-infusion results in increased SVR and MAP[366]. This supports the idea that basal endothelium-derived NO is a major determinant of SVR and MAP. Despite a lack of PNS innervation, systemic arterioles express AChRs, and exogenous administration of ACh results in vasodilation by both NO-dependent and NO-independent pathways[367]. The role of endogenous ACh on the regulation of SVR remains unclear, although it is produced by circulating lymphocytes[368].

Determinants of cardiac output

Cardiac output (CO) is the product of heart rate (HR), the number of times the heart beats per minute, and stroke volume (SV), the volume of blood ejected from the left ventricle (LV) when the heart contracts during systole. Stroke volume is thus the difference between left ventricular end-diastolic volume (EDV) and end-systolic volume (ESV).

$$\mathbf{CO = HR \times SV}$$

This means that cardiac output can be altered by changes in heart rate and/or stroke volume.

3.1 | Introduction

Extrinsic regulation of cardiac output

The heart is innervated by both the sympathetic (SNS) and parasympathetic (PNS) branches of the autonomic nervous system. Sympathetic nerves release NA, which acts on cardiac beta-1-adrenoreceptors (β_1Rs) to increase both heart rate, a positive chronotropic effect, and cardiac contractility, a positive ionotropic effect. Increased contractility of cardiac muscle results in a greater percentage of the blood in the LV at the end of diastole being ejected during systole, an increase in ejection fraction (EF), which results in an increase in stroke volume. Cardiac β_1Rs can also be activated by circulating adrenaline, which is released from the adrenal medulla in response to sympathetic stimulation and thus represents the endocrine effector arm of the SNS. Increased SNS activity thus acts to increase cardiac output by increasing both heart rate and stroke volume. Parasympathetic nerves release ACh, which acts on cardiac M_2 mAChRs to reduce heart rate, a negative chronotropic effect. PNS activity does not affect cardiac contractility, and so increased PNS activity acts to decrease cardiac output by decreasing heart rate only.

Increasing heart rate or contractility in isolation will not result in a significant increase in cardiac output because venous collapse will limit the return of blood to the heart, known as venous return, and thus decrease EDV and stroke volume. This reveals that maximum cardiac output is limited by mean systemic filling pressure (MSFP), the blood pressure that would exist throughout the circulatory system if the heart were to stop beating and the circulating blood were to redistribute. In order to significantly increase cardiac output, MSFP must also increase. MSFP is determined by total blood volume and the mean tension in the walls of the venous system, which is increased by venoconstriction, the SNS-mediated contraction of VSMCs in the walls of the veins.

Intrinsic regulation of cardiac output

Independent of extrinsic inputs, the length of a relaxed cardiac muscle fibre affects the force it generates during contraction. This means that increased stretch of the heart in diastole as a result of an increase in LV end-diastolic pressure (LVEDP) results in increased cardiac contractility and increased stroke volume. This is known as the Frank-Starling mechanism or Starling's Law of the Heart. Therefore, as well as MSFP limiting maximum cardiac output, increasing MSFP causes an increase in cardiac output. This is because an increase in MSFP increases venous return and thus increases EDV, resulting in an increase in LVEDP and thus cardiac contractility, stroke volume and cardiac output due to the Frank-Starling mechanism. Therefore, blood volume is a major determinant of MAP. The kidneys are primarily responsible for regulating blood volume.

3.1.2 Cardiovascular adaptation to pregnancy

As introduced in Section 1.4.2, pregnancy causes significant changes to the maternal cardiovascular system (Figure 3.2). These changes must occur in order to meet the increased metabolic demands of the mother as pregnancy progresses, and to ensure adequate uteroplacental perfusion to support the healthy growth and development of the fetus.

The key haemodynamic changes that occur during normal pregnancy are a decrease in systemic vascular resistance, a transient decrease in arterial blood pressure, and increases in blood volume and cardiac output[369]. The first change reported is an increase in heart rate, which starts as early as between the second and fifth weeks of gestation and continues into the third trimester[370,371]. This increase in heart rate is thought to be driven by an increase in SNS activity. Stroke volume also increases as pregnancy progresses, albeit not as early as heart rate[371]. The increase in stroke volume is primarily driven by an increase in blood volume. Plasma volume begins to increase after six weeks of gestation in humans[372], reaching an increase of more than 10% above pre-pregnancy levels by 12 weeks and as much as a 40% increase by 24 weeks of gestation. This increase in plasma volume is mediated by increased activation of the renin-angiotensin-aldosterone system[373] related to increased SNS activity. The increase in blood volume results in an increase in MSFP, and therefore an increase in EDV[374] and subsequently stroke volume. The increases in both heart rate and stroke volume result in an increase in cardiac output. Cardiac output increases progressively throughout the first and second trimesters, reaching almost 50% more than pre-conception levels by 24 weeks of gestation[371]. The increase in cardiac output is associated with physiological cardiomyocyte hypertrophy[369], resulting in an increase in maternal LV mass during pregnancy[374]. The expansion of plasma volume is accompanied by a disproportionately smaller increase in the number of circulating red blood cells (RBCs), resulting in relative haemodilution and a decrease in haematocrit[369], the percentage of total blood volume made up of RBCs.

In non-pregnant individuals, a progressive increase in cardiac output should drive an increase in MAP, however, this is not what is seen in pregnancy. Arterial blood pressure decreases in early to mid-pregnancy. Decreases in both DBP and MAP are widely reported, while different studies have found that SBP decreases to a similar magnitude as DBP and MAP[375], that it decreases but not to the same extent as DBP and MAP[376], or that it does not change at all[371]. Arterial blood pressure reaches its nadir during the second trimester, and then increases again from the third trimester until delivery[377]. The decrease in arterial blood pressure in the presence of an increase in blood volume and cardiac output is due to a large decrease in SVR. SVR decreases at the same time as cardiac output is increasing, and can drop to 40% of pre-conception levels by the middle of the second trimester[377].

3.1 | Introduction

A major contributor to the decreased vascular resistance is the development of the low resistance uteroplacental unit. However, in humans, vascular resistance begins to drop as early as five weeks of gestation, several weeks before placentation is fully established, thus suggesting that other factors underlie the initial drop in SVR. Indeed, significant systemic vasodilation occurs during early pregnancy, resulting in a drop in SVR. This decrease in SVR is due to both an increased release of and vascular sensitivity to vasodilators, and an increased vascular resistance to vasoconstrictors[378]. A key driver of the systemic vasodilation in early pregnancy is the hormone relaxin, which is released from the corpus luteum. Serum relaxin concentration rapidly increases in early pregnancy and peaks at the end of the first trimester[379]. Relaxin induces endothelium-dependent vasodilation[380], and has also been found to antagonise the action of the vasoconstrictor endothelin-1 (ET-1)[381]. Pregnant women are also resistant to the vasoconstrictive effects of both NA[382] and angiotensin II (AngII)[383]. Increases in circulating NO are reported in pregnant rodents[384] and sheep[385], and this increase in NO bioavailability may contribute to low systemic vascular resistance during pregnancy in these species. However, whether NO bioavailability changes in human pregnancy is unclear[386].

While the earliest decreases in SVR during pregnancy are a result of systemic vasodilation, the remodelling of the spiral arteries and subsequent development of the low resistance uteroplacental unit contribute to the continued reduction in SVR as gestation progresses. The spiral arteries are tertiary branches of the uterine arteries that supply blood to the upper layer of the endometrium[387], and therefore to the placenta during pregnancy. In non-pregnant individuals, the spiral arteries are relatively narrow, coiled blood vessels[387] with significant SNS innervation[388] and the blood flow through the upstream uterine arteries is highly pulsatile[389]: high during systole and almost zero during diastole. The spiral arteries are extensively remodelled during pregnancy. This remodelling is stimulated by a combination of hormonal changes, signalling by immune cells in the maternal decidua and trophoblast cells from the placenta migrating and invading the maternal uterine wall[390]. The remodelling involves a reduction in sensitivity to vasoconstrictors[391] and the loss of smooth muscle in the vessel walls[388], with subsequent enormous vasodilation such that uteroplacental resistance plummets. This results in increased blood flow through the uterine arteries. Indeed, uterine artery blood flow increases from around 50 mL/min in a non-pregnant woman, to nearly 1 L/min in late pregnancy[392]. This increased blood flow through the uterine arteries includes an increased systolic flow, however, more importantly, the drop in uteroplacental resistance results in a high velocity of blood flow during diastole such that there is continuous blood flow through the uterine arteries during both systole and diastole[389]. This results in the blood flow through the uterine arteries becoming less pulsatile. The pulsatility of uterine artery blood flow is described by calculation of the pulsatility index (PI). Therefore, as gestation progresses, uterine artery PI decreases.

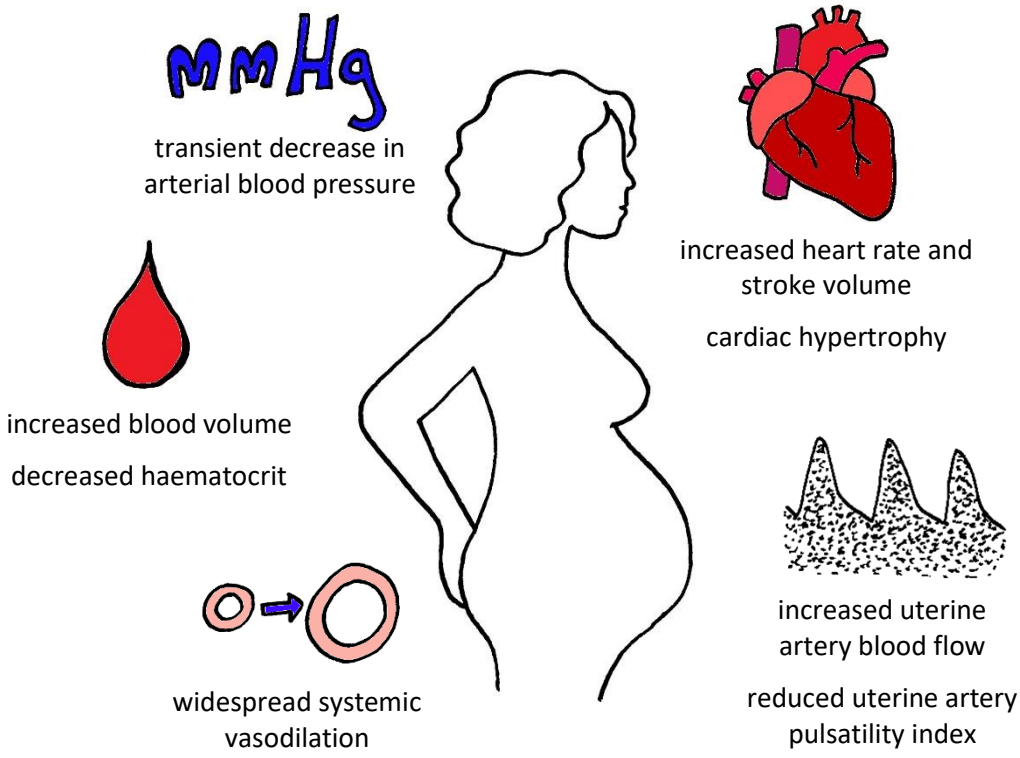


Figure 3.2: Maternal cardiovascular adaptations to pregnancy.

3.1.3 Obesity and cardiovascular adaptation to pregnancy

In non-pregnant individuals, obesity is associated with increased SNS activity[393], and one result of this is fluid retention which acts to increase blood volume. Indeed, blood volume is greater in non-pregnant overweight and obese individuals than in lean people[149], resulting in an increase in stroke volume[394]. The increased blood volume is not typically proportional to the increased body weight, resulting in reduced blood volume per unit body weight in obese people[395]. This is because fat mass is relatively under perfused compared to lean mass. Blood volume of obese women increases during pregnancy[396]. However, obese pregnant women have been found to have a similar total blood volume as lean pregnant women[396], suggesting that plasma volume expansion in response to pregnancy is impaired in obese women. In support of this, one study found that there was no difference in stroke volume between lean pregnant women and morbidly obese pregnant women[397]. Obese people also typically have elevated resting heart rate both before[398,399] and during pregnancy[397,400]. However, both obesity[401] and diabetes[402] have been found to result in a reduced increase in heart rate during pregnancy. Together, these data suggest that overweight and obese women enter pregnancy with an already elevated baseline SNS activity, increased blood volume and increased heart rate compared to lean women, and that this may result in a lesser capacity for cardiovascular adaptation to pregnancy.

Women with a pre-pregnancy BMI >25 kg/m^2 are more likely to enter pregnancy with elevated arterial blood pressure[149], and have higher blood pressure at any point during pregnancy[403,404]. Increases in either SBP or DBP in the first trimester are associated with an increased risk of going on to develop hypertensive disorders of pregnancy[405], therefore, as discussed in Section 1.4.2, obesity is associated with an increased risk of gestational hypertension and pre-eclampsia[406]. Moreover, in pregnancies not complicated by hypertensive disorders, several studies have found that the transient drop in arterial blood pressure seen in a normal pregnancy is not observed in obese pregnancy[401,407], and that obese pregnant women have higher SVR than lean women[404]. This suggests that obesity impairs vascular adaptation to pregnancy. In non-pregnant individuals, obesity is associated with reduced systemic vascular vasodilation in response to both ACh, which is indicative of endothelial dysfunction, and the NO-donor sodium nitroprusside (SNP), which is indicative of vascular dysfunction at the level of the VSMCs[408], and one study found the same impairment in vasodilatory response to ACh and SNP in obese pregnant women[409]. Obesity in non-pregnant women is also associated with reduced NO bioavailability[410] and the increased SNS activity in obese people promotes systemic vasoconstriction. Therefore, obesity is associated with vascular dysfunction which may contribute to impaired vascular adaptation to pregnancy. As well as impacting the adaptation of systemic vasculature to pregnancy, obesity impairs spiral artery remodelling during pregnancy[411] and the development of the low resistance uteroplacental unit. This results in the physiological drop in PI over the course of gestation being reduced in overweight and obese women[404,412].

3.1.4 Effects of exercise during pregnancy

Regular endurance exercise training in humans results in increased blood volume and stroke volume, which result in an increase in cardiac output in spite of a concurrent decrease in resting heart rate. It also stimulates physiological cardiac hypertrophy, resulting in increased heart weight. Both regular exercise and pregnancy therefore result in significant adaptive changes in cardiovascular structure and function, with some of these changes acting in the same direction and others not. For example, although regular exercise training is associated with decreased resting heart rate, pregnancy is associated with an increased heart rate. These two opposing directions of change have been found to modulate one another, with women who continue aerobic exercise training during pregnancy having lower resting heart rates than matched sedentary controls[413]. Stroke volume, on the other hand, is increased both by exercise training and during normal pregnancy, and women who continue aerobic exercise training during pregnancy have been found to have an augmented pregnancy-associated increases in stroke volume compared to matched sedentary controls[413].

However, this reflects the cardiovascular phenotype of lean women who already engage in regular exercise training before pregnancy, whereas this chapter will investigate how the introduction of an exercise intervention during pregnancy in otherwise sedentary overweight and obese women affects cardiovascular function.

Wolfe et al.[414] used echocardiography to investigate the impact of moderate-intensity cycling sessions three times per week throughout the second and third trimester on cardiac structure and systolic function in previously sedentary lean pregnant women. This study found no significant difference in either ventricular dimensions or resting heart rate between the exercising and sedentary groups. This suggests that any effects of the exercise training were likely subtle and thus masked by the large physiological changes in response to pregnancy. However, while the exercise intervention did not result in any differences in resting heart rate, women in the exercise group had a smaller increase in heart rate in response to moderate or intense exercise at the end of the third trimester than sedentary women, which is indicative of improved aerobic fitness. Other studies have also found that new-onset maternal exercise training during pregnancy results in increased aerobic capacity[415], another indicator of improved aerobic fitness. No studies assessing cardiac structure and function with an exercise intervention during obese pregnancy using echocardiography were found in literature searches. However, as described in Section 1.7.1, both the DALI[309] and IMPROVE[311] studies found an improvement in maternal aerobic fitness with an exercise-only intervention during obese pregnancy, even in the absence of a reduced risk of negative obstetric outcomes. These studies suggest that an exercise intervention during pregnancy can improve maternal aerobic fitness and therefore benefit maternal health in both lean and obese women. While new-onset exercise training in pregnancy has not been found to alter baseline cardiac function during pregnancy in lean women, this does not exclude the possibility that it may benefit obese women. Given that maternal pre-pregnancy obesity impairs normal cardiovascular adaptation to pregnancy, an exercise intervention may have a greater effect on maternal cardiovascular function during pregnancy in the context of obesity.

3.1.5 Exercise during obese pregnancy: evidence from animal models

Human trials of exercise intervention during obese pregnancy offer conflicting results on maternal and neonatal outcomes. Of the RCTs discussed in Section 1.7.1, some found that maternal exercise reduces GWG[308,313], while others found no effect on maternal body weight[310,315]. Some trials found decreased incidence of GDM, with[313] or without[315] reductions in GWG, while others found no difference in GDM incidence between intervention and control groups[308,310,311]. The potential benefit of exercise intervention during pregnancy on the incidence of hypertensive disorders of pregnancy is also unclear.

3.1 | Introduction

Despite the ETIP trial finding that an exercise intervention during obese pregnancy reduced SBP in late gestation[316], none of the individual RCTs discussed found that maternal exercise decreased the incidence of hypertensive disorders of pregnancy. However, a large systematic review and meta-analysis of 106 studies, including more than 270,000 women, found that exercise during pregnancy does reduce the incidence of both GDM and pre-eclampsia[416].

Animal models of exercise intervention during pregnancy enable faster and more comprehensive study of the effects of exercise intervention on maternal cardiometabolic function, as well as easier postnatal follow-up of mothers and offspring. Numerous models of maternal exercise intervention during obese pregnancy have been developed in rodents. Different protocols for maternal exercise have been used in different studies, including voluntary wheel running[417–420], in which a free-spinning wheel is put into the animal's home cage, being run on a treadmill[421], and swimming[422]. Studies using animal models have found that maternal exercise during pregnancy can prevent the negative effects of exposure to maternal obesity during pregnancy on offspring health, including reducing adiposity[419,422,423] and improving glucose tolerance[420,422–424].

The Ozanne group has developed a treadmill-running exercise intervention using our mouse model of maternal diet-induced obesity. While it is more labour-intensive for researchers and may be more stressful for the mice, treadmill running has the advantage over voluntary wheel running of controlling the amount of exercise that different dams do. Previous studies using this exercise intervention have found that while it does not alter maternal body weight or fat mass[292,301], it improves maternal insulin sensitivity in late gestation[292]. Therefore, this protocol of treadmill running is considered equivalent to a moderate aerobic exercise intervention in humans, similar to those discussed in Section 1.7.1. It is not such an intense intervention that it results in changes in maternal body weight, but it is sufficient to improve metabolic fitness. It also rescues the increased placental hypoxia and lipid deposition seen in obese pregnancy[292]. Follow-up studies have shown that maternal exercise prevents the programming of increased insulin resistance[292], and pathological cardiomyocyte hypertrophy and the associated cardiac dysfunction[301] in 8-week-old male offspring of obese dams. However, maternal exercise did not rescue the programmed increase in SBP in 8-week-old offspring of obese dams[301]. Therefore, this intervention has been shown to improve the metabolic health of both obese dams and their offspring, and the cardiac structure and function of their offspring. However, the cardiovascular phenotype of the dams in this model has yet to be characterised, either with or without this exercise intervention. Given that obesity impairs maternal cardiovascular adaptation to pregnancy in humans, and that this likely contributes to altered fetal development and thus to poor offspring health, it is paramount that we investigate maternal cardiovascular function in our model.

3.1.6 Chapter aims

1) To establish if changes in heart rate and SBP are seen in early pregnancy in lean dams in our mouse model, and whether this is altered by maternal obesity.
2) To assess maternal cardiac and systemic vascular function during pregnancy in lean and obese dams for the first time in this model.
3) To investigate the effect of maternal obesity on both uterine artery blood flow *in vivo* and uterine artery reactivity *ex vivo.*
4) To investigate whether a maternal exercise intervention offsets any negative effects of obesity on maternal cardiovascular adaptation to pregnancy.

3.2 Methods

3.2.1 Animal Model

Data in this chapter come from female mice undergoing their second pregnancy (P2), with dams being studied from before they were mated (PreP2) until the morning of gestational day 19 (E18.5). Three groups of dams were generated: control dams ('Con), obese dams ('Ob) and obese-exercised dams ('Ob-Ex). All animals had undergone a first pregnancy (see Section 2.1) and thus were considered PreP2.

All PreP2 females fed a control diet from weaning became 'Con dams and were mated for P2 for up to three attempts from one week after weaning of their first litter. Once PreP2 females fed the HF-HS obesogenic diet reached a pre-determined threshold of obesity of 10 g body fat as measured by TDNMR (Bruker LF90II), they were randomly assigned to either 'Ob or 'Ob-Ex. Due to the time taken for PreP2 females fed the obesogenic diet to reach this body fat threshold, 'Ob and 'Ob-Ex females were an average of approximately 16 days older than 'Con females when they plugged for P2 (average age at P2 plug was 123, 122 and 107 days old respectively).

Upon reaching 10 g body fat, 'Ob dams were mated for P2 for up to three attempts. After reaching 10 g body fat but before mating for P2, 'Ob-Ex dams were subjected to five days of training to run on the mouse treadmill, as described in Section 3.2.2. The day after the final training day, 'Ob-Ex dams were mated for P2 for up to four days. 'Ob-Ex dams were only allowed one mating attempt in order to avoid confounding effects of different durations of the exercise intervention. Seven obese-exercised females that were recorded as plugged were found to be not pregnant at E18.5. However, they completed the entire experimental protocol and were culled 18 days after a plug was noted and thus were considered matched non-pregnant obese-exercised females.

A subset of 'Con and 'Ob PreP2 animals were used to generate offspring. Data from these dams and their offspring will be presented in Chapter 4.

3.2.2 Treadmill running of obese-exercised females

Once HF-HS diet-fed females assigned to the 'Ob-Ex dam group reached 10 g body fat as measured by TDNMR and before they were mated for P2, they were trained to run on a treadmill (Exer 3/6, Columbus Instruments). Training consisted of five days over which both the speed and duration of running was gradually increased until mice were comfortably running the full protocol on the fifth training day (see Table 3.1). During training, mice were housed in pairs. The day after mice completed the training protocol, they were mated for P2.

	Time at each Speed and Total (Cumulative) Time (minutes)									
	Train Day 1		Train Day 2		Train Day 3		Train Day 4		Train Day 5 / Full Protocol	
Speed (m/min)	Time	Total	Time	Total	Time	Total	Time	Total	Time	Total
5	1	1	1	1	1	1	0.5	0.5	0.5	0.5
7.5	13	14	18	19	1	2	1.5	2	1.5	2
10	-	-	-	-	16	18	3	5	1	3
12.5	-	-	-	-	-	-	10	15	14	17
10	-	-	-	-	-	-	3	18	1	18
7.5	-	-	-	-	1	19	1.5	19.5	1.5	19.5
5	1	15	1	20	1	20	0.5	20	0.5	20

Table 3.1: Exercise protocol with speeds and durations of treadmill running.

From the day of plug (E1) up to and including the 17th day of gestation (E17), obese-exercised females ran the full protocol five days per week (Monday to Friday). The exercise protocol was completed in red light and began no earlier than 6pm each evening, with mice being returned to their home cages without being re-exposed to white light until the next morning. In the event that mice were not able to run at the maximum speed without stopping, the treadmill speed would be transiently decreased and gradually increased so that the mouse would run for the full 20 minutes at the highest speed that they were capable of achieving.

3.2.3 Non-invasive blood pressure measurement

Blood pressure was measured in PreP2 dams once they reached the relevant threshold for P2 mating and prior to starting treadmill training or being paired with a male. As described in Section 2.2, blood pressure measurements were made in the conscious animal using non-invasive restraint tail cuff plethysmography (BP-200 system, Visitech). PreP2 dams were subjected to at least two days training and a minimum of 10 SBP and heart rate measurements were recorded on the measurement day. Pregnant dams also had SBP and heart rate measurements recorded at E5 and E10. All sessions were performed at the same time of day, between 17:00 – 18:30, to control for the natural circadian rhythm of blood pressure.

3.2.4 TDNMR and fasted blood sample

Dam body composition was measured by TDNMR (Bruker LF90II) on the day of plug (E0.5) and the morning of the 18th day of gestation (E17.5). Immediately after the TDNMR measurement was made on E17.5, the animal was subjected to a four-hour fast. At the end of the fast, a nick was made at the end of the tail, and blood was collected for glucose measurement (AlphaTRAK2, Zoetis, USA).

3.2.5 Echocardiography and uterine artery doppler

In vivo cardiac and uterine artery function were assessed by ultrasound (Vevo 3100 system with MX400 probe, Fujifilm Visualsonics) under general anaesthesia. On the morning of the 19th day of gestation (E18.5), pregnant mice were anaesthetised by inhalation of 2% isofluorane in O_2. Once unconscious, mice were placed in the supine position on a heated platform and anaesthesia maintained by inhalation of between 1.5-2% isofluorane with an O_2 flow rate of 0.6 - 0.8 L/min through a nose cone. Real time recordings of ECG and respiration were made by placing the mouse's paws on electrode plates, and body temperature was monitored using a rectal probe. Body temperature was maintained between 36.5-37.5^{o}C. Lubricant was placed on the mouse's eyes to ensure they did not dry out and hair on the chest and abdomen were removed using a depilating cream.

Images of the left ventricle (LV) of the maternal heart were obtained in the parasternal long-axis view (PSLAX) and apical four-chamber view (Apic-4). Mitral valve (MV) flow was recorded using Pulsed Wave (PW) Doppler in Apic-4. Pulmonary artery (PA) flow was recorded using PW Doppler in a modified Apic-4 view and also in the parasternal short-axis view (PSAX). Flow through both uterine arteries was also recorded using PW Doppler. How each of these views were obtained, representative images from them and what measurements were taken and calculated from each are described below.

Data from recorded clips was extracted using VevoLAB software version 5.6.1 (Fujifilm Visualsonics). A minimum of nine cardiac cycles were analysed for each measurement in every animal. In B-mode, measurements were made across multiple clips in order to achieve this, while in all other modes multiple measurements were made per clip, avoiding breaths. All files were blinded and randomised prior to image analysis.

<u>PSLAX B-mode</u>

To obtain the PSLAX view, the ultrasound probe was placed so that both the apex of the heart and the left ventricular outflow tract (LVOT) were in the plane of the image. Cineloops of beating hearts were obtained in PSLAX using 2D B-mode imaging (Figure 3.3(A)) and the manual LV trace tool was used to outline the LV lumen. For the LV trace, marker points were placed at either side of the LVOT and at the apex, after which the endocardium was traced. In a given clip, this was done at end-diastole (Figure 3.3(B)) and end-systole (Figure 3.3(C)) for as many consecutive cardiac cycles as possible between two breaths. The inner diameter and area of the LV lumen were then used to calculate systolic parameters including stroke volume and ejection fraction (Table 3.2 and Appendix 1).

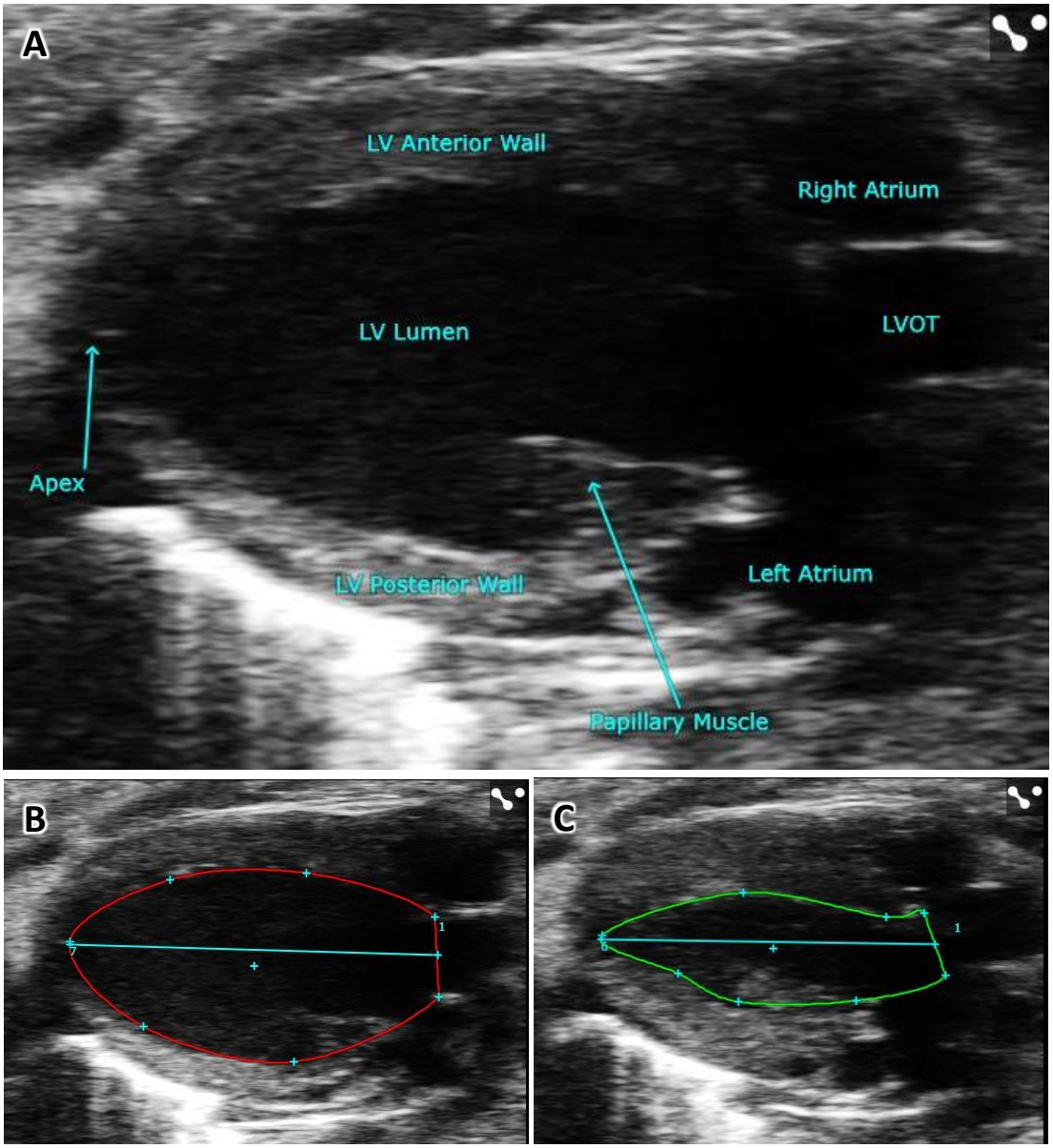


Figure* 3.3*: PSLAX B-mode analysis. *Annotated left ventricle (LV) image in B-mode (A). B-mode images at end-diastole (B) and end-systole (C) showing manual LV trace outlines (red and green) and LV endocardial length measurements (light blue).*

Longitudinal fractional shortening (FS) is the percentage change in the length of the LV between diastole and systole. In order to assess longitudinal FS, the left ventricular endocardial length (LVEnL), the distance between the apex and the mid-point of the LVOT opening, was measured at end-diastole and end-systole in B-mode (Figure 3.3(B,C)).

PSLAX M-mode

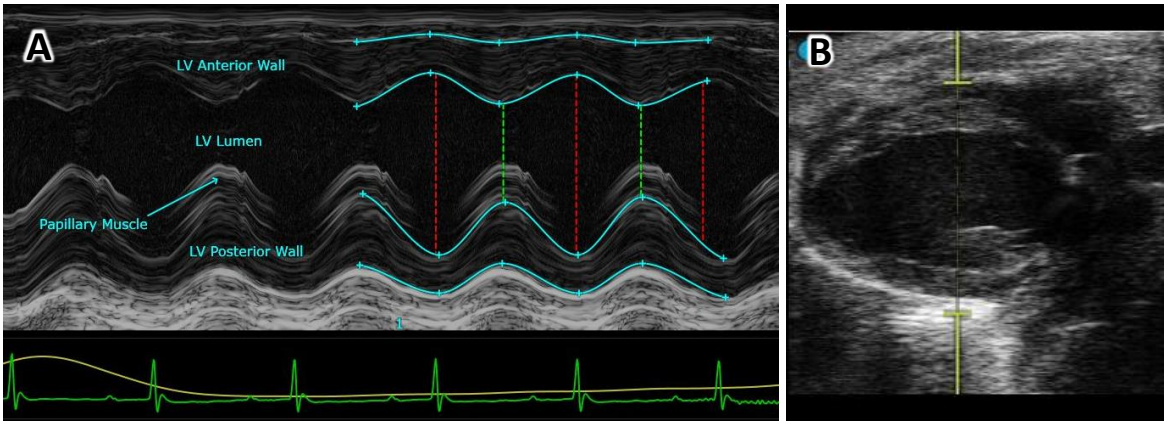


Figure* 3.4*: PSLAX M-mode analysis. *Annotated representative M-mode clip (A); blue lines show manual LV trace while red and green lines indicate points of end-systole (green) and end-diastole (red). Physiological traces shown below M-mode clip: respiration in yellow and ECG in green. Corresponding B-mode image (B) with yellow line indicating M-mode position.*

Cineloops recorded in M-mode have a higher temporal resolution than B-mode clips. However, measurements must be perpendicular to the LVOT-apex axis and consistently taken at the mid-point of the LV in order to produce accurate data that can be compared between animals (Figure 3.4(B)). Measurements were once again obtained by using the manual LV trace tool (Figure 3.4(A)). In M-mode this involves tracing the upper and lower boundaries of each the anterior and posterior LV walls over as many consecutive cardiac cycles as possible between two breaths, resulting in the measurement of LV wall widths.

Apic-4 mitral valve flow

The Apic-4 view was obtained by placing the probe on the abdomen such that the image plane is oriented cranially, looking up through the heart with the apex closest to the probe and the atria furthest away (Figure 3.5(A)). Colour Doppler was then be used to optimise placement of the probe, such that LV filling through the MV from the left atria was clearly be seen during diastole (Figure 3.5(B)) with no backflow into the atria during systole (Figure 3.5(C)). The PW Doppler measurement was taken at the point where the flow was greatest. The resulting waveform is shown in Figure 3.5(D)).

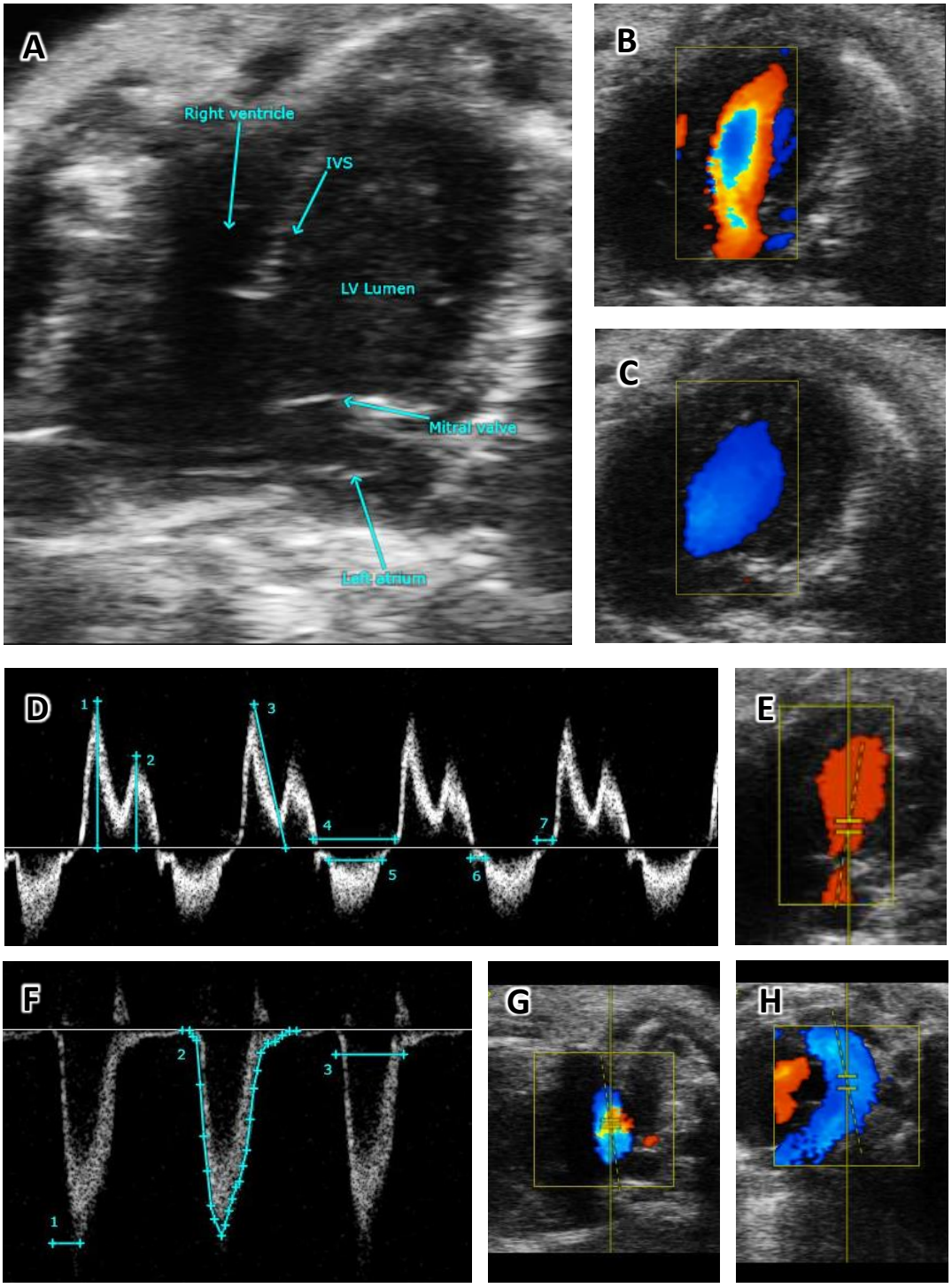


Figure* 3.5*: Apic-4 and PSAX analysis of mitral valve (MV) and pulmonary artery (PA) flow.
Labelled Apic-4 B-mode image of the heart (A), with colour Doppler images of left ventricle (LV) during diastole (B) and systole (C). Representative MV PW Doppler trace (D) annotated for 1. E peak, 2. A peak, 3. MV deceleration time, 4. Non-filling time, 5. Aortic ejection time, 6. Isovolumetric contraction time and 7. Isovolumetric relaxation time. Corresponding colour Doppler image (E) with yellow gates indicating MV flow measurement position. Representative PW Doppler trace of PA (F) showing annotations of 1. PA acceleration time, 2. PA velocity time integral and 3. PA ejection time. Corresponding colour Doppler images in Apic-4 (G) and PSAX (H) with yellow gates indicating PA flow measurement position. The red area in (H) is the beginning of the aortic arch, and you can see where the PA branches and one branch runs down left from the measurement point.

From the MV PW Doppler waveform (Figure 3.5(D)), the maximum velocity of the E peak and A peak were measured. The E peak represents the maximum velocity of blood flowing through the MV into the LV by passive filling during early diastole and therefore it is affected by LV filling pressures, and the rate of relaxation and compliance of the LV. The A peak represents the maximum velocity flow in late diastole caused by atrial contraction and is therefore affected by atrial contractility and LV compliance. The ratio between the E and A wave peaks (E:A) is a commonly described measurement of diastolic function. A series of time measurements were also made, including the LV non-filling time (NFT) and its components: the isovolumetric contraction time (IVCT), aortic ejection time (AET) and isovolumetric relaxation time (IVRT). IVCT is the time between the MV closing and the aortic valve opening, AET is the time that the aortic valve is open and blood is being ejected from the LV into the aorta, and IVRT is the time between the aortic valve closing and the MV opening. An increase in IVRT with no change in LV filling pressures suggests prolonged (and likely impaired) LV relaxation, but an increase in filling pressure will result in a decrease in IVRT. The downward slope of the trace from the E peak was traced to calculate the MV deceleration rate and deceleration time. The MV deceleration time is the time it takes for the pressure in the left atrium and LV to equilibrate.

PSAX pulmonary artery flow

The PSAX view was obtained by rotating the probe 90^{o} the position used to obtain the PSLAX view. The probe was then moved cranially until the PA came into view before it was crossed over by the aortic arch. Colour Doppler was used to guide placement of the probe and the PW Doppler measurement was taken in the centre of the flow, immediately before the PA bisected to form the left and right pulmonary arteries (Figure 3.5(G)). Minor adjustments were made to obtain a trace with minimal turbulent flow. This generated the waveform in Figure 3.5(F), from which PA ejection time (PET) and PA acceleration time (PAT) were measured. PET is the total time that blood is being ejected from the right ventricle through the pulmonary artery, while PAT is the time between the pulmonary valve opening and the blood being ejected from the right ventricle reaching peak velocity. A decrease in the ratio of PAT:PET is associated with increased PA blood pressure. The PW Doppler trace was then traced to calculate the PA velocity time integral (VTI), from which the mean pressure gradient and mean and peak velocity of flow through the PA were calculated.

Apic-4 pulmonary artery flow

From the position in which MV flow was recorded in Apic-4, the probe was moved cranially until the PA came into view (Figure 3.5(H)). PA flow was recorded and annotated as described above.

Uterine artery flow

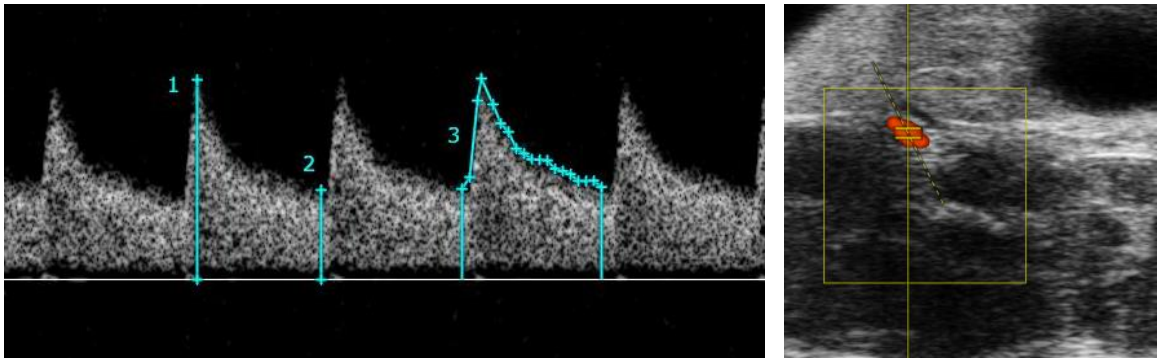


Figure* 3.6*: Uterine artery (UtA) PW Doppler analysis. *Representative PW Doppler trace of UtA (A) with annotations for 1. Peak systolic velocity, 2. End diastolic velocity and 3. UtA velocity time integral. Corresponding colour Doppler image (B) showing flow through the UtA in red, with yellow gates indicating UtA flow measurement position. The circular shadow seen in the top right corner of (B) is the mouse's bladder.*

In order to locate the uterine arteries, the probe was placed horizontally across the mouse level with the groin and then gradually moved cranially. When this was done, the bladder appeared clearly as a large, black circle, which was used as a landmark. Moving cranially then brought the bottom of the abdominal aorta into the image plane. From here, the probe was slowly moved caudally so that the abdominal aorta was be followed down until it split into the two common iliac arteries. Then, in order to locate each uterine artery (UtA) in turn, one common iliac artery was followed until the UtA branched off and looped back on itself such that the blood through it flowed towards the probe, which was shown by Colour Doppler (Figure 3.6(B)). The PW Doppler recording was then taken parallel to the flow through the UtA, and from the resulting waveform (Figure 3.6(A)) peak systolic velocity (PSV) and end-diastolic velocity (EDV) were measured, and the waveform was traced to calculate the VTI. These measures are then used to calculate the pulsatility index (PI) and resistive index (RI) (Table 3.2 and Appendix 3.1). The RI is another parameter used to assess uterine artery blood flow and it is closely related to the PI.

View	Imaging Mode	Primary Measurement	Secondary Measurement
PSLAX	B-Mode LV Trace	LV inner diameter (LVID) (systole and diastole) LV area (systole and diastole)	*LV volume (systole and diastole) *Stroke volume (SV) Cardiac output (CO) *Ejection fraction (EF)
	B-Mode Linear	Endocardial length (LVEnL) (systole and diastole)	*Fractional shortening (FS) (longitudinal)
	M-Mode LV Trace	LVID (systole and diastole) Anterior wall width (LVAW) (systole and diastole) Posterior wall width (LVPW) (systole and diastole)	*Fractional Shortening (FS) (radial)
Apic-4	Mitral valve (MV) PW Doppler	E wave peak velocity (E wave) A wave peak velocity (A wave) MV deceleration (MV Decelerⁿ) rate and time Aortic ejection time (AET) Isovolumetric contraction time (IVCT) Isovolumetric relaxation time (IVRT) Non-filling time (NFT)	E:A ratio *Myocardial performance index (MPI)
	Pulmonary artery (PA) PW Doppler	PA acceleration time (PAT) PA ejection time (PET) PA velocity time integral (VTI)	PAT:PET ratio *Mean and Peak PA flow velocity *Mean PA pressure gradient
PSAX	PA PW Doppler	PAT, PET, and PA VTI	As above
UtA	Uterine artery (UtA) PW Doppler	Peak systolic velocity (PSV) End diastolic velocity (EDV) UtA VTI	UtA pulsatility index (PI) UtA resistive index (RI)

Table 3.2: Summary of ultrasound measurements made in each view.
*Calculations for secondary measurements are described in Appendix 3.X. Those marked with * are calculated by VevoLAB automatically*

3.2.6 Femoral and uterine artery myography

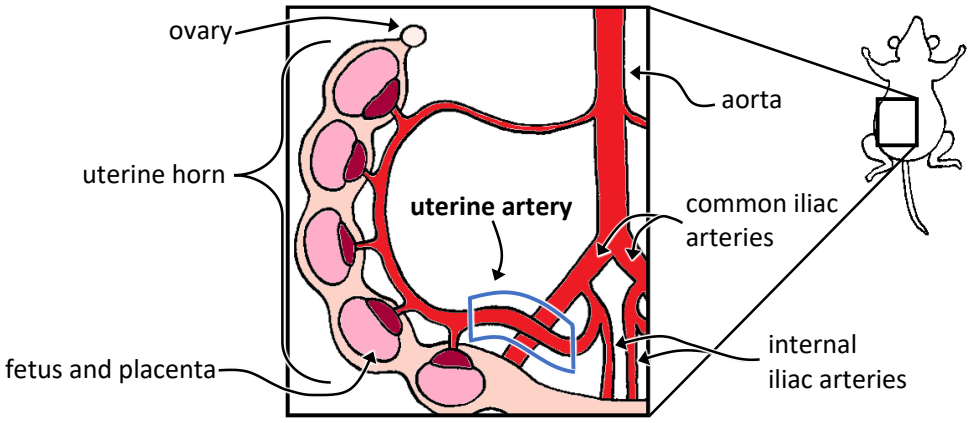


Figure 3.7: Diagram illustrating segment of uterine artery excised for wire myography.

One femoral artery was excised and mounted on the wire myograph as described in Section 2.3.

The uterine arteries supply blood to the uterus. There are two uterine arteries, one on each side, and they each branch off from an internal iliac artery (Figure 3.7). In order to excise the uterine arteries, the euthanised mouse was first pinned in the supine position, the abdominal cavity opened, and the gravid uterus exposed. The point where the aorta splits into the two common iliac arteries was located, and the vessels followed until the uterine artery branched from the internal iliac artery. The artery was separated from the surrounding fat by blunt dissection and then excised and placed in Krebs. After any remaining adipose tissue was removed, a segment approximately 2 mm in length was mounted on two 40 µm diameter stainless steel wires in the myograph chamber and subjected to the same normalisation procedure and K^+ curve as the femoral artery, as described in Section 2.3.

As the 420A Dual Wire Myograph system used has two chambers, when performing both femoral and uterine artery myography on vessels from a single animal, one femoral and one uterine artery were selected at random and mounted in the two chambers. Unfortunately, one of the force transducers broke part way through this cohort and could not be repaired without the myograph being shipped to Denmark. Therefore, myography could not be performed on both a femoral and uterine artery in all animals, resulting in lower numbers than planned.

The vasomotive agents used to generate dose-response curves are described in Table 3.3.

Agent	Doses (M)
Phenylephrine (PE)	10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}
Sodium Nitroprusside (SNP)	10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}
Acetylcholine (ACh)	10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}

***Table 3.3: Vasomotive agents used in wire myography in dam cohort**. Drugs and doses used in myography experiments with femoral and uterine arteries from Control, Obese, and Obese-Exercised dams on the 19th day of gestation.*

3.2.7 Tissue collection

At the end of the ultrasound protocol, dams were killed by cervical dislocation while still anaesthetised. Blood was collected by cardiac puncture. Immediately after culling, body weight was recorded and then the gravid uterus was exposed, and the uterine arteries excised (see Section 3.2.6) before the gravid uterus was removed and placed on ice. Maternal tissues were excised, weighed and either fixed in formalin or snap-frozen on dry ice. Fetal and placental tissues were taken by Dr I. Inzani for further processing and analysis (not presented in this thesis).

3.2.8 Statistical analysis

All data were analysed using Prism 9.0 software (GraphPad) and are presented as mean ± standard error of the mean (SEM). N (numbers) refer to an individual dam. Data points were excluded as statistical outliers prior to statistical analysis if they were more than two standard deviations from the mean of the group.

There were three groups of pregnant dams, ‘Con, ‘Ob and ‘Ob-Ex. A Shapiro-Wilk test was used to determine if data were normally distributed, and then data were analysed by either one-way ANOVA or Kruskal-Willis test as appropriate to test for an effect of group. When data were analysed by ANOVA, the Brown-Forsythe test was used to verify if groups had equal variances. If groups did not have equal variances, a Welch ANOVA was used. If a significant effect was identified, Tukey’s multiple comparison test was used to identify pairwise differences between groups. While maximal response femoral and uterine artery wire myography data was analysed according to this protocol, dose-response curves were analysed as described in Section 2.3.3.

When investigating the effect of pregnancy on the ‘Ob-Ex group, there were two groups of animals, pregnant (Preg) and non-pregnant (NP) therefore data was analysed by t-test if normally distributed or by Mann-Whitney test if not. Data analysed by t-test were checked for equal variance using an F test and a Welch correction used if groups did not have equal variances.

In the echocardiography, the same PA measurements were made in both PSAX and Apic-4 views. These data were analysed by mixed effects analysis (View and Group), matched by animal. In the uterine artery flow analysis, the same measurements were made from PW Doppler traces taken of the left and right uterine arteries in a single animal. These data were also analysed by mixed effects analysis (Left/Right and Group), matched by animal. If there was either a significant effect of Group or a significant interaction between the two factors, data were separated and analysed by a View- or Left/Right- specific one-way ANOVA or Kruskal-Wallis test as appropriate.

3.3 Results and Discussion

This study set out to investigate whether maternal cardiovascular adaptation to pregnancy was altered by obesity, and to assess whether an exercise intervention during pregnancy would offset any negative effects. This would be achieved by comparing cardiovascular function in three groups of pregnant dams, 'Con, 'Ob, and 'Ob-Ex, that would be culled in late gestation. However, seven of the intended 'Ob-Ex females did not go on develop an established pregnancy. This could only be confirmed by ultrasound imaging on the day that the animals were due to be culled, 18 days after a copulatory plug was found. These animals thus represent non-pregnant (NP) obese females that underwent the same exercise intervention protocol and were exposed to the same HF-HS diet as the pregnant 'Ob-Ex dams. Therefore, this study can be used to assess the effects of pregnancy on obese female mice undergoing a three-week exercise intervention by comparing pregnant and NP obese-exercised females. This is in addition to its original aim of investigating the effects of maternal obesity and exercise during pregnancy by comparing 'Con, 'Ob, and 'Ob-Ex dams. Data comparing the seven NP obese-exercised females with the 14 pregnant 'Ob-Ex dams will be presented first in this chapter. Any discussion of NP females in this chapter is specifically referencing these obese females who underwent the exercise intervention but were not found to be pregnant 18 days after a copulatory plug was found, and they are exclusively compared with pregnant 'Ob-Ex dams.

3.3.1 Effect of pregnancy on body composition

There was no difference in body weight, fat mass or lean mass between 'Ob-Ex dams and NP females on the day that a copulatory plug was found (Figure 3.8(A,D,E)). This suggests that body weight at mating was not a contributing factor to whether these females went on to develop an established pregnancy. There was no change in fat mass as measured by TDNMR between the day of plug and 17 days later in either 'Ob-Ex dams or NP females (Figure 3.8(E)), but pregnant 'Ob-Ex dams were both heavier (Figure 3.8(B)) and had an increased lean mass (Figure 3.8(D)) than NP females 17 days after plugging. Therefore, 'Ob-Ex dams gained more body weight between the two time points than NP females (Figure 3.8(C)). When females were culled 18 days after a copulatory plug was found and the uterus excised, there was no difference in hysterectomised weight between 'Ob-Ex dams and NP females (Figure 3.8(F)). Together, this suggests that the increased body weight in the pregnant 'Ob-Ex dams can be explained entirely by the increased mass of the gravid uterus.

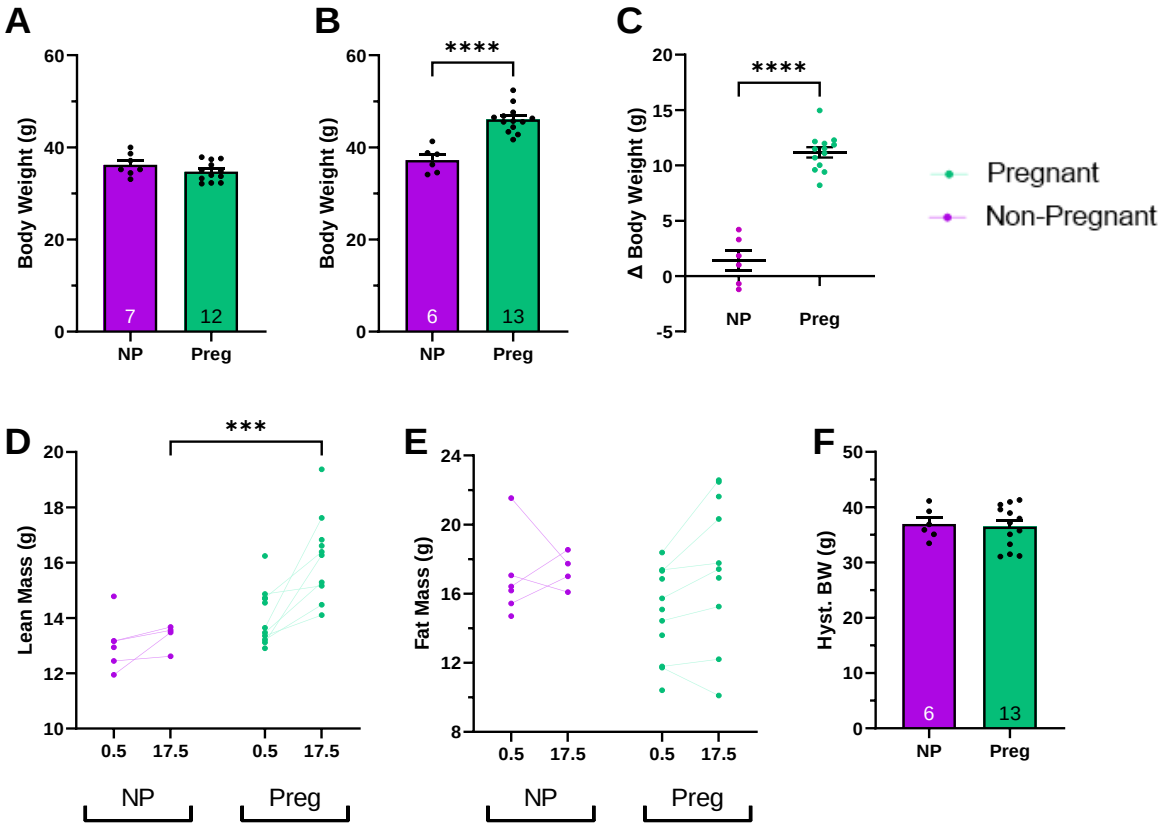
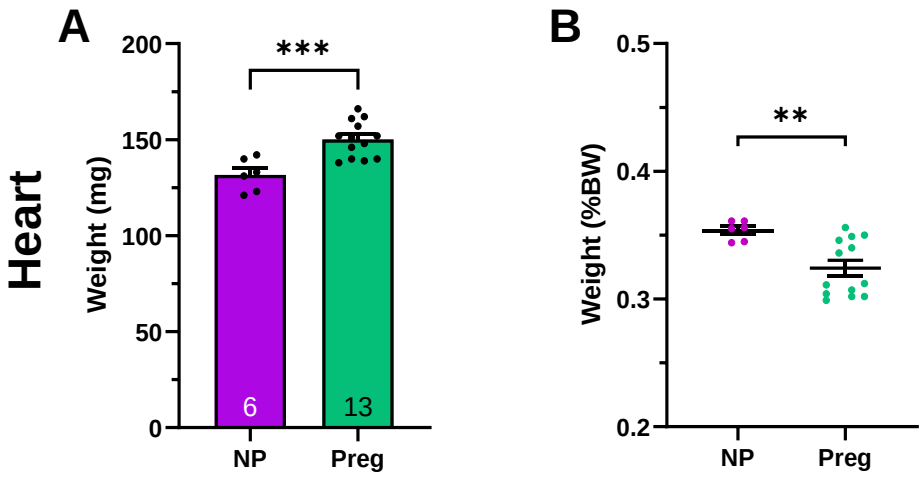


Figure 3.8: Body weight and composition of pregnant 'Ob-Ex dams and non-pregnant age-, diet- and exercise-matched females. *Body weight of pregnant (Preg) dams and non-pregnant (NP) females on the day of plug (A) and 17 days later (B), and the difference between the two (C). Lean (D) and fat (E) mass measurements on the same days. Hysterectomised body weight (F) measured the following morning. In (B) and (C), ****p<0.0001 using student's t-test. In (D), ***p<0.001 using Sidak's multiple comparisons test after mixed-effects analysis. In (D) and (E), E0.5 = day of plug and E17.5 = 17 days later, in late gestation. Numbers presented in bars in (A), (B), and (F) are matched with data in (C)-(E).*

Pregnant 'Ob-Ex dams had increased absolute heart weights relative to NP females 18 days after plugging (Figure 3.9(A)). This is consistent with the well-established cardiac hypertrophy that occurs in pregnancy[374]. However, when normalised to body weight, relative heart weight was lower in pregnant animals (Figure 3.9(B)) and thus the increase in heart weight as pregnancy progressed was not proportional to the increase in body weight. This finding is consistent with other studies published in rodents[425]. The same pattern of increased absolute and decreased relative weight was found in the kidneys of pregnant 'Ob-Ex dams compared to NP females (Figure 3.9(C)). Absolute liver weight was also increased in pregnant 'Ob-Ex dams, but unlike the heart and kidneys, relative weight was also increased, although this did not reach statistical significance (Figure 3.9(C)).



C

	Unit	Non-Pregnant (n=6)	Pregnant (n=13)	p-value
Liver	mg	1434 ± 67	1969 ± 59	**<0.0001**
	%BW	3.92 ± 0.14	4.25 ± 0.10	*0.0886*
Kidney	mg	151 ± 4	169 ± 2	**0.0004**
	%BW	0.4010 ± 0.007	0.3684 ± 0.008	**0.0216**

Figure 3.9: Absolute and relative organ weights of pregnant and non-pregnant obese-exercised females 18 days after plug*. Heart weight expressed as absolute weight (A) and relative to body weight (%BW) (B). **p<0.01, ***p<0.001 using student's t-test. Absolute and relative weights of liver and kidney (C) analysed by student's t-test. NP = non-pregnant. Preg = pregnant. Numbers: NP=6, Preg=13.*

3.3.2 Effect of pregnancy on haematocrit

Pregnant 'Ob-Ex dams had a lower haematocrit than NP females (Figure 3.10(A)). Pregnancy is associated with plasma volume increases with proportionally smaller increases in RBC mass, resulting in relative haemodilution[369]. Therefore, this reduced haematocrit indicates that the blood volume of the pregnant females increased as expected. Haematocrit correlated with body weight change between day of plug and 17 days later, when haematocrit was measured (Figure 3.10(B)).

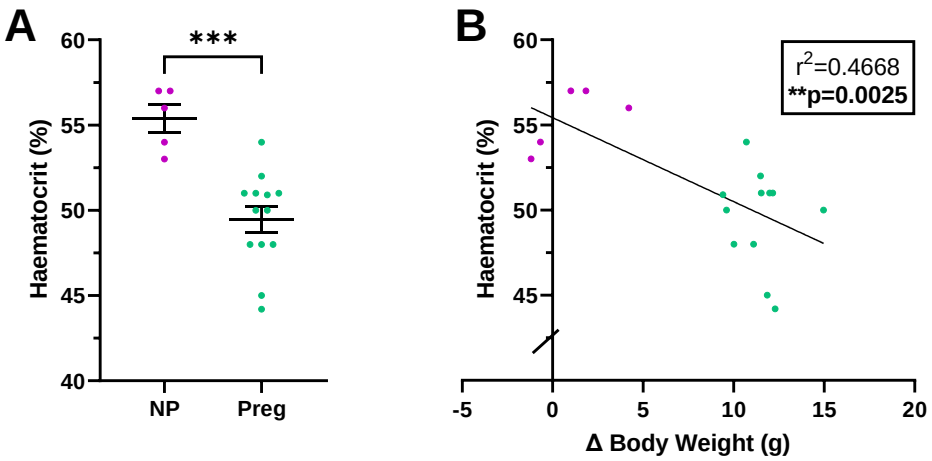


Figure 3.10: Haematocrit of pregnant and non-pregnant obese-exercised females. *Haematocrit (A) 17 days after plug, ***p<0.001 by student's t-test. Haematocrit plotted against body weight change between day of plug and 17 days later (B) analysed by simple linear regression. NP = non-pregnant. Preg = pregnant. Numbers: NP=5, Preg=13.*

3.3.3 Left ventricular function in pregnant and non-pregnant females

While there was no difference in LV volume at end-systole, EDV was increased in pregnant females (Figure 3.11(A)). This resulted in a significant increase in stroke volume (Figure 3.11(B)). These volume changes are discussed below. It also resulted in increases in ejection fraction and radial fractional shortening, consistent with pregnancy-associated increases in cardiac contractility. There was no difference in heart rate between pregnant and NP females in this study (Figure 3.11(C)). This was unexpected as heart rate is reported to increase during pregnancy[370,371]. However, heart rate here was measured in the anaesthetised animal and thus differences in heart rate in the conscious animal may have been masked by the cardiorespiratory suppressant effects of the anaesthetic isofluorane.

There was an increase in LV endocardial length at end-diastole (Figure 3.11(G)) in pregnant 'Ob-Ex dams, but not at end-systole, compared to NP females resulting in a non-significant increase in longitudinal FS (p=0.0592) (Figure 3.11(H)). Once again, this is consistent with a pregnancy-associated increase in cardiac contractility.

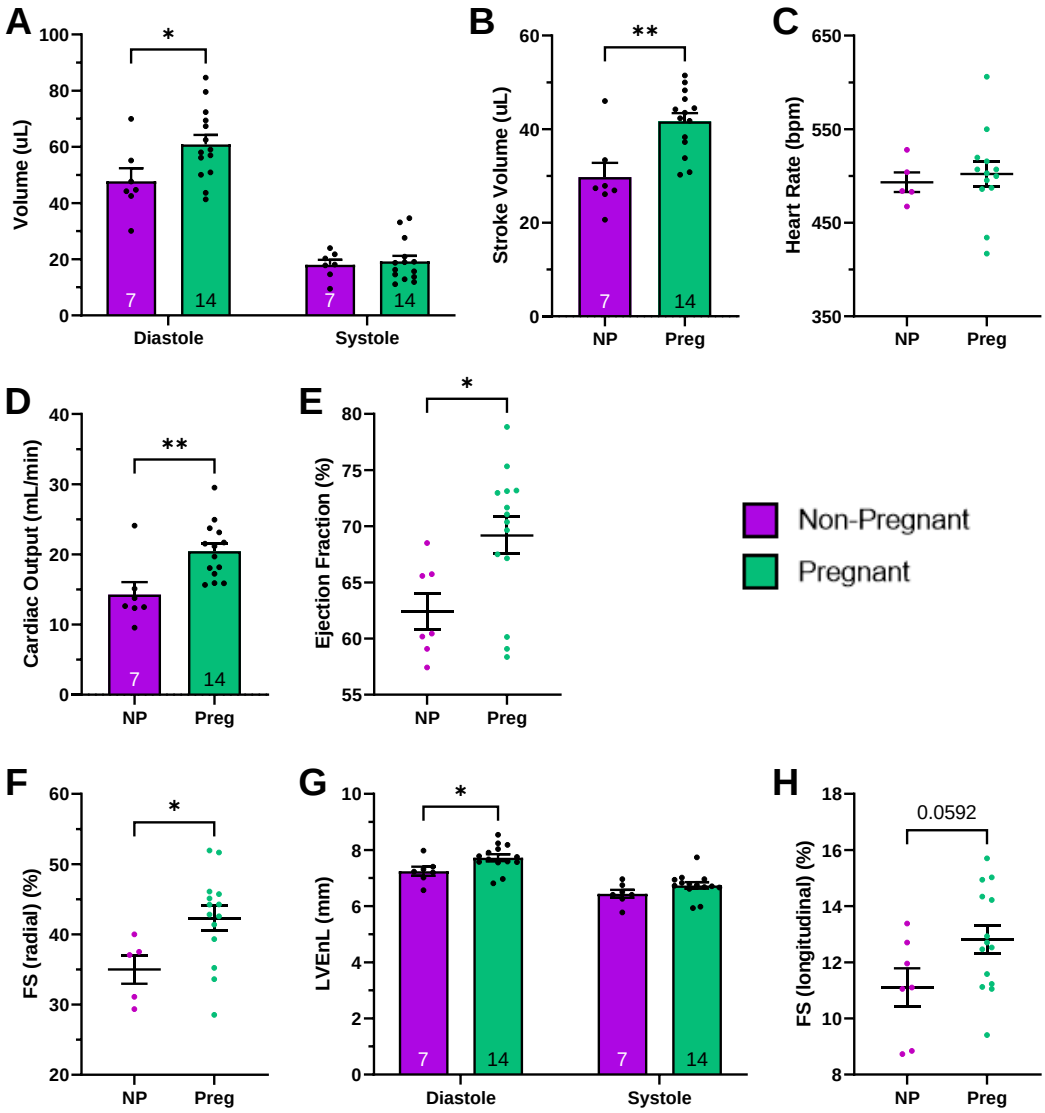


Figure 3.11: Echocardiographic measures of systolic function in pregnant and non-pregnant obese-exercised animals*. End-systolic and end-diastolic left ventricle (LV) volumes (A), stroke volume (B), heart rate (C), cardiac output (D), ejection fraction (E), radial fractional shortening (FS) (F), end-systolic and end-diastolic LV endocardial length (LVEnL) (G) and longitudinal FS (H), all measured 18 days after plug. *p<0.05, **p<0.01 using student's t-test. NP = non-pregnant. Preg = pregnant. Numbers: NP = 7, Preg = 14.*

As described in Section 3.1.2, it is well-established that blood volume increases during pregnancy[369371372]. This increase in blood volume results in an increase in MSFP, and therefore an increase in EDV[374] and subsequently stroke volume. Therefore, the increases in end-diastolic LV endocardial length and EDV, plus the subsequent increase in stroke volume, in the pregnant females are consistent with an increase in blood volume and thus in line with expectation. This is further supported by the fact that both EDV and stroke volume correlate with the change in body weight between the day of plug and 17 days later, and with haematocrit on the 17th day (Figure 3.12).

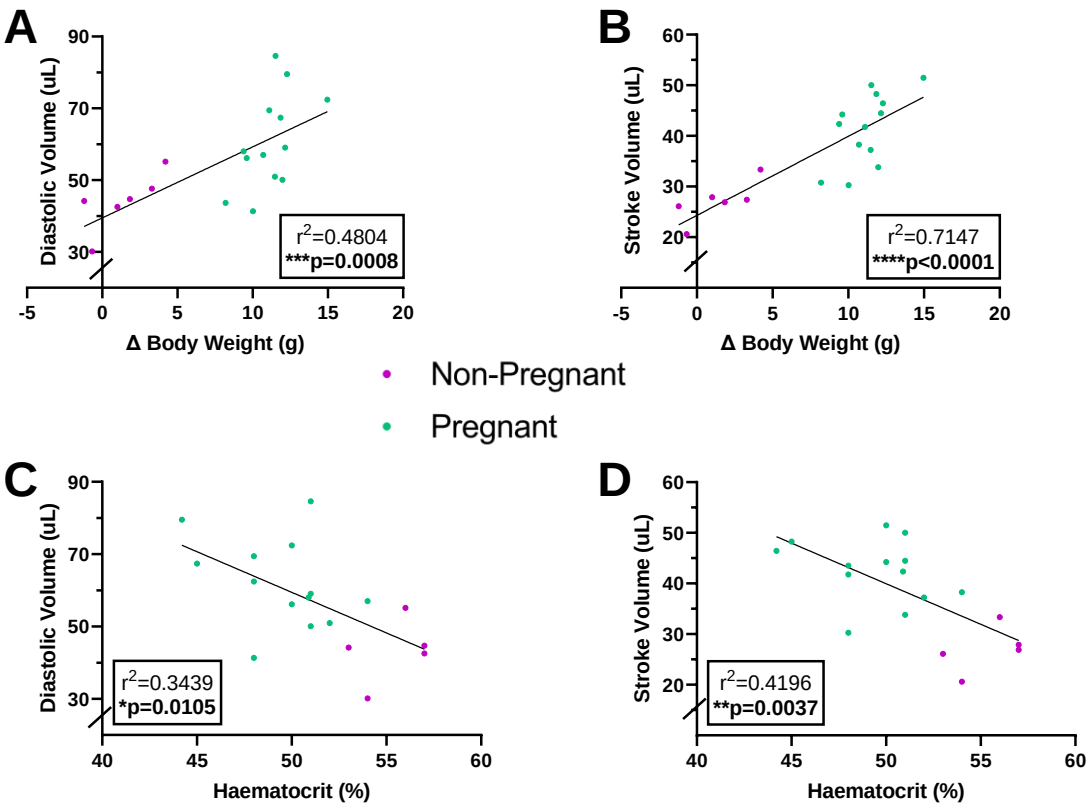


Figure 3.12: Correlations between heart volume and measures associated with increased blood volume in pregnant and non-pregnant females. *Diastolic volume (A,C) and stroke volume (B,D) plotted against change in body weight in the 17 days after plug (A,B) and haematocrit (C,D). r^2 and p-values from simple linear regression analyses. Numbers: Non-Pregnant = 6-5, Pregnant = 13.*

Pregnant 'Ob-Ex dams had a greater peak E wave velocity than NP females, and no difference in either peak A wave velocity (Figure 3.13(A)) or the resulting E:A ratio (Figure 3.13(B)). Echocardiographic data on the changes in mitral valve blood flow during pregnancy is limited, but the few studies that are published find that both the maximum E and A wave peaks are increased during pregnancy, and that the A wave increase is greater than that of the E wave by late gestation[426,427]. This results in the E:A ratio in the third trimester usually being reduced relative to the non-pregnant state. The increased maximum velocity of the E wave in pregnant 'Ob-Ex dams in this study is therefore in line with published data, but that there was no difference in peak A wave velocity between pregnant and NP females is not. Moreover, peak E-wave velocity, but not peak A-wave velocity, correlated with the change in body weight between the day of plug and 17 days later (Figure 3.14). This finding will be discussed further in combination with echocardiographic measurements of diastolic function in pregnant 'Con, 'Ob, and 'Ob-Ex dams in Section 3.3.10.

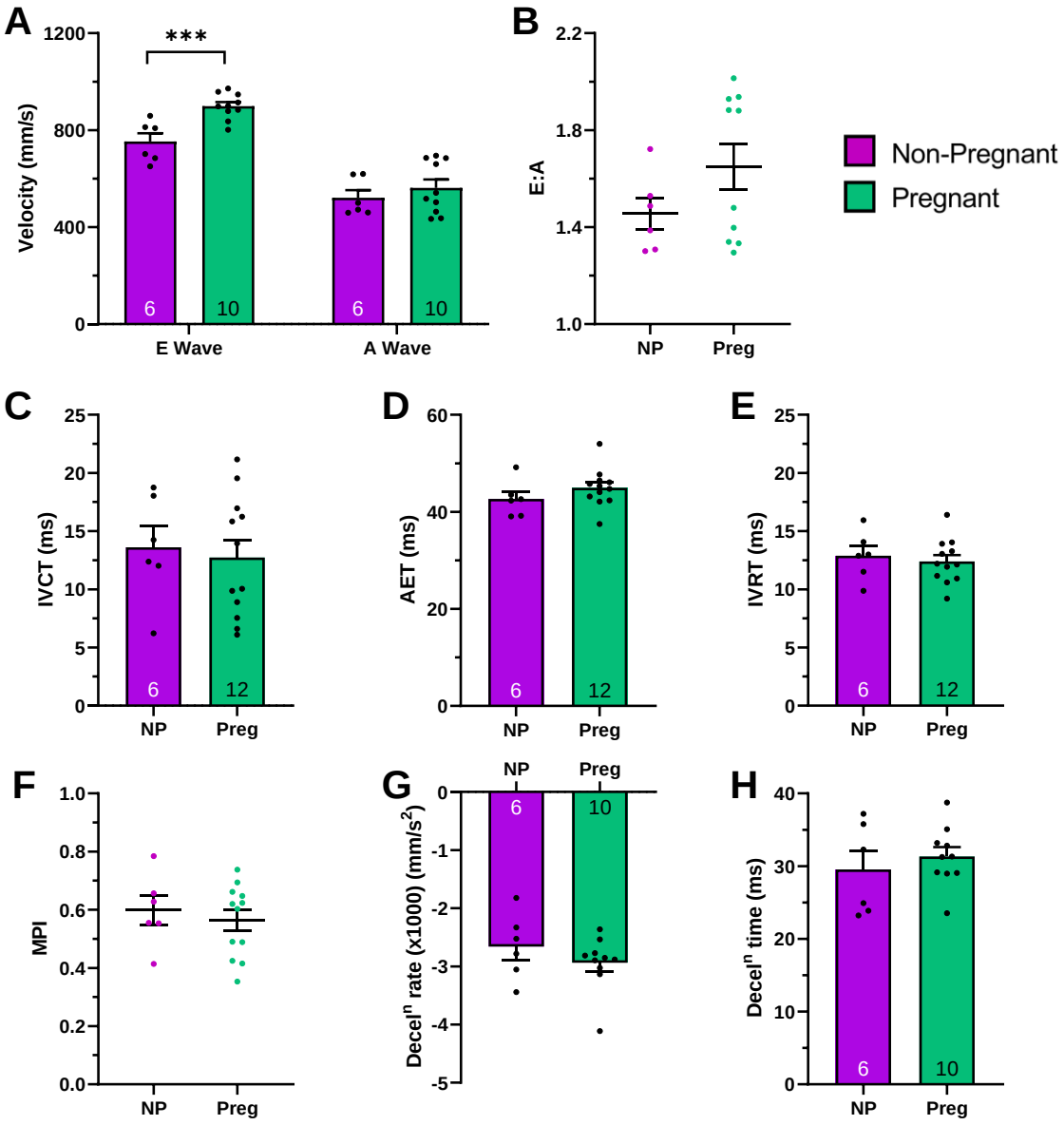


Figure 3.13: Echocardiographic measures of left ventricular diastolic function in pregnant (Preg) and non-pregnant (NP) obese-exercised females 18 days after plug. *Peak E-wave and A-wave velocities (A) and E:A ratio (B). Isovolumetric contraction time (IVCT) (C), aortic ejection time (AET) (D), isovolumetric relaxation time (IVRT) (E), myocardial performance index (MPI) (F), and mitral valve deceleration rate (G) and time (H). ***p<0.001 using student's t-test. Numbers: NP = 6, Preg = 10-12.*

No other measures derived from the MV PW Doppler were different between pregnant and NP females (Figure 3.13(C-H)), suggesting that adaptation of the maternal heart to pregnancy in 'Ob-Ex dams did not result in major changes in diastolic function or ventricle stiffness. This is not in line with expectation, as pregnancy is usually associated with decreases in both in IVRT and deceleration time[428].

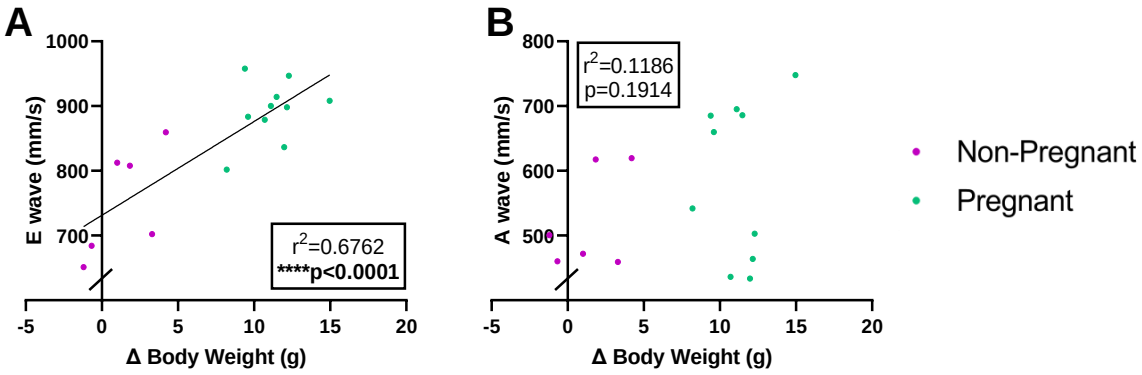


Figure 3.14: Correlation between peak E- and A- wave velocities and change in body weight in pregnant and non-pregnant females. *Peak E-wave velocity (B) and A-wave velocity (B) plotted against change in body weight in the 17 days after plug. r^2 and p-values from simple linear regression analyses. Numbers: Non-Pregnant = 6-5, Pregnant = 13.*

3.3.4 Pregnancy and pulmonary artery flow

When analysed by two-way ANOVA for the effect of view (PSAX or Apic-4) and group (Preg or NP), view had a significant effect on every measure of PA flow other than PAT. There was also no effect of group on PAT (Figure 3.15(A)). Measures that were also affected by group were analysed by view-specific one-way ANOVA. There was no effect of group on PAT:PET (Figure 3.15(C)). An increase in PET in pregnant compared to NP females was found only when analysing traces taken in Apic-4 and not in PSAX (Figure 3.15(B)).

Mean (Figure 3.15(E)) and peak (Figure 3.15(F)) velocity, as well as mean gradient (Figure 3.15(D)), of flow through the PA were greater in pregnant 'Ob-Ex dams compared to NP females irrespective of the view from which the PA PW Doppler recordings were made. No published data on the effect of pregnancy on blood flow through the pulmonary artery was found in literature searches. That mean and peak velocity of blood flow through the pulmonary valve and the mean gradient of the PA blood flow trace were greater in pregnant females is most likely due to increases in blood volume and CO.

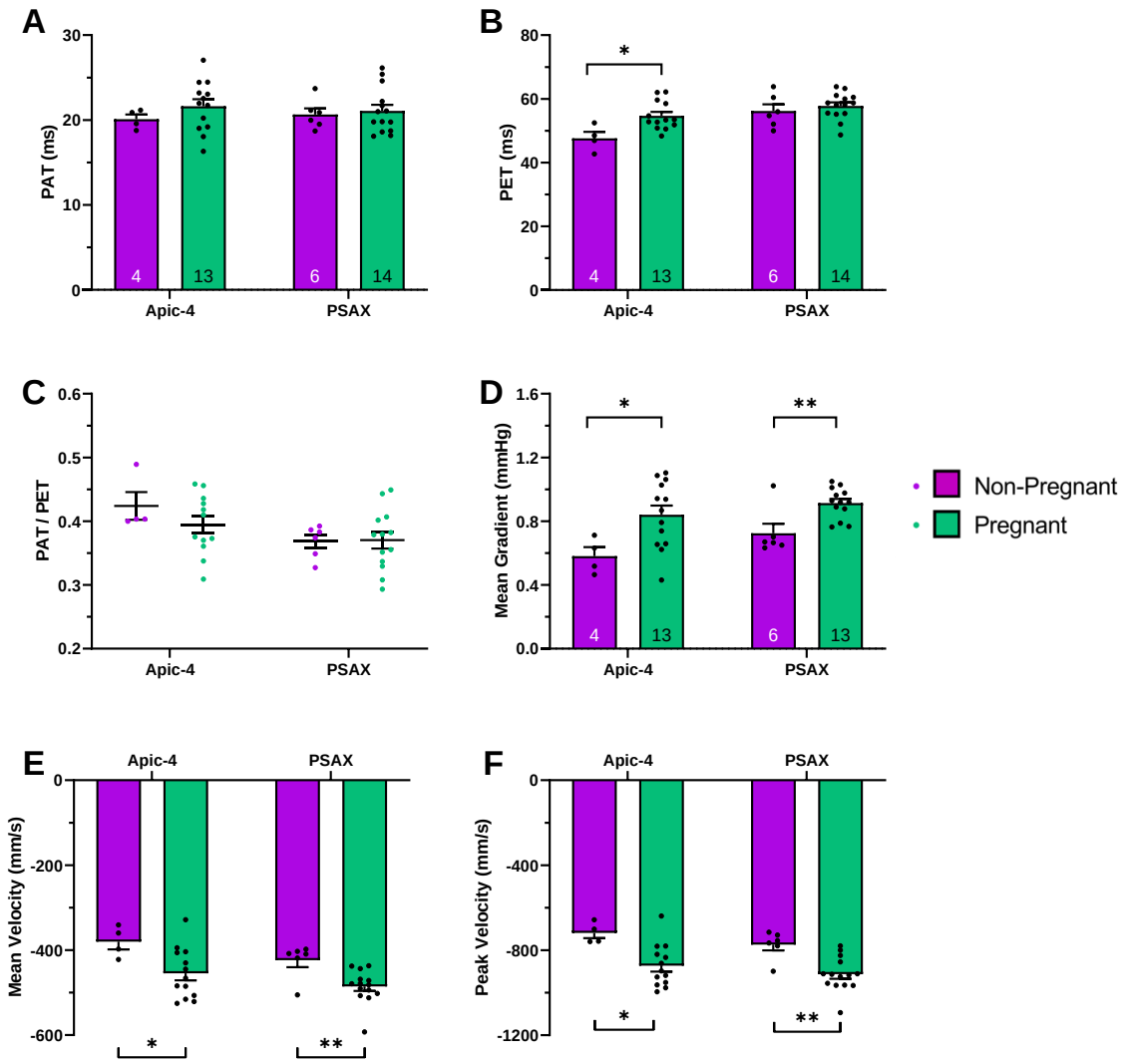


Figure 3.15: Echocardiographic measures of pulmonary artery (PA) flow in pregnant and non-pregnant obese-exercised females 18 days after plug. *PA acceleration time (PAT) (A), PA ejection time (PET) (B) and PAT:PET ratio (C). Mean gradient (D), mean velocity (E) and peak velocity (F) calculated from PA velocity time integral, all measured in both PSAX and Apic-4 views. *p<0.05, **p<0.01 using Tukey's multiple comparison after mixed-effects analysis for both View and Group. NP = non-pregnant. Preg = pregnant. Numbers: Non-Pregnant = 4-6, Pregnant = 13-14.*

This chapter will now move on to discuss the three groups of dams who did develop an established pregnancy after mating, 'Con, 'Ob, and 'Ob-Ex. The prior comparisons of pregnant versus NP obese-exercised females will be referenced where relevant to aid data interpretation.

3.3.5 Body weight and composition of pregnant dams

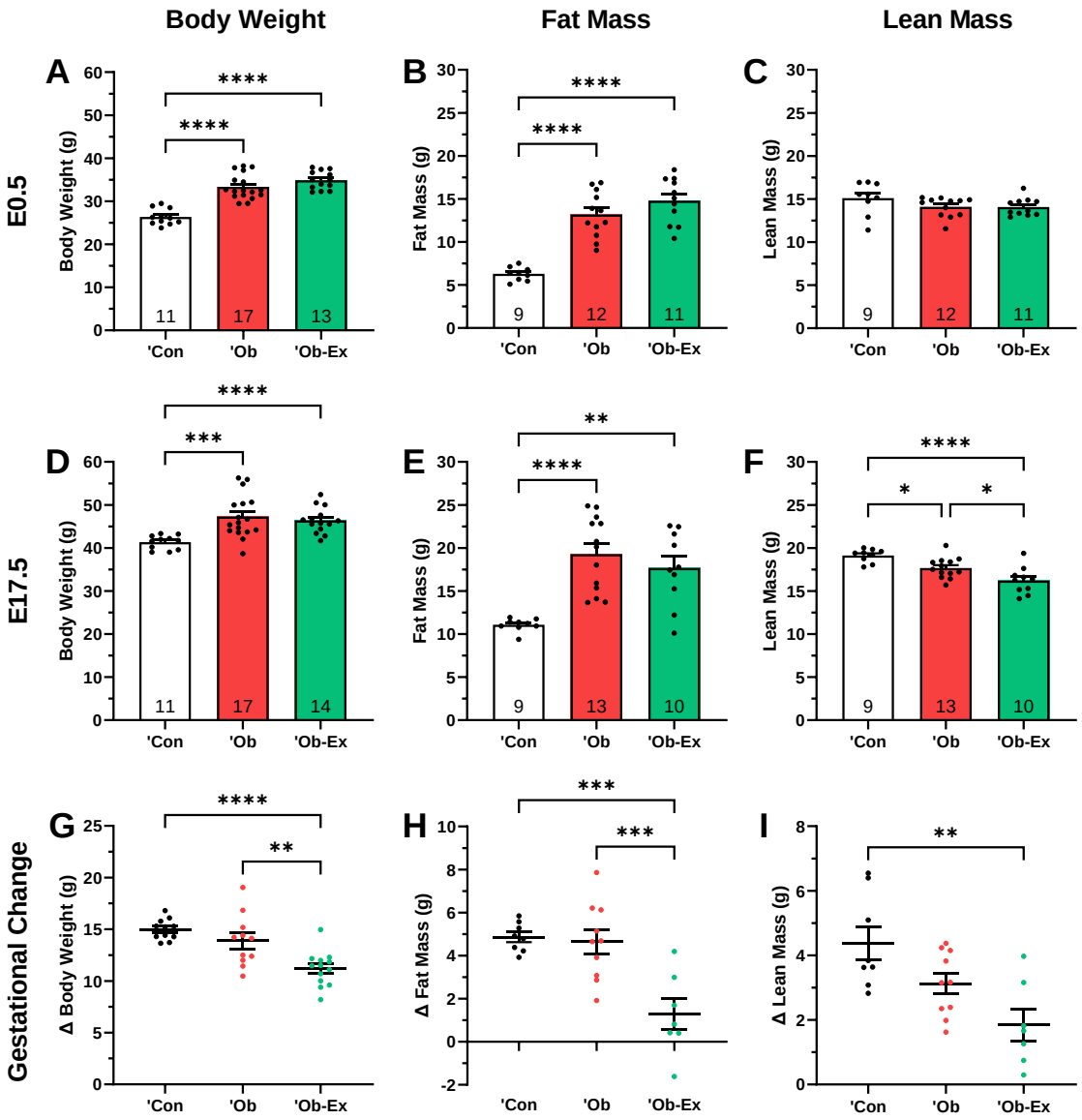


Figure 3.16: Gestational change in body weight and composition of dams. *Body weight (A,D) and composition (B,C,E,F) as measured by TDNMR, on day on plug (E0.5) and morning of 18th day of gestation (E17.5), and the difference between the two (G-I). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 using Tukey's multiple comparison test after a significant effect of Group identified by one-way ANOVA. Numbers presented inside bars in (A)-(F), which are matched with data in (G)-(I).*

'Ob dams were heavier (Figure 3.16(A)) and had a greater fat mass (Figure 3.16(B)) than 'Con on the day of plug. Therefore, feeding of the HF-HS obesogenic diet from weaning did result in an obesity phenotype. There was no difference in the increase in either body weight (Figure 3.16(G)) or fat mass (Figure 3.16(H)) between 'Con and 'Ob over the course of pregnancy, and so the increased body weight and fat mass in 'Ob remained at E17.5 (Figure 3.16(D,E)).

There was no significant difference in body weight, fat mass, or lean mass between 'Ob and 'Ob-Ex dams on the day of plug (Figure 3.16(A-C)), meaning that the one week of exercise training prior to mating had no effect on body composition and so exercised and non-exercised obese dams entered pregnancy with comparable body compositions. There was also no difference in body weight or fat mass between 'Ob and 'Ob-Ex dams at E17.5 (Figure 3.16(D,E)). However, 'Ob-Ex dams had a lower increase in body weight over the course of pregnancy compared to both 'Con and 'Ob dams (Figure 3.16(G)), representing a reduction in GWG in response to maternal exercise. This has also been found in some human RCTs of maternal exercise during obese pregnancy[308,313], as discussed in Section 1.7.1. This was in part due to any increase in fat mass over the course of pregnancy being reduced to a negligible amount in 'Ob-Ex dams (Figure 3.16(H)). That the 'Ob-Ex dams gained less body weight and fat mass during pregnancy (Figure 3.16(G,H)) but this was not sufficient to result in a significant difference in total fat mass or body weight compared to non-exercised 'Ob dams in late gestation (Figure 3.16(D,E)) may be due to the 'Ob-Ex dams entering pregnancy with a non-significant 1.6 g higher average body weight and fat mass than 'Ob (Figure 3.16(A,B)).

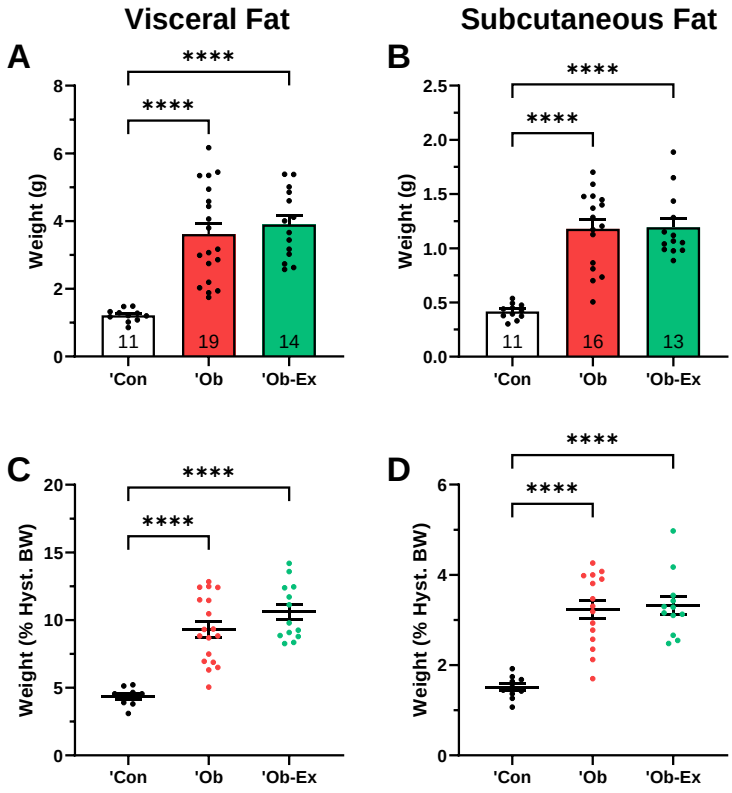


Figure 3.17: Dam visceral and subcutaneous white adipose tissue (WAT) at E18.5. *Combined mass of visceral fat depots collected expressed as absolute weight (A) and relative to hysterectomised body weight (Hyst. BW) (C), and the same measurements for subcutaneous fat (B,D). Data analysed by one-way ANOVA with post hoc Tukey's multiple comparisons. Numbers presented inside bars in (A) and (B), which are matched with data in (C) and (D).*

	mg	'Con (n=11)	'Ob (n=19)	'Ob-Ex (n=14)	p-value	post hoc
Gonadal Fat		638 ± 35.0	2329 ± 209	2564 ± 190	**<0.0001**	**'Con vs 'Ob, p<0.0001 'Con vs 'Ob-Ex, p<0.0001**
IP Fat		494 ± 25	948 ± 101	981 ± 71	**0.0002**	**'Con vs 'Ob, p=0.0009 'Con vs 'Ob-Ex, p<0.0001**
RP Fat		85.4 ± 3.9	342 ± 25	352 ± 26	**<0.0001**	**'Con vs 'Ob, p<0.0001 'Con vs 'Ob-Ex, p<0.0001**

Table 3.4: Dam visceral white adipose tissue (WAT) depots at E18.5. *Absolute masses of individual visceral fat depots collected. Data analysed by one-way ANOVA with post hoc Tukey's multiple comparisons. IP = intraperitoneal, RP = retroperitoneal.*

While there were no differences in lean mass between groups on the day of plug (Figure 3.16(C)), 'Ob dams had reduced lean mass compared to 'Con at E17.5, and 'Ob-Ex dams had reduced lean mass compared to both 'Con and 'Ob (Figure 3.16(F)). 'Ob-Ex dams also gained less lean mass than 'Con over the course of pregnancy (Figure 3.16(I)). This finding is best interpreted alongside dam weight measurements made the following morning, on E18.5, after the animal had been culled (Figure 3.16).

As at E17.5 (Figure 3.16), 'Ob and 'Ob-Ex dams were heavier than 'Con at E18.5 (Figure 3.18(A)), and this pattern of increased body weight in obese dams irrespective of exercise was also present following hysterectomy (Figure 3.18(B)). Both 'Ob and 'Ob-Ex dams had reduced gravid uterus weight compared to 'Con at E18.5 (Figure 3.18(C)). Given that there was no significant effect of maternal group on litter size (Figure 3.18(D)), exposure to maternal obesity *in utero* therefore resulted in a decrease in the average combined weight of the fetuses and their placentas (fetoplacental unit weight) which was not corrected by maternal exercise (Figure 3.18(E)). This decrease in the average weight of the fetoplacental unit was due to a decrease in fetal weight only and there was no difference in placental weight between groups (data from the thesis of Dr I. Inzani). This decrease in fetal weight was associated with symmetrical growth restriction (data from the thesis of Dr I. Inzani). This study therefore found that maternal obesity resulted in a reduced fetal growth, or IUGR, phenotype. This is consistent with previous studies using this model[293,297] and human data showing that babies born to obese mothers are more likely to be born SGA due to IUGR[104,112]. The exercise intervention did not prevent the IUGR in response to maternal obesity in this study.

Consistent with previous studies[292], there was no effect of group on maternal plasma glucose after a 4-hour fast at E17.5 (Table 3.4).

'Con (n=11)	'Ob (n=18)	'Ob-Ex (n=14)	p-value
10.2 ± 0.2	10.3 ± 0.4	10.2 ± 0.3	0.9426

Table 3.5: Maternal plasma glucose on E17.5 after a 4-hour fast. *All units are mmol/L. p-value calculated by one-way ANOVA.*

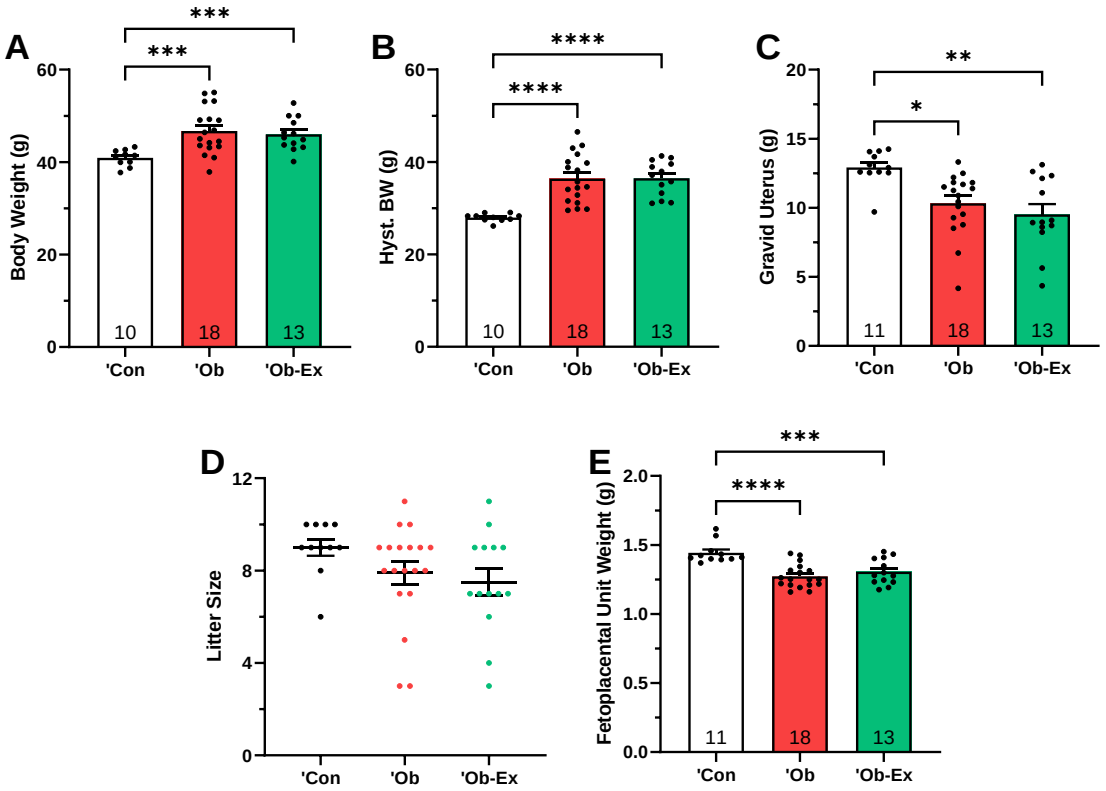


Figure 3.18: Dam and fetoplacental unit weights at E18.5. *Total body weight (A), hysterectomised body weight (Hyst. BW) (B) and weight of the gravid uterus (C), alongside litter size (D) and average fetoplacental unit weight (E). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 using Tukey's multiple comparison test after a significant effect of Group found by one-way ANOVA or Kruskal-Wallis test. Numbers: 'Con = 10-11, 'Ob = 18, 'Ob-Ex = 13.*

3.3.6 Blood pressure before and during pregnancy

PreP2 SBP data from 'Ob and 'Ob-Ex females were combined in PreP2 SBP and heart rate analyses because measurements were taken before 'Ob-Ex had started treadmill running and thus they were equivalent to 'Ob. In humans, obesity is associated with an increased risk of hypertension independent of pregnancy[149], therefore overweight and obese women are more likely to enter pregnancy with elevated blood pressure. However, rodents are relatively resistant to the development of hypertension, including in the context of diet-induced obesity. As a result of this, studies typically having to induce hypertension in rodents by genetic or pharmacological manipulation of components of blood pressure regulation such as the renin-angiotensin system[429,430]. Therefore, it is unsurprising that immediately prior to mating for P2, there was no difference in SBP between lean ('Con) females and those fed a HF-HS obesogenic diet from weaning ('Ob and 'Ob-Ex) (Figure 3.19(A)) in this study. This was not the case for heart rate. Obese females (combined 'Ob and 'Ob-Ex) had an elevated heart rate compared to 'Con before pregnancy (Figure 3.19(C)). This is consistent with human data, as overweight and obese individuals have an higher resting heart rate[398,399] than lean people.

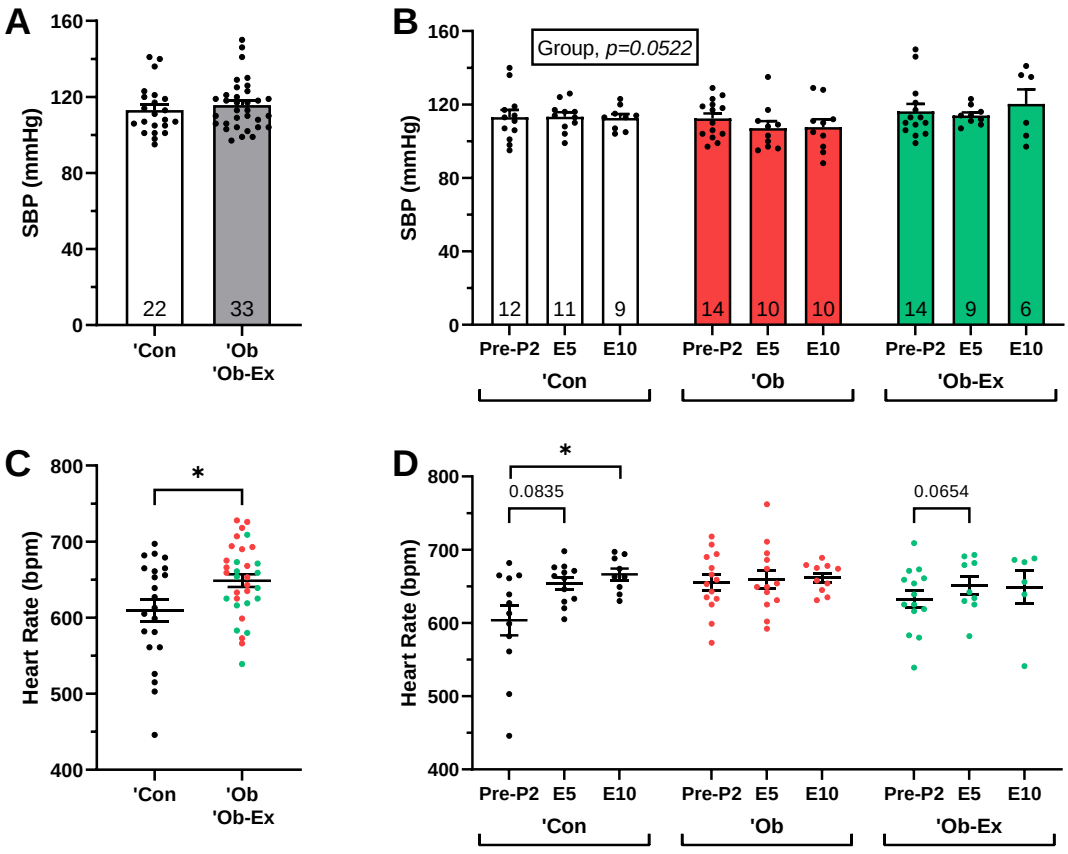


Figure 3.19: Systolic blood pressure (SBP) and heart rate of dams before and during pregnancy. *Pre-pregnancy (PreP2) SBP (A) and heart rate (C), and longitudinal SBP (B) and heart rate (D). For PreP2 data, *p<0.05 by student's t-test. For longitudinal heart rate data, *p<0.05 using Tukey's multiple comparison test after a significant effect of Timepoint found by mixed-effects analysis. Box in (B) presents trending effect of group identified by mixed-effects analysis. Numbers presented inside bars in (A) and (B), which are matched with data in (C) and (D).*

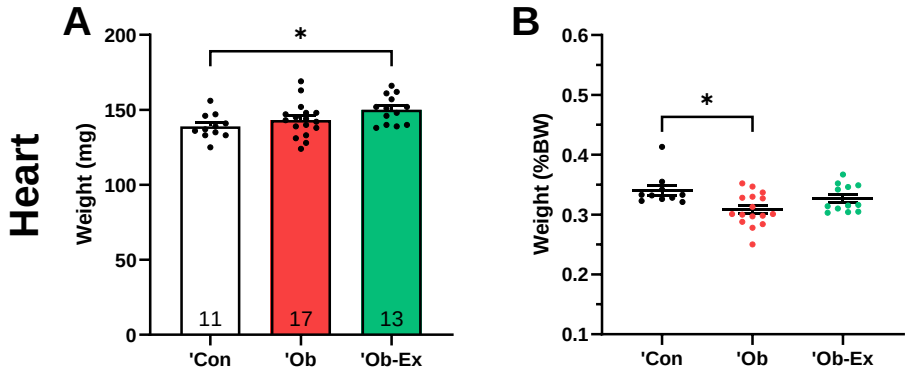
Dam SBP and heart rate were also measured in early pregnancy (E5) and mid-gestation (E10). When analysed by mixed-effects analysis for timepoint and group ('Con, 'Ob, or 'Ob-Ex), there was a trending effect of group on longitudinal SBP from PreP2 to E10 (p=0.0522) (Figure 3.19(B)), but this did not reach statistical significance. It is well established that arterial blood pressure transiently decreases during pregnancy in humans[377], although significant drops in DBP and MAP are more widely reported than changes in SBP. Comparable data in rodents is limited, although one longitudinal arterial blood pressure study in pregnant mice using radiotelemetry identified decreases in both SBP and DBP that reached their nadir around E9[431]. While no differences in SBP in early gestation were found in this study, blood pressure was measured using tail cuff plethysmography which is both less accurate than radiotelemetry and means that measurements are made at a single time of day rather than across a 24-hour period. Therefore, the trending effect of group found by mixed-effects analysis may suggest that subtle changes in SBP were present but not large enough to have been detectable by this method and/or at the time of day when measurements were made.

When longitudinal heart rate was analysed by mixed-effects analysis, a significant effect of timepoint was found (p=0.0148) (Figure 3.19(D)). Post hoc Tukey's multiple comparisons testing revealed a stepwise increase in heart rate in 'Con dams from pre-pregnancy to mid-gestation, resulting in a significant difference in heart rate between PreP2 and E10. This is consistent with published data as changes in heart rate are well documented in early pregnancy, with heart rate increasing as early as two to five weeks of gestation in humans[370,371]. Once again, longitudinal data is limited in mice, but radiotelemetry data showing an increase in heart rate between conception and mid-gestation has been published[431]. No such increase in heart rate between the different timepoints was found in 'Ob dams (Figure 3.19(D)). However, heart rate was similar between 'Con and 'Ob dams at E10, with the lack of comparable increase between pre-pregnancy and mid-gestation in 'Ob dams being due to their elevated pre-pregnancy heart rate. This suggests that 'Ob dams entering pregnancy with an elevated heart rate resulted in a reduced capacity to raise their heart rate as pregnancy progressed. This is consistent with human data that shows that obese women typically enter pregnancy with an elevated heart rate[398,399], and are unable to raise their heart rate with advancing gestation to the same extent as lean women[401]. While a trend for an increase in heart rate between PreP2 and E5 was seen in both 'Con and 'Ob-Ex dams (Figure 3.19(D)), the significant increase in heart rate between PreP2 and E10 seen in 'Con was not replicated in 'Ob-Ex. Therefore, the pregnancy-associated increase in heart rate seen in 'Con dams and abolished in 'Ob was not rescued by maternal exercise intervention.

The decreasing numbers in Figure 3.19 between PreP2 and E10 in all three groups should be noted, with 12 'Con, 14 'Ob, and 14 'Ob-Ex PreP2 measurements decreasing to nine, ten, and six respectively by E10. SBP measurements from a dam are not presented for one of two reasons. For some dams, no blood pressure measurements were made on E5 or E10 due to scheduling issues or equipment failures. However, some data were excluded due to either fewer than ten successful measurements being obtained, or the coefficient of variation (%CV) of the measurements made that day being greater than 15%. An increased rate of exclusion in a particular group would be noteworthy as it may indicate that these animals are more stressed as they are less likely to remain calm and still while restrained on the tail cuff plethysmography system. As well as impairing data interpretation, as stress would result in an acute increase in SBP and heart rate, this is particularly important in developmental programming studies as maternal stress can programme the cardiometabolic health of adult offspring[90–92]. However, there was no significant difference in the number of dams for whom blood pressure measurements were excluded between groups.

3.3.7 Organ weights of pregnant dams

There was no difference in absolute heart weight between 'Con and 'Ob dams at E18.5 (Figure 3.20(A)). Given that 'Ob dams were heavier than 'Con, this resulted in a decrease in the heart weight of 'Ob dams relative to total body weight (Figure 3.20(B)). This may suggest that maternal obesity limits the adaptive physiological cardiac hypertrophy that usually occurs in response to pregnancy in this model. Absolute heart weight was increased in 'Ob-Ex dams relative to 'Con (Figure 3.20(A)), resulting in comparable heart weight relative to total body weight (Figure 3.20(B)). Therefore, this exercise intervention may promote physiological hypertrophy of the heart in response to pregnancy. Absolute and relative weights of dam liver and kidney are shown in Figure 3.20(C). Neither maternal obesity nor maternal exercise affected absolute liver weight, but relative liver weight was reduced in 'Ob dams compared to 'Con whether expressed relative to total or hysterectomised body weight. Absolute kidney weight was increased in both 'Ob and 'Ob-Ex dams compared to 'Con, but these differences were lost when normalised to total body weight.



C	Unit	'Con (n=10-11)	'Ob (n=16-19)	'Ob-Ex (n=11-14)	p-value	post hoc
Liver	mg	1881 ± 39	1945 ±55	1969 ± 59	0.5747	n/a
	%BW	4.60 ± 0.07	4.13 ± 0.10	4.26 ± 0.10	**0.0112**	**'Con vs 'Ob, p=0.0083** *'Con vs 'Ob-Ex, p=0.0930*
Kidney	mg	155 ± 3	167 ± 4	169 ± 2	**0.0100**	**'Con vs 'Ob, p=0.0229** **'Con vs 'Ob-Ex, p=0.0154**
	%BW	0.378 ± 0.006	0.359 ± 0.008	0.375 ± 0.010	0.2176	n/a

Figure 3.20: Dam organ weights at E18.5*. Heart weight expressed as absolute weight (A) and relative to total body weight (%BW) (B). Absolute and relative weights of other organs (C). *p<0.05, **p<0.01, using post hoc Tukey's multiple comparison test after a significant effect of Group found by one-way ANOVA or Kruskal-Wallis test. Numbers presented inside bars in (A) are matched with data in (B).*

While absolute heart weight was increased in 'Ob-Ex dams compared to 'Con (Figure 3.20 (A)), this was not associated with differences in LV wall width at either end-diastole or end-systole as measured in PSLAX M-mode during echocardiography (Figure 3.21). This suggests that the increase in heart weight in 'Ob-Ex dams is due to an increase in the overall size of the heart rather than a thickening of the ventricle walls. The only difference in wall width found between groups was an increased LVPW width in 'Ob dams at end-diastole compared to 'Con (Figure 3.21).

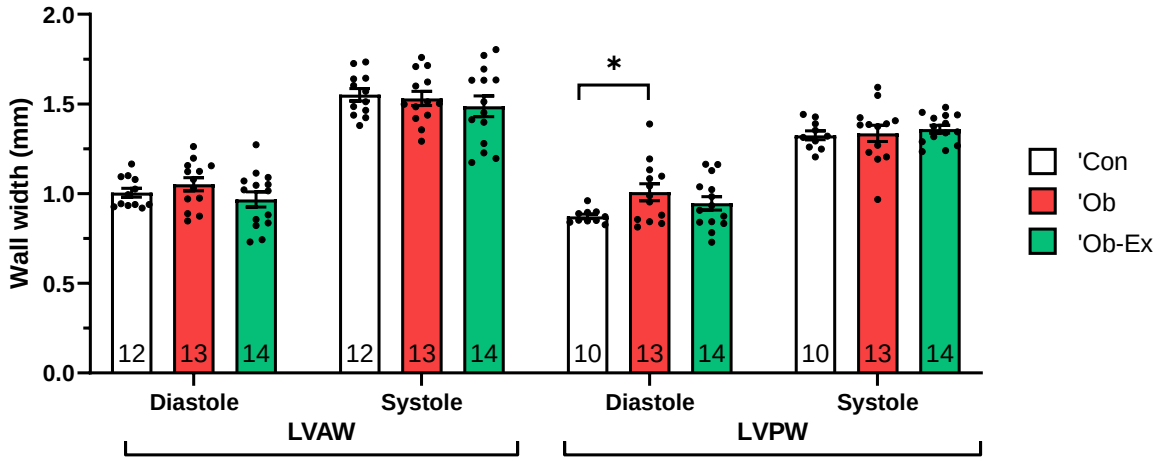


Figure 3.21: Left ventricle (LV) wall widths of E18.5 dams. *Width of LV anterior wall (LVAW) and LV posterior wall (LVPW) in diastole and systole, measured by echocardiography using M-mode images taken in PSLAX. *p<0.05 using Tukey's multiple comparison test after a significant effect of Group found by Welch ANOVA. Numbers presented inside bars.*

3.3.8 Haematocrit of pregnant dams

There was no difference in haematocrit between 'Con and 'Ob dams in late pregnancy (Figure 3.22(A)). Given that pregnancy-associated increases in blood volume result in relative haemodilution and a decrease in haematocrit[369], this could suggest that maternal obesity does not affect blood volume expansion in this model, although further data is needed. Measuring the haematocrit of 'Con and 'Ob dams both before pregnancy and in late gestation would enable the relative gestational changes in haematocrit to be calculated which would provide further information on plasma volume expansion.

Haematocrit was increased in 'Ob-Ex dams compared to 'Con at E17.5 (Figure 3.22(A)). This suggests that despite exercise training stimulating increases in blood volume in non-pregnant humans[432], and that lean women who exercise regularly before and throughout pregnancy have augmented increases in stroke volume compared to sedentary women[413], blood volume expansion was reduced in 'Ob-Ex dams compared to 'Con in this study. Blood volume did increase over the course of pregnancy in 'Ob-Ex dams, as their haematocrit was lower than NP obese-exercised females (see Section 3.3.2).

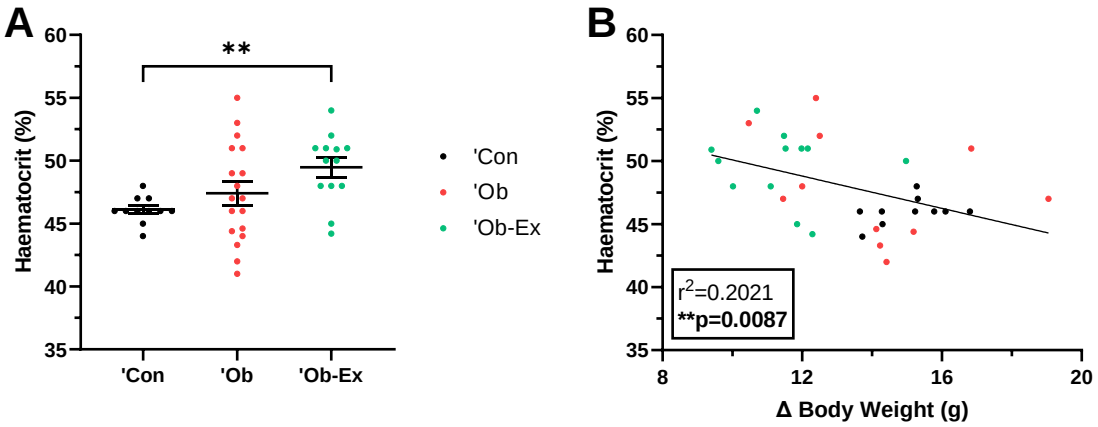


***Figure 3.22: Dam haematocrit at E17.5**. Dam haematocrit (A), **p<0.01, using post hoc Tukey's multiple comparison test after a significant effect of Group found by Welch ANOVA. Dam haematocrit plotted against gestational weight gain (B) analysed by simple linear regression. Numbers: 'Con = 11, 'Ob = 18, 'Ob-Ex = 13*

Across all three groups of pregnant dams, haematocrit at the end of gestation negatively correlated with GWG (Figure 3.22(B)). This suggests that the reduced haematocrit in 'Ob-Ex compared to 'Con dams is because the increase in blood volume in the 'Ob-Ex dams was proportional to their smaller increase in body weight during pregnancy compared to non-exercised females. These data complement those presented in Figure 3.10(B), as haematocrit correlated with body weight change between day of plug and 17 days later in pregnant 'Ob-Ex dams and NP obese-exercised females.

3.3.9 Systolic function in late pregnancy

There was no effect of group on any of the indices of systolic dysfunction measured on E18.5 by echocardiography (Table 3.5). Therefore, while the decreased relative heart weight of 'Ob dams (Figure 3.20(B)) and the increased absolute heart weight of 'Ob-Ex dams (Figure 3.20(A)), both compared to 'Con, suggest that maternal obesity and exercise may affect pregnancy-associated cardiac hypertrophy, any such differences between groups were not sufficient to result in differences in systolic function. While no significant differences were found between groups, both EDV and stroke volume correlated with GWG (Figure 3.23), just as they did with body weight change between day of plug and 17 days later in pregnant and NP obese-exercised females in Figure 3.12. Together, these data further support that blood volume has increased in these pregnant dams with advancing gestation, as expected, that this is proportional to GWG, and that this increase in blood volume is having the anticipated effect on increasing MSFP, EDV[374], and subsequently stroke volume.

	'Con (n=12)	'Ob (n=14)	'Ob-Ex (n=14)	p-value
Heart Rate (bpm)	492 ± 14	507 ± 14	487 ± 14	0.6621
Diastolic Area (mm^2)	24.2 ± 0.6	22.7 ± 0.8	23.8 ± 0.8	0.3563
Systolic Area (mm^2)	12.7 ± 0.3	11.2 ± 0.5	12.2 ± 0.8	0.1544
Diastolic Volume (uL)	64.5 ± 2.6	58.3 ± 3.4	62.7 ± 3.6	0.3735
Systolic Volume (uL)	20.9 ± 0.9	17.1 ± 1.2	20.2 ± 2.2	0.1742
Stroke Volume (uL)	43.6 ± 2.5	41.2 ± 2.3	42.5 ± 1.9	0.7403
Cardiac Output (mL/min)	21.2 ± 1.0	20.7 ± 1.0	20.8 ± 1.2	0.9218
Ejection Fraction (%)	67.2 ± 1.6	70.7 ± 0.8	68.5 ± 1.9	0.2374
FS (radial) (%)	43.7 ± 0.7	41.5 ± 1.4	42.3 ± 1.7	0.5252
LVEnL (diastole) (mm)	7.82 ± 0.11	7.65 ± 0.13	7.80 ± 0.13	0.1710
LVEnL (systole) (mm)	6.94 ± 0.12	6.78 ± 0.12	6.80 ± 0.12	0.6024
FS (longitudinal) (%)	11.3 ± 0.6	11.2 ± 0.6	12.8 ± 0.5	0.1268

Table 3.6: Echocardiographic indices of left ventricular systolic function in E18.5 dams.
Stated p-values reflect results of one-way ANOVA or Kruskal-Wallis test for effect of Group. FS = fractional shortening, LVEnL = Left ventricle endocardial length.

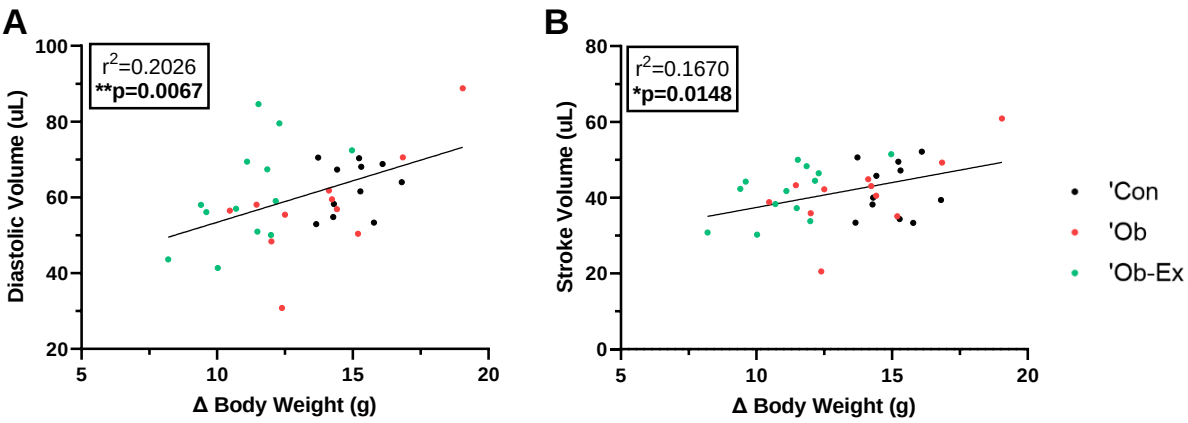


Figure 3.23: ***Correlations between heart volume at E18.5 and gestational weight gain.*** *Diastolic volume (A) and stroke volume (B) plotted against gestational weight gain. r^2 and p-values from simple linear regression analyses. Numbers: 'Con = 12, 'Ob = 14, 'Ob-Ex = 14*

3.3.10 Diastolic function in late pregnancy

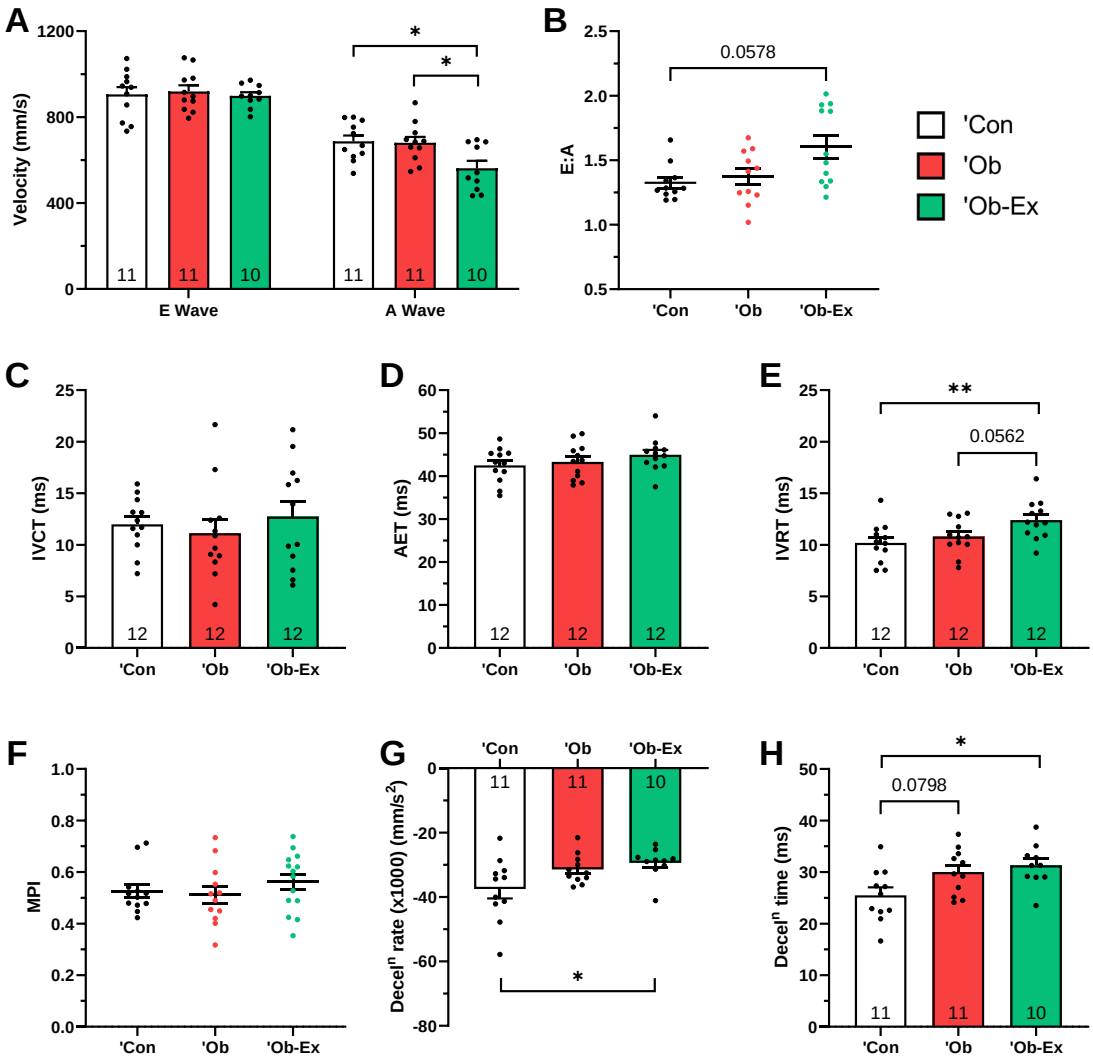


Figure 3.24: Echocardiographic indices of left ventricular diastolic function in E18.5 dams.
*Peak E and A wave velocities (A), E:A ratio (B), isovolumetric contraction time (IVCT) (C), aortic ejection time (AET) (D), isovolumetric relaxation time (IVRT) (E), myocardial performance index (MPI) (F), and mitral valve deceleration rate (G) and time (H). *p<0.05 and **p<0.01, using Tukey's multiple comparison test after a significant effect of Group found by one-way ANOVA or Kruskal-Wallis test. Numbers in (B) match those presented in bars in (A), numbers in (F) match those in bars in (C)-(E).*

All echocardiographic measurements relating to dam diastolic function taken at E18.5 are presented in Figure 3.24. No significant differences in indices of diastolic function between 'Con and 'Ob dams were found. Therefore, obesity during pregnancy did not result in overt diastolic dysfunction in late gestation in this study. However, differences in diastolic function were identified in 'Ob-Ex dams.

While there was no effect of group on peak E wave velocity in late gestation, peak A wave velocity was decreased in 'Ob-Ex dams compared to both 'Con and 'Ob (Figure 3.24(A)). This resulted in a non-significant increase in E:A ratio in 'Ob-Ex dams compared to 'Con (Figure 3.24(B)).

No differences in IVCT or AET were found between groups (Figure 3.24(C,D)), but IVRT was increased in 'Ob-Ex dams compared to 'Con (Figure 3.24(E)). There was also a trend for increased IVRT in 'Ob-Ex dams compared to 'Ob dams, but this did not reach statistical significance (p=0.0562). No effect of group on MPI was identified (Figure 3.24(F)). E wave deceleration rate was decreased in 'Ob-Ex dams compared to 'Con, resulting in a concurrent increase in deceleration time (Figure 3.24(G,H)). These findings must be considered alongside the limited published echocardiographic data on how blood flow through the mitral valve changes during pregnancy and what was found in Section 3.3.3 of this thesis when comparing pregnant 'Ob-Ex dams and NP obese-exercised females.

As discussed in Section 3.3.3, the few published studies of pregnancy-associated changes in mitral valve blood flow find that the peak velocity of both the E and A waves increases during pregnancy[426,427], and that the A wave increase is greater than that of the E wave by late gestation[426,427]. This results in the E:A ratio usually being reduced in late gestation relative to the non-pregnant state. Pregnancy is also associated with decreases in both in IVRT and deceleration time[428]. The decreased peak A wave velocity, non-significantly decreased E:A ratio, increased IVRT, and increased deceleration time in 'Ob-Ex dams therefore all suggest that diastolic function is not changing as expected or to the same extent in 'Ob-Ex dams compared to 'Con and 'Ob. This is supported by the data presented in Section 3.3.3 of this thesis. Peak E wave velocity was increased in pregnant 'Ob-Ex dams relative to NP females, but peak A wave velocity was not (Figure 3.13(A)). Moreover, no differences in IVRT or deceleration time were found between pregnant and NP obese-exercised females (Figure 3.13(E,H)). Taken together, these data suggest that the typical pregnancy-associated increase in peak E wave velocity occurred in all three groups of dams, but the other expected changes in indices of diastolic function: peak A wave velocity, IVRT, and deceleration time, likely occurred in 'Con and 'Ob dams, but not 'Ob-Ex. This is a novel and unexpected finding.

An increased IVRT is a common marker of diastolic dysfunction as it is indicative of impaired LV relaxation. However, a decrease in LV filling pressures will also result in an increase in IVRT. Therefore, the increases in both E and A wave peak velocities, decreased deceleration time, and decreased IVRT observed in normal pregnancy are likely all due to the increases in blood volume and subsequently MSFP. However, these changes were not seen in 'Ob-Ex dams compared to NP obese-exercised females, and this likely underlies the differences in these measures between 'Ob-Ex and 'Con dams. Together with the increased haematocrit in 'Ob-Ex dams, this is consistent with the 'Ob-Ex dams having a reduced blood volume expansion compared to both 'Con and non-exercised 'Ob dams, resulting in differences in indices of diastolic function.

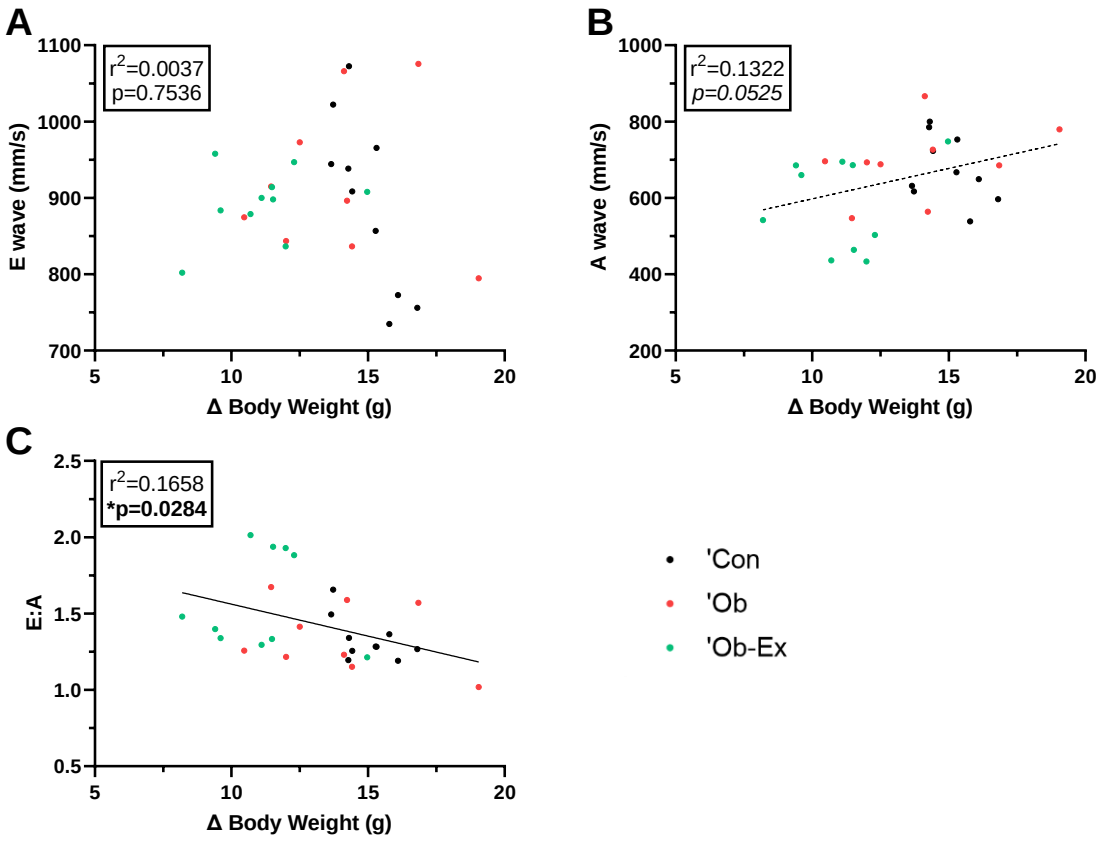


Figure 3.25: Correlation between both peak E and A wave velocities and E:A ratio at E18.5, and gestational weight gain. *Peak E-wave velocity (A), peak A-wave velocity (B), and E:A ratio (C) plotted against change in body weight in the 17 days after plug. r^2 and p-values from simple linear regression analyses. Numbers: 'Con = 10, 'Ob = 9, 'Ob-Ex = 10.*

In Section 3.3.3, peak E wave velocity was found to correlate with body weight change between the day of plug and 17 days later in pregnant and NP obese-exercised females (Figure 3.14(A)), but peak A wave velocity did not (Figure 3.14(B)). Conversely, when considering the same measurements made at E18.5 across the three groups of pregnant dams, there is no correlation between GWG and peak E wave velocity (Figure 3.25(A)), a non-significant correlation between GWG and peak A wave velocity (Figure 3.25(B)), and a significant negative correlation between GWG and E:A ratio (Figure 3.25(C)). These data further support that the decreased peak A wave velocity in 'Ob-Ex dams compared to 'Con, and subsequent increase in E:A, are due to reduced blood volume expansion in the 'Ob-Ex dams proportional to their lower GWG.

Increased deceleration time is also seen in obese non-pregnant women[149], and it is postulated to be the result of the increased blood volume, rather than changes in cardiac relaxation[394]. Deceleration rate was not significantly reduced in 'Ob dams compared to 'Con (p=0.1109) but 'Ob dams tended to have a longer deceleration time (p=0.0798). This may suggest that the obesity phenotype of the 'Ob dams in this study was not severe enough to result in changes in diastolic function.

3.3.11 Pulmonary artery flow in late pregnancy

					Mixed-Effects Analysis		
	View	'Con (n=11-12)	'Ob (n=11-12)	'Ob-Ex (n=12-14)	View	Group	Inter^{n}
Mean Velocity (mm/s)	Apic-4	-437 ± 18	-451 ± 15	-455 ± 17	**<0.0001**	0.8420	0.2522
	PSAX	-514 ± 14	-506 ± 13	-485 ± 10			
Peak Velocity (mm/s)	Apic-4	-825 ± 22	-869 ± 31	-873 ± 28	**0.0003**	0.9151	0.2193
	PSAX	-955 ± 28	-940 ± 33	-913 ± 22			
Mean Gradient (mmHg)	Apic-4	0.778 ± 0.061	0.825 ± 0.055	0.840 ± 0.058	**<0.0001**	0.8081	0.2288
	PSAX	1.066 ± 0.060	1.032 ± 0.056	0.949 ± 0.043			
PAT (ms)	Apic-4	21.1 ± 0.7	21.8 ± 0.8	21.6 ± 0.8	0.4458	0.9561	0.7656
	PSAX	21.2 ± 0.6	20.9 ± 0.8	21.1 ± 0.7			
PET (ms)	Apic-4	53.7 ± 1.1	51.5 ± 1.6	54.7 ± 1.2	**<0.0001**	0.3331	0.3545
	PSAX	59.1 ± 1.0	57.8 ± 1.2	57.8 ± 1.1			
PAT/PET	Apic-4	0.393 ± 0.012	0.425 ± 0.015	0.396 ± 0.015	**<0.0001**	0.3711	0.1498
	PSAX	0.358 ± 0.008	0.362 ± 0.010	0.372 ± 0.013			

Table 3.7: Echocardiographic measures of pulmonary artery (PA) flow in E18.5 dams.
Right-hand columns present the results of mixed-effects analysis for the effect of view (PSAX or Apic-4), group, and the interaction between the two (inter^{n}). FS = fractional shortening, LVEnL = Left ventricle endocardial length. PAT = PA acceleration time. PET = PA ejection time.

All echocardiographic measurements relating to blood flow through the PA in E18.5 dams are presented in Table 3.6. In line with what was found when comparing PA flow between pregnant 'Ob-Ex dams and NP females (Section 3.3.4, Figure 3.15), when analysed by two-way ANOVA for view (PSAX or Apic-4) and group ('Con, 'Ob, or 'Ob-Ex), view had a significant effect on all measure except PAT. There was no effect of group on any of the measures of pulmonary flow analysed. Therefore, while pregnancy itself was associated with changes in PA flow in obese-exercised females (Section 3.3.4, Figure 3.15), there was no effect of either maternal obesity or the exercise intervention on PA flow in late gestation in pregnant dams.

3.3.12 Reactivity of systemic vasculature in late pregnancy

In a normal human pregnancy, a parallel increase in CO and decrease in arterial blood pressure occur during early gestation, which is associated with a significant drop in SVR[377]. While no significant changes in SBP between pre-conception and mid-gestation were identified in 'Con dams in this study (see Section 3.3.6), that heart rate increased without a concurrent increase in blood pressure is consistent with SVR decreasing as expected. As discussed in Section 3.1.3, several studies have found that the arterial blood pressure of obese women does not transiently decrease during pregnancy as expected[401,407], and that obese pregnant women have higher SVR than lean pregnant women[404], suggesting that obesity may impair pregnancy-associated changes in SVR in humans. That no changes in either SBP or heart rate were observed in 'Ob dams in Section 3.3.6 could suggest that there are impairments in cardiovascular adaptation to pregnancy in these dams, including in reducing SVR.

While the pregnancy-associated drop in SVR is in part due to the addition of the low resistance uteroplacental unit, significant systemic vasodilation is responsible for much of this change. This systemic vasodilation is due to both an increased release of and vascular sensitivity to vasodilators, resulting in endothelium-dependent vasodilation[380], and an increased vascular resistance to vasoconstrictors[378] including both NA[382] and AngII[383]. Defects in these changes in systemic vascular reactivity are associated with the development of pre-eclampsia[433]. Changes in reactivity of mesenteric arteries to both vasoconstrictors and vasodilators have been found during pregnancy using rodent models[434,435].

In this study, responses to vasomotive agents were assessed in isolated femoral arteries from pregnant dams. This means that these data cannot be used to make comparisons between the pregnant and non-pregnant state. Rather, this study set out to investigate whether femoral artery reactivity to vasoconstrictors and vasodilators was different between lean and obese pregnant dams in order to infer whether maternal obesity altered systemic vascular function in pregnancy, as this could contribute to the increased incidence of pre-eclampsia in overweight and obese women.

Response to depolarisation

There was no significant effect of group on the contractile response of isolated femoral arteries to VSMC depolarisation by exposure to Krebs with an increasing concentration of K^+ (Figure 3.26). When analysed by one-way ANOVA, there was a trending effect of group on the AUC derived from the K^+ response curves (data not shown), but this did not reach significance ($p=0.0729$). This trending effect was likely driven by a non-significant increase in reactivity of vessels from 'Ob dams compared to both 'Con and 'Ob-Ex. Therefore, neither diet-induced obesity nor the exercise intervention resulted in a significant difference in the overall contractile capacity of femoral arteries isolated from E18.5 dams.

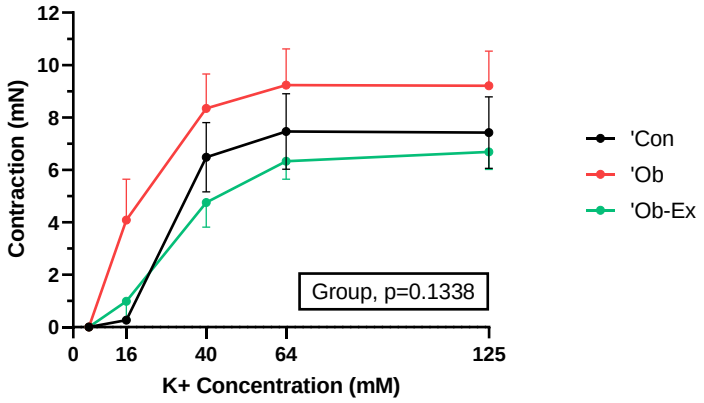


Figure 3.26: Femoral response to increasing concentration of K^+. *Response curves of femoral arteries from E18.5 dams to Krebs with an increasing concentration of K^+, with box showing group effect p-value from mixed-effects analysis. Numbers: 'Con = 6, 'Ob = 7, 'Ob-Ex = 6.*

Response to increasing concentration of PE

There was no effect of group on femoral artery contractile response to an increasing concentration of PE, whether expressed as absolute change in tension from a normalised baseline (Figure 3.27(A)) or relative to the matched vessel's maximal response to K^+ (Figure 3.27(B). Therefore, neither diet-induced obesity nor the exercise intervention altered the response of femoral arteries from E18.5 dams to alpha-adrenergic stimulation in this study.

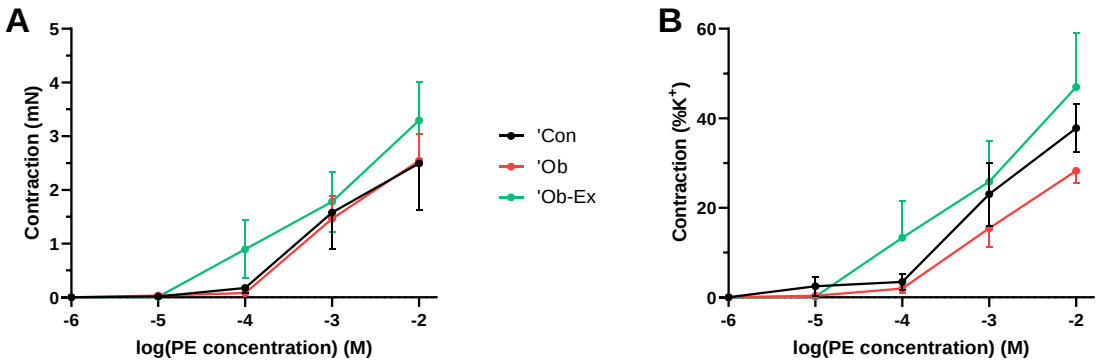


Figure 3.27: Femoral vascular response to increasing concentration of phenylephrine (PE). *Femoral artery dose-response curves to PE from E18.5 dams expressed as absolute change in tension normalised to 10^{-6}M PE (A). Data from these dose-response curves was then transformed according to the vessel's maximal contraction to elevated K^+ (%K^+) (B). Numbers: 'Con = 6, 'Ob = 6, 'Ob-Ex = 5.*

However, the femoral response to PE did not plateau within the range of doses given, and therefore group differences in reactivity may not have been detectable in as they may only become apparent at higher concentrations of PE.

Response to increasing concentration of SNP

No effect of group on isolated femoral artery relaxation in response to the NO donor SNP was found (Figure 3.28). However, vessels did not tend to relax until they were exposed to more than 10^{-4}M SNP, the mean maximal relaxation to SNP across the three groups was 60%, and the response did not plateau within the range of doses administered.

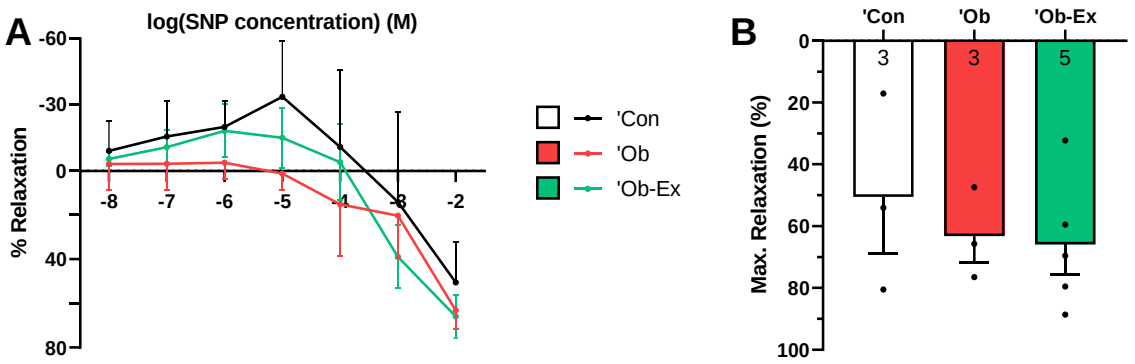


Figure 3.28: Femoral vascular response to increasing concentration of sodium nitroprusside (SNP). *Dose-response curves to SNP by arteries from E18.5 dams (A), expressed as %Relaxation following pre-constriction by exposure to noradrenaline with corresponding values of maximal %Relaxation (B). Numbers: 'Con = 3, 'Ob = 3, 'Ob-Ex = 5.*

The SNP used in this study was made from a 10^{-2}M stock in distilled water that had been stored -20°C for up to 18 months prior to being used. Unfortunately, this may have resulted in the stock deteriorating such that the concentrations that the vessels were exposed to in these experiments were far lower than intended.

Response to increasing concentration of ACh

No effect of group on isolated femoral artery relaxation in response to ACh was found (Figure 2.29). Vessels did not tend to relax until they were exposed to more than 10^{-5}M ACh.

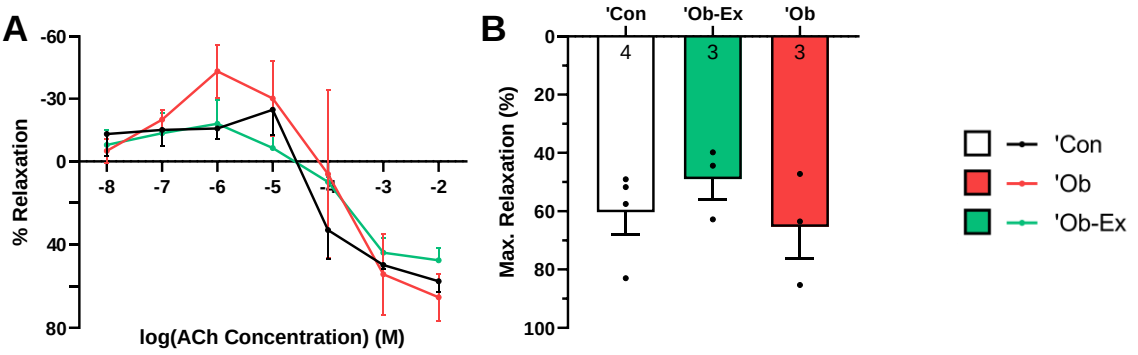


Figure 3.29: Femoral vascular response to increasing concentration of acetylcholine (ACh). *Dose-response curves to ACh by arteries from E18.5 dams (A), expressed as %Relaxation following pre-constriction by exposure to noradrenaline with corresponding values of maximal %Relaxation (B). Numbers: 'Con = 4, 'Ob = 3, 'Ob-Ex = 3.*

Although no changes in either SBP or heart rate were observed in 'Ob dams in Section 3.3.6, which might suggest that there are impairments in vascular adaptation to pregnancy in these dams, neither maternal diet nor the exercise intervention affected the vascular reactivity of femoral arteries isolated from E18.5 dams in this study. However, interpretation of these data is made more difficult by several factors including the lack of plateau in the PE dose response curves, that the SNP used was likely at a far lower dose than intended due to storage issues, and that one of the myograph transducers being broken resulted in numbers being lower than they should have been.

When searching the literature, one study investigating the effect of ten days of maternal high-fat feeding before mating and throughout pregnancy on mesenteric artery reactivity in late-gestation rats was identified. This study found a decreased reactivity to both ACh and NO in mesenteric arteries from dams fed the HFD, but no effect on vessel contraction in response to K^+ or NA[436]. The next closest study used the same model with or without diabetes to assess femoral vascular reactivity in dams 16 days after they had birthed a litter[284]. High-fat feeding alone had no effect on femoral reactivity in these postpartum dams, whereas combined high-fat feeding and diabetes increased femoral reactivity to NA and decreased reactivity to ACh. However, these studies may not be clinically relevant to obese women as the diet alone had no effect on maternal body weight within either study period and the maternal diabetes was chemically induced by injection with streptozotocin at the start of pregnancy in the study of postpartum dams

Even if differences in femoral vascular reactivity were identified in this study or others, this would not guarantee that SVR would have been altered in these animals, as myography data alone are not sufficient to draw such conclusions. Measurement of SVR requires simultaneous measurement of CO and MAP, which is typically achieved in mice by catheterisation of the left ventricle and carotid artery[437]. Applying this to pregnant dams in a model of diet-induced obesity would provide further insight into the effects of maternal obesity on cardiovascular adaptation to pregnancy.

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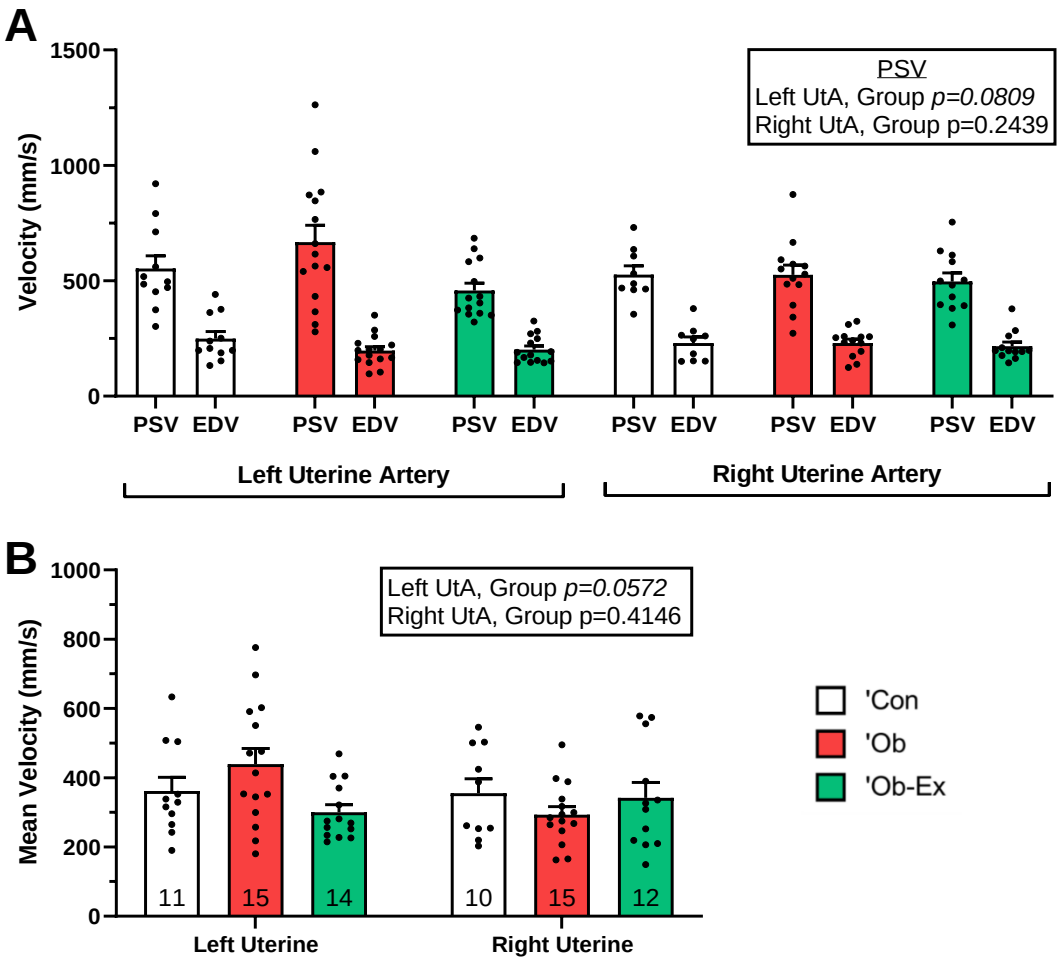
3.3.13 Uterine artery blood flow in late pregnancy

It is well established that over the course of pregnancy, uterine artery blood flow increases and becomes less pulsatile due to remodelling of the spiral arteries. This results in decreases in pulsatility index (PI) over the course of gestation[389]. In overweight and obese women, this physiological decrease in PI is often attenuated[438,439], which is indicative of impaired development of the low resistance uteroplacental unit. Increased uterine artery PI is associated with an increased risk of hypertensive disorders of pregnancy and IUGR[440]. In order to investigate whether this impairment in changes in uterine artery blood flow in obese women may also be present in the obese dams in this study, blood flow through both uterine arteries was measured in anaesthetised dams using PW Doppler in late pregnancy.

Data from the analysis of uterine artery flow are presented in Figure 3.30. When analysed by two-way ANOVA for the effect of group or side (left versus right UtA (L/R)), there was a significant interaction between these two factors on both PSV ($p=0.0146$) and mean velocity ($p=0.0298$). These data were therefore separated and analysed by side-specific one-way ANOVA for an effect of group. One-way ANOVA analysis revealed no effect of group on either PSV or mean velocity in the right UtA, and a non-significant trend for an effect of group on both measures in the left UtA ($p=0.0809$ and $p=0.0572$ respectively). There was no effect of either side (L/R) or group on EDV, PI or resistive index (RI). That there was no significant effect of group on any of the measurements of uterine artery blood flow was unexpected, not only because impairments in the physiological decrease in PI over the course of gestation is reported in humans[438,439], but because an increase in PI in late gestation has been identified in obese dams using our mouse model of maternal diet-induced obesity previously. These data therefore do not replicate previous data from our group using the same model.



C

					Mixed-Effects Analysis		
	L/R	'Con (n=10)	'Ob (n=15)	'Ob-Ex (n=13)	L/R	Group	Inter^{n}
Pulsatility Index (PI)	Left	0.864 ± 0.054	0.839 ± 0.027	0.857 ± 0.033	0.5360	0.6066	0.6967
	Right	0.938 ± 0.116	0.854 ± 0.039	0.845 ± 0.046			
Resistive Index (RI)	Left	0.553 ± 0.019	0.552 ± 0.012	0.559 ± 0.012	0.6831	0.8848	0.6540
	Right	0.576 ± 0.040	0.558 ± 0.017	0.548 ± 0.018			

***Figure 3.30: Uterine artery (UtA) flow in E18.5 dams**. Peak systolic velocity (PSV) and EDV (end diastolic velocity) (A), and mean velocity (B) through left and right UtAs. Table (C) shows pulsatility index (PI) and resistive index (RI) of UtA flow through each uterine artery and right-hand columns present the results of mixed-effects analysis for the effect of left versus right UtA (L/R), group, and the interaction between the two (inter^{n}). Boxes in (A) and (B) show results of left- or right-UtA specific one-way ANOVA or Kruskal-Wallis test for an effect of group after a significant interaction was found by mixed-effects analysis. Numbers in (A) match those presented in bars in (B).*

Hufnagel *et al*. found increased PI and RI in obese dams compared to lean dams at E18.5[293]. However, in the data included in this thesis, no effect of maternal obesity was found on either PI or RI. When comparing the data from this study with that from Hufnagel *et al*. it seems that this is predominantly due to higher PI and RI values in lean dams in this study (Table 3.7). It should be noted that in the Hufnagel et al. study, both PI and RI correlated with maternal heart rate and thus the authors corrected for this. No correlation with heart rate was found in this study and so the data presented in this thesis is not corrected for heart rate. In the interest of comparing like for like, none of the data presented in Table 3.7 is corrected for maternal heart rate.

	Pulsatility Index		Resistive index	
Cohort	Lean dam	Obese dam	Lean dam	Obese dam
Wilsmore	0.901 ± 0.085	0.847 ± 0.033	0.565 ± 0.030	0.555 ± 0.015
Hufnagel *et al*.	0.671 ± 0.006	0.796 ± 0.004	0.477 ± 0.003	0.545 ± 0.002

Table 3.8: Pulsatility and resistive indexes of uterine artery blood flow in E18.5 lean and obese dams across two cohorts.

There are three differences between these cohorts of animals that could explain why the same differences in uterine artery PI and RI between lean and obese dams in late pregnancy were not found in this study. Most notably, there was a smaller difference in body weight between the lean and obese dams in this study compared to those in Hufnagel *et al*., the details of which are presented in Table 3.8. In the current study the controls were heavier and the obese dams lighter than previously.

g	Day of Plug Body Weight		Late Gestation Body Weight	
Cohort	Lean dam	Obese dam	Lean dam	Obese dam
Wilsmore	26.3 ± 0.6	33.3 ± 0.7	41.3 ± 0.5	47.3 ± 1.0
Hufnagel *et al*.	24.5 ± 0.2	36.9 ± 0.1	39.0 ± 0.3	50.5 ± 0.2

Table 3.9: Body weight of lean and obese dams at the start and end of pregnancy across two cohorts.

While obese dams were on average 12.4 g heavier than lean dams in late gestation in Hufnagel *et al*., in this study the difference was only 6 g – meaning that the difference in body weight between the groups in this study was half that in Hufnagel *et al*. In this study, the threshold used for the mating of obese dams was 10 g of body fat as measured by TDNMR, and this threshold has been used in this model previously[301]. However, in the Hufnagel *et al*. study, 35 g body weight was used as the threshold for mating obese dams. This was used due to recurrent problems with the TDNMR machine at the time that that study was being run making it not possible to use a mating threshold based on body fat.

As a result of these differences, the obesity phenotype is weaker in obese dams in this study than in Hufnagel *et al*. It is therefore particularly notable that in spite of this reduced maternal obesity phenotype and lack of uterine artery PI increase, the IUGR phenotype consistently seen in our model was still seen in this study. This is discussed further in Section 3.5.2.

While this huge discrepancy in body weight difference is most likely the reason that the difference in PI found in Hufnagel *et al.* was not replicated in this study, there are two further differences between the cohorts that should be noted. Firstly, the uterine artery PW Doppler measurements were the first ultrasound measurements made in Hufnagel *et al*., and thus were made within 15 minutes of the mouse being anaesthetised. In this study, maternal echocardiographic measures were taken before the uterine artery PW Doppler and so mice had been anaesthetised for approximately 40 minutes before these measures were made. It is well established that the anaesthetic used, isofluorane, is a cardiorespiratory suppressant and causes vasodilation. Therefore, the greater exposure to isofluorane before measurements were made may have masked any differences in PI and RI in this study. Secondly, the average heart rate during the uterine PW Doppler measurements was very different between the two cohorts, being higher in this study in both lean (531 ± 2 bpm) and obese dams (532 ± 4 bpm) compared to the Hufnagel *et al*. study (lean: 492 ± 4, obese: 465 ± 1 bpm).

3.3.14 Uterine artery myography

While much of the increase in blood flow through the uterine arteries with advancing gestation is due to significant remodelling of the downstream spiral arteries, the reactivity of the uterine arteries to vasomotive agents changes during pregnancy just as the reactivity of the systemic vasculature does[441]. In this study, responses to vasomotive agents were assessed in isolated uterine arteries from pregnant dams. As with the femoral artery myography data presented in Section 3.3.12, these data cannot be used to make comparisons between the pregnant and non-pregnant state. Rather, uterine artery reactivity to vasoconstrictors and vasodilators was investigated in both lean and obese pregnant dams in order to infer whether maternal obesity altered uterine vascular function in pregnancy, as this could result in reduced blood flow to the fetus and might contribute to the observed IUGR phenotype.

Response to depolarisation

There was no effect of group on the contractile response of isolated uterine arteries from E18.5 dams to VSMC depolarisation by exposure to Krebs with an increasing concentration of K^+ (Figure 3.31).

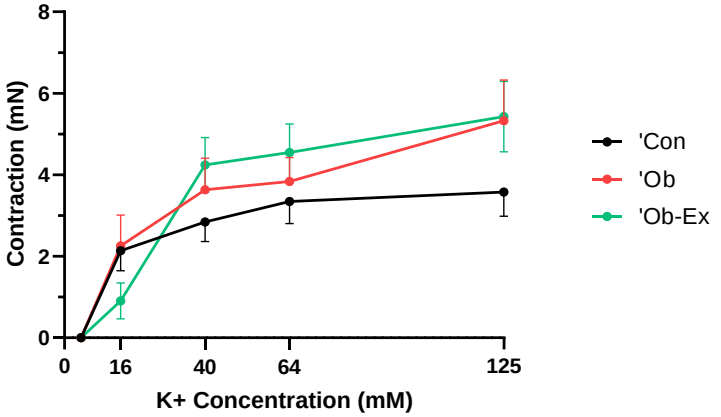


Figure 3.31: Uterine vascular response to increasing concentration of K^+. *Response curves of uterine arteries from E18.5 dams to Krebs with increasing K^+. Numbers: 'Con = 6, 'Ob = 8, 'Ob-Ex = 6.*

Response to increasing concentration of PE

When uterine artery PE dose-response curves where analysed by mixed-effects analysis, there was a trending effect of group whether expressed as absolute change in tension from a normalised baseline (p=0.0793) (Figure 3.32(A)) or relative to maximal K^+ response (p=0.0620) (Figure 3.32(B)). However, this did not reach statistical significance. Uterine arteries from 'Con and 'Ob dams contracted more forcefully in response to PE than the maximum contraction elicited to K^+ (mean maximal contraction to PE: 119 ± 6 %K^+ and 102 ± 4 %K^+ respectively) (Figure 3.32(B)). This was not the case for uterine arteries from 'Ob-Ex dams (86 ± 9 %K^+), resulting in a reduced maximal contraction in response to PE when expressed relative to K^+-induced contraction in 'Ob-Ex compared to 'Con (p=0.0063) when analysed by one-way ANOVA with Tukey's post hoc testing.

The femoral response to PE did not plateau within the range of doses given in any group, and therefore significant differences in reactivity may not have been detectable in as they may only become apparent at higher concentrations of PE. Regardless, that when expressed relative to the maximal contraction in response to depolarisation, the maximal response to the selective α1R-agonist PE was decreased in uterine arteries from 'Ob-Ex dams compared to 'Con is indicative of reduced uterine artery reactivity to alpha-adrenergic vasoconstriction in 'Ob-Ex dams.

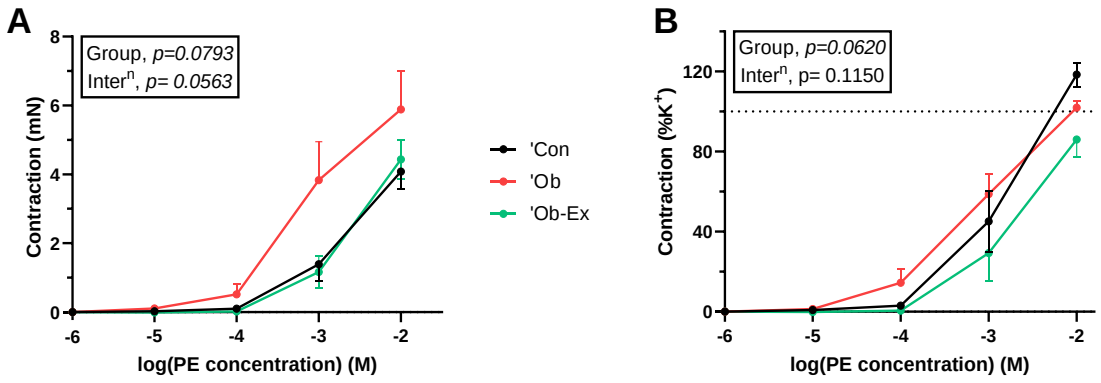


Figure 3.32: Uterine vascular response to increasing concentration of phenylephrine (PE). *Uterine artery dose-response curves to PE from E18.5 dams expressed as absolute change in tension normalised to 10^{-6}M PE (A). Data from these dose-response curves was then transformed according to the vessel's maximal contraction to elevated K^+ (%K^+) (B). Boxes show p-value of effect of group and interaction (inter^n) from mixed-effects analysis. Numbers: 'Con = 6, 'Ob = 7, 'Ob-Ex = 6.*

Response to increasing concentration of SNP

No effect of group on isolated uterine artery relaxation in response to the NO donor SNP was found (Figure 3.33). Vessels did not tend to relax until they were exposed to more than 10^{-4}M SNP, the mean maximal relaxation to SNP across the three groups was less than 30%, and the response did not plateau within the range of doses administered. As described in Section 3.3.12, the stock that the SNP used in this study was made from may have deteriorated due to being stored at -20°C for up to 18 months prior to being used such that the concentrations that the vessels were exposed to in these experiments were far lower than intended.

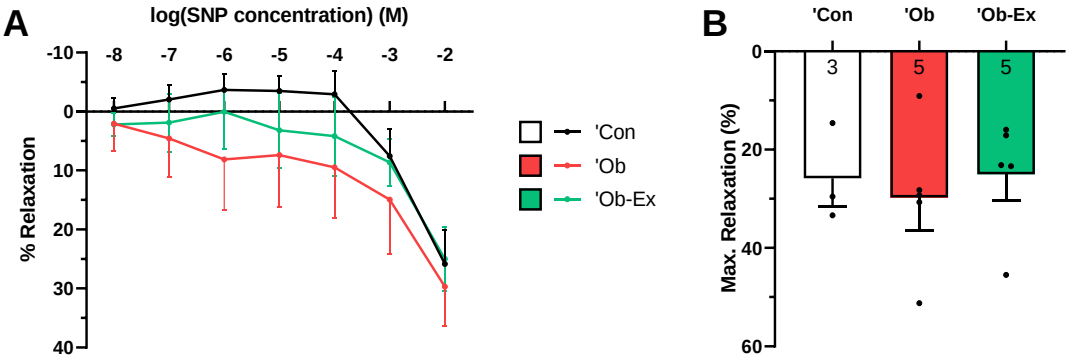


Figure 3.33: Uterine vascular response to increasing concentration of sodium nitroprusside (SNP). *Dose-response curves to SNP by arteries from E18.5 dams (A), expressed as %Relaxation following pre-constriction by exposure to noradrenaline with corresponding values of maximal %Relaxation (B). Numbers: 'Con = 3, 'Ob = 5, 'Ob-Ex = 5.*

Response to increasing concentration of ACh

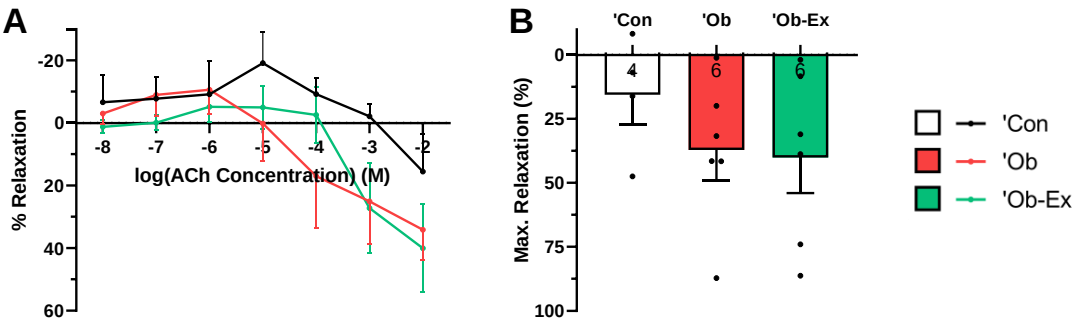


Figure 3.34: Uterine vascular response to increasing concentration acetylcholine (ACh).
Dose-response curves to ACh by arteries from E18.5 dams (A), expressed as %Relaxation following pre-constriction by exposure to noradrenaline with corresponding values of maximal %Relaxation (B). Numbers: 'Con = 4, 'Ob = 6, 'Ob-Ex = 6.

No effect of group on isolated uterine artery relaxation in response to ACh was found (Figure 3.34). Vessels from 'Con and 'Ob dams did not tend to relax until they were exposed to more than 10^{-6}M ACh, while those from 'Ob-Ex did not tend to relax until they were exposed to more than 10^{-4}M ACh. Moreover, the data were highly variable and no clear pattern of ACh-induced vasodilation was seen in any group. As discussed in Section 3.1.1, ACh-mediated vasodilation is endothelium-dependent. Therefore, that the uterine arteries in this study did respond to SNP (albeit at higher doses than expected) but had almost no response to ACh is indicative of endothelial dysfunction. However, it is highly likely that rather than endothelial dysfunction, this may be an artefact of the endothelium being damaged during the mounting process. One must take great care when mounting vessels on a wire myography to avoid touching the endothelium with the tip of the wire during mounting, as this will mechanically damage the endothelium. This is delicate but perfectly possible to achieve when mounting femoral arteries on a 40um wire once you are proficient at mounting vessels. However, a uterine artery is much narrower than a femoral artery and therefore it is much more difficult to mount a uterine artery on two 40um wires on a myograph without damaging the endothelium. It is highly likely that damage being done to the endothelium during mounting of the narrow uterine arteries in this study contributed to the poor and highly variable ACh-responses recorded. Some researchers use 25um wire to mount murine uterine arteries in order to reduce this issue, but this introduces problems of its own, not least that 25um wires are much weaker than 40um wires and thus more prone to snapping, and they also have a greater tendency to twist or bend, making inserting the wire through a vessel much more difficult to achieve without damaging the endothelium anyway.

Therefore, while this study found no significant differences in uterine artery endothelium-dependent relaxation (response to ACh) or endothelium-independent relaxation (response to the NO-donor SNP), this may have been due to experimental issues in both cases.

Only one study assessing the impact of maternal high-fat feeding on uterine artery reactivity during pregnancy was identified in literature searches. While this study did find changes in mesenteric artery reactivity, no differences in uterine artery reactivity to either vasoconstrictors or vasodilators in pregnant rats fed a HFD for ten days before pregnancy were identifed[436].

3.4 Key findings

1) The increase in maternal heart rate in early gestation in lean dams was abolished in obese dams, indicative of impaired cardiovascular adaptation to pregnancy. This was not rescued by maternal exercise intervention.
2) Obesity during pregnancy did not alter dam *in vivo* cardiac or *ex vivo* vascular function in late pregnancy.
3) Neither maternal obesity nor the exercise intervention altered blood flow through the uterine arteries in late gestation.
4) Even in the absence of a reduction in uterine artery PI, maternal obesity resulted in fetal growth restriction which was not corrected by maternal exercise.
5) The exercise intervention reduced gestational weight gain compared to both lean and obese dams. This was associated with changes in haematocrit and cardiac function consistent with reduced blood volume expansion.

3.5 Future work

While the data presented in this chapter are primarily physiological *in vivo* and functional data, both fixed and frozen tissues were collected from dams upon culling. These tissues are available for subsequent molecular and histological work to investigate the underlying mechanisms and structural changes associated with the functional differences identified in this thesis and thus address some of the outstanding questions from this work.

3.5.1 What underlies the differences in heart weight?

There was no difference in absolute heart weight between 'Con and 'Ob dams in this study, resulting in the heart weight of 'Ob dams relative to total body weight being reduced. Absolute heart weight was increased in 'Ob-Ex dams relative to 'Con, resulting in comparable relative heart weights between the two groups. This could suggest that 'Ob dam hearts did not undergo pregnancy-associated cardiac hypertrophy as expected, and that the exercise intervention may have corrected this by promoting physiological hypertrophy of the heart.

Whether the differences in cardiac hypertrophy between the three groups of dams are associated with differences in cardiomyocyte hypertrophy could be investigated by sectioning fixed hearts and staining them using wheat germ agglutinin. Wheat germ agglutinin binds glycoproteins on the cardiomyocyte surface membrane, allowing for visualisation of cell boundaries and determination of cell cross-sectional area[442]. This technique has been used previously by our group to establish that the cardiac hypertrophy seen in male 8-week-old offspring of obese dams is associated with cardiomyocyte hypertrophy[290,295,301].

Unlike pathological hypertrophy, pregnancy-induced cardiac hypertrophy is not associated with the re-expression of fetal genes[443]. Rather, pregnancy-induced cardiac hypertrophy is associated with decreased transcript and protein levels of Kv4.3, a K^+ channel expressed by ventricular cardiomyocytes, but not with changes in the expression of similar proteins including Kv4.2 and Kv1.4[443]. Assessing Kv4.3 expression, either by RT-qPCR or measuring protein level, in the frozen dam hearts collected in this study could therefore provide insight into potential mechanisms underlying the reduced cardiac hypertrophy seen in 'Ob dams.

3.5.2 What is causing the fetal growth restriction?

In this study, maternal obesity was associated with fetal IUGR. This is consistent with previous studies from the Ozanne group in which offspring of obese dams have been growth restricted in late gestation[293] or at birth[297] compared to those of lean dams.

Hufnagel *et al*.[293] suggested that reduced UtA compliance in obese dams was a contributing factor to this fetal growth restriction, as UtA PI correlated with fetal body weight. This is a perfectly reasonable assumption as reductions in uterine blood flow are associated with impaired fetal growth[444]. However, the reduction in UtA PI was not replicated in this study, likely due to a less severe obesity phenotype of the obese dams, but the fetal IUGR persisted. This suggests that there are other factors contributing to the growth restriction of fetuses of obese dams.

The placenta is the primary interface between the mother and fetus and ultimately controls nutrient supply to the fetus. Placental malfunction is the most common cause of IUGR[445]. Therefore, the next step in determining why the fetuses of 'Ob dams are growth restricted would be to turn to their placentas, which were fixed at the point of culling. Previous studies from our group have found that maternal obesity is associated with the downregulation of transcripts involved in the development of the labyrinth zone in placentas from obese dams[446] resulting in reduced labyrinth area[293,446]. This would be expected to impact fetal growth as the labyrinth zone is the primary site of nutrient exchange in the murine placenta. In line with this, in Hufnagel *et al*., placental labyrinth zone area also correlated with fetal body weight[293]. The next step in investigating what underlies the fetal IUGR found in this study would be to analyse placental structure, particularly to see if labyrinth zone area was reduced in the placentas from obese dams.

3.5.3 Is cardiovascular function in late gestation impaired in dams with a more severe obesity phenotype?

The lack of an increase in dam heart rate between conception and mid-gestation in 'Ob dams in this study is indicative of impaired cardiovascular adaptation to pregnancy. Nevertheless, any impairments in cardiovascular adaptation in these dams was not sufficient to result in overt diastolic or systolic dysfunction in late gestation, as assessed by echocardiography. Given that the obese dams in this study have been found to have a milder obesity phenotype than dams in previous studies from our group, a possible next step would be to investigate whether cardiovascular function is impaired in late gestation in dams with a more severe obesity phenotype. If this is the case, it could be particularly interesting to investigate whether impaired UtA compliance is associated with cardiac dysfunction, as both are indicative of impaired cardiovascular adaptation to pregnancy. Furthermore, it remains to be established whether the exercise intervention applied to obese dams in this study would improve maternal cardiovascular function in a more obese cohort. These queries would need to be assessed in a new cohort of fatter dams by using the same protocols used in this chapter.

Chapter 4: Cardiovascular Function in Young Adult Offspring

4.1 Introduction

4.1.1 Sex differences in cardiovascular disease

As introduced in the General Introduction of this thesis (see Section 1.2), CVD is the leading cause of death worldwide[11]. However, there are sex differences in both the morbidity and mortality of CVD. Pre-menopausal women are relatively protected against CVD, meaning that CVD is less common in younger women compared to age-matched men. The age of onset of CVD therefore tends to be later in women. Nevertheless, across their life course, men are at an increased risk of developing numerous CVDs, including coronary heart disease[54,447–449], stroke[449,450], and atrial fibrillation[451]. Women typically have a better prognosis than men, usually having a longer time between heart failure diagnosis and either requiring a heart transplant[452] or dying[305]. This improved prognosis in women with CVD is associated with reduced pathological LV hypertrophy[453] and cardiac remodelling[454,455], and reduced cardiomyocyte necrosis and apoptosis[452,455] compared to men. This relative protection is lost at menopause. The impact of ageing and menopause on cardiovascular function is discussed in Chapter 5, whereas this chapter will focus on young adults. Therefore, unless stated otherwise, in this chapter any reference to "women" is specifically meaning pre-menopausal women.

Much of the relative protection against CVD in pre-menopausal women is attributed to oestrogen. Most of the work performed to interrogate the role of oestrogen in female cardiac health uses ovariectomised rodents, to remove endogenous oestrogen, with or without exogenous oestrogen administration. Such studies have identified that oestrogen reduces pathological remodelling in response to experimentally-induced pressure-[456] and volume-overload[457], two key mechanisms of heart failure. Oestrogen has been found to confer protection against ischaemia-reperfusion injury *ex vivo*[458,459] in rats. This may in part be due to oestrogen maintaining cardiac mitochondrial function[460]. Mitochondria are a major source of ROS, and while low levels of ROS are required for normal cell function, elevated ROS can result in reduced NO bioavailability[461], as well as oxidative damage to both DNA, lipids, and proteins. The reduced NO bioavailability is of note because endogenous cardiac NO acts to prevent stiffening of the ventricle[462]. Oestrogen upregulates antioxidant enzymes and mitochondria from female rats produce half the amount of ROS that mitochondria from male rats do[463], this results in males having much higher levels of mitochondrial DNA oxidative damage. Therefore, circulating oestrogen confers protection against cardiac damage and pathological remodelling, which likely contributes to the reduced morbidity and mortality of CVD in pre-menopausal women.

4.1.2 Sex differences in hypertension and blood pressure homeostasis

The single greatest risk factor for the development of CVD is hypertension[55]. In The Framingham Heart study, 91% of patients who developed heart failure over a 20 year follow-up study first developed hypertension[305]. As with CVD, there are sex differences in the prevalence of hypertension with numerous studies finding that hypertension is more common in men than in women until approximately 40 years of age[464–466]. As introduced in Section 1.2.2 and expanded upon in Section 3.1.1, factors that affect systemic vascular resistance (SVR) affect blood pressure homeostasis and have been implicated in the pathogenesis of hypertension[60]. Numerous sex differences in factors that contribute to SVR regulation and blood pressure homeostasis have been identified and therefore may contribute to the difference in the prevalence of hypertension between young men and women.

As introduced in Section 3.1.1, basal endothelium-derived NO is a major determinant of SVR and MAP, with experimental decreases in plasma NO levels by eNOS-inhibitor-infusion resulting in increased SVR and MAP in humans[366]. As well as increasing NO bioavailability in the heart, oestrogen also directly increases vascular endothelial NO production via Oestrogen Receptor alpha (ERα)[467]. This promotes a more vasodilatory baseline state in the peripheral vasculature of women than men.

Increased activity of sympathetic vasoconstrictor nerves that innerve VSMCs, known as muscle sympathetic nerve activity (MSNA), acts to increase SVR in an individual by stimulating vasoconstriction via noradrenaline (NA) binding to α1Rs (alpha-adrenergic vasoconstriction) (see Section 3.1.1). However, while administration of NA via the brachial artery causes significant vasoconstriction in men, this response is reduced or not seen at all in women[468]. This suggests that NA may not influence SVR as expected in women. MSNA can be directly recorded from human peripheral nerves using microneurography[469], the insertion of tungsten microelectrodes into the nerve. At a population level, SVR positively correlates with MSNA in young men[470], however, the same relationship is not seen in women[471]. This supports the idea that factors other than sympathetic vasoconstrictor nerve activity modulate SVR in women. In addition to α1Rs, VSMCs express beta-2-adrenoreceptors (β2Rs), which preferentially bind circulating adrenaline. Activation of β2Rs results in VSMC relaxation and subsequent vasodilation (beta-adrenergic vasodilation). Both the vasoconstrictive-response to NA administration and the positive correlation between MSNA and SVR are unmasked in women following IV administration of a non-selective beta-adrenoreceptor antagonist[468]. IV administration of the same antagonist in men has no effect on either the relationship between MSNA and SVR, or vasoconstrictive response to NA.

Therefore, in men, sympathetic vasoconstrictor nerve activity is a major regulator of SVR, whereas in women, enhanced beta-adrenergic vasodilation acts to offset the vasoconstrictive effects of sympathetic vasoconstrictor nerve activity. This enhanced beta-adrenergic vasodilation may once again be attributable to oestrogen, as Ferrer et al.[472] found that oestrogen treatment increased vasodilatory responses to β2R stimulation in isolated mesenteric arteries from ovariectomised rats.

The renin-angiotensin-aldosterone system (RAAS) affects blood pressure homeostasis both by modulating vascular tone and by regulating blood volume, and increased RAAS activity has been implicated in heart failure[473] and obesity-associated hypertension[474]. This is because increased activation of the classical RAAS pathway results in increased circulating AngII and subsequently increased plasma aldosterone. AngII binds to Angiotensin Type 1 receptors (AT_1Rs) on VSMCs and is a potent vasoconstrictor, thereby increasing SVR, and aldosterone acts to increase fluid resorption in the kidney, resulting in increased blood volume. However, female mice are more resistant to developing hypertension in response to chronic AngII infusion[475] than male mice. Once again, this difference has been attributed to oestrogen. When considering vascular reactivity, ovariectomy results in increased contractile responses to AngII in rat aortic rings *ex vivo* which is associated with increased VSMC AT_1R expression[476]. These changes in vascular function are prevented with oestrogen administration, suggesting that oestrogen reduces vascular reactivity to AngII. Indeed, one study in humans found that men have an exaggerated increase in arterial blood pressure in response to IV administration of AngII compared to women[477], although other studies have found no such difference in response[478].

ET-1 is widely considered to be a potent vasoconstrictor. However, the vasomotive effect of ET-1 depends on the type and location of its receptor[479]. VSMCs predominantly express Endothelin-1 Receptor Type A (ET_AR), but also express Endothelin-1 Receptor Type B (ET_BR). Binding of ET-1 to either of these receptors on VSMCs results in vasoconstriction. However, the endothelium also expresses ET_BRs, and activation of endothelial ET_BRs results in endothelium-dependent vasodilation. Therefore, the location of ET_BR expression affects whether their activation stimulates vasoconstriction or vasodilation. Women typically have a greater proportion of endothelial ET_BRs relative to vascular smooth muscle ET-1 receptors than men[480]. As a result of this, subcutaneous administration of an ET_BR antagonist results in localised vasoconstriction in women, but vasodilation in men[481]. This suggests that ET_BR-activity contributes to tonic vasoconstriction in men, but to vascular relaxation in women.

Therefore, sex differences have been identified in several different systems that affect blood pressure homeostasis. These differences may contribute to the difference in the prevalence of hypertension between young men and woman.

4.1.3 Sex differences in programming of offspring cardiovascular dysfunction

As discussed in Section 1.5.9, while maternal obesity has been found to programme changes in cardiovascular structure and function in both male and female offspring at various ages, there is also evidence of sex-specific effects of maternal obesity or high-fat feeding on the offspring cardiovascular system from animal models. This includes that exposure to maternal HFD results in pathological cardiomyocyte hypertrophy in 12-week-old male offspring only. This sex-specific phenotype has been identified both in offspring of obese dams fed a HFD from weaning[275] and in offspring of dams only fed a HFD between mating and delivery[222]. This suggests that male offspring may be more susceptible to negative cardiac effects of exposure to maternal high-fat feeding. Previous studies by the Ozanne group have found increased heart weight[290,295,298,301] which is associated with pathological cardiomyocyte hypertrophy[290,295,301] in 8-week-old male offspring of obese dams, but whether this same phenotype is present in female offspring has not previously been investigated.

Meta-analyses of animal studies conclude that exposure to maternal obesity *in utero* increases SBP independent of both species and current age[224]. However, few studies have published blood pressure data in both male and female offspring, and those that have found that maternal obesity has both age- and sex-dependent effects on the programming of offspring hypertension. Both male and female offspring of rats fed a high-fat diet from ten days before pregnancy were found to have comparable SBP and DBP to offspring of control dams until 12 weeks of age[219], with increased arterial blood pressure only developing later in this model. This suggests that maternal high-fat feeding during pregnancy alone is not sufficient to result in overt differences in blood pressure in offspring at this age. Studies investigating offspring blood pressure and vascular function beyond 12 weeks of age are discussed in Chapter 5. A study in which female rats were fed the same obesogenic diet that we use in the Ozanne group from five weeks before pregnancy onwards found that arterial blood pressure was elevated in both male and female offspring at 4 and 12 weeks of age, suggesting that a greater maternal obesity phenotype is sufficient to alter offspring arterial blood pressure at this age. This was associated with a female-specific decrease in heart rate in 4-week-old offspring and a male-specific decrease in heart rate in 12-week-old offspring[282], indicating sex-specific differences in cardiovascular function. Using tail cuff plethysmography, previous studies in the Ozanne group have found an increase in evening SBP in 8-week-old male offspring of obese dams compared to controls[298,301]. These experiments have not been performed in female offspring.

Therefore, studies on the impact of exposure to maternal obesity *in utero* on cardiovascular structure and function that include both male and female offspring are limited and to date, all *in vivo* and *ex vivo* functional experiments assessing cardiovascular function by the Ozanne group in our model have only been performed in male offspring.

As introduced in Section 1.6.2, cardiac sympathetic dominance, dysregulation of the autonomic control of the heart with a shift towards greater sympathetic input and reduced parasympathetic modulation, is a marker of heart failure[304]. Blackmore et al. [295] previously found evidence of cardiac sympathetic dominance in 12-week-old male offspring of obese dams in our model. This study used the Langendorff preparation to investigate the ionotropic and chronotropic responses of the isolated heart. Hearts from 12-week-old male offspring of obese dams exhibited both increases in the inotropic response to sympathetic stimulation and decreases in the chronotropic and inotropic response to parasympathetic stimulation, therefore indicating sympathetic dominance. However, evidence of cardiac sympathetic dominance has not previously been investigated *in vivo* in our model of maternal diet-induced obesity.

4.1.4 Chapter aims

When it was first conceived, the main aim of this chapter was to investigate whether the cardiovascular function of young adult offspring of obese dams was different to those of lean dams, and to establish if there were sex differences in any programmed effects. Due to poor breeding outcomes, insufficient numbers of offspring of obese dams were generated to compare cardiovascular function between groups as intended. This is discussed in detail in Section 4.3.1.

Aims that are addressed in this chapter are:

1) To establish whether the body and heart weight phenotypes previously seen in male offspring of obese dams in our mouse model of maternal diet-induced obesity are also found in female offspring.
2) To assess diastolic function and pulmonary artery flow in young adult offspring for the first time in this model.
3) To develop a protocol to assess cardiac reactivity to sympathetic stimulation *in vivo.*
4) To assess peripheral vascular reactivity to a range of vasomotive agents.

4.2 Methods

4.2.1 Animal model

Data in this chapter comes from two groups of 8-week-old offspring: Con (P2 offspring of 'Con dams) and Ob (P2 offspring of 'Ob dams). PreP2 dams were generated as described in Section 2.1. All PreP2 females fed a control diet from weaning became 'Con dams and were mated for P2 for up to three attempts from one week after weaning of their first litter. Once PreP2 females fed the HF-HS obesogenic diet ('Ob dams) reached a body weight of 35 g, they were mated for P2 for up to three attempts. Body weight was used as the mating threshold for this cohort, rather than fat mass as measured by TDNMR, due to a change in the availability of equipment (discussed in Section 4.3.1).

Dams were allowed to litter naturally and, where necessary, litter sizes were reduced to six pups at PND2. Pups were then left with their mother to suckle. Both Con and Ob offspring were weaned at PND21, and weaned animals were housed in same-sex littermate pairs and given *ad libitum* access to standard RM1 laboratory chow. Cage mates were randomly assigned to Animal A or Animal B, and the procedures carried out on each are summarised in Table 4.1. Offspring were culled at 8 weeks of age. In this study, offspring body composition was measured using a different TDNMR machine to the one used in Chapters 3 and 5 (EchoMRI-100H).

	Animal A	Animal B
From 4 weeks of age	Weekly weighing and TDNMR	
At 7 weeks of age	Blood pressure measured	
At 8 weeks of age	16 hour overnight fast	Basal and stimulated echocardiography
Tissues dissected and weighed	Yes - fasted	No
Femoral artery myography	Yes	No

Table 4.1: Summary of procedures carried out on Con and Ob offspring.

4.2.2 Non-invasive blood pressure measurements

Non-anaesthetised blood pressure measurements of Animal A were made using non-invasive restraint tail cuff plethysmography (BP-200 system, Visitech) as described in Section 2.2 at 7 weeks of age. Animals were subjected to two days training and a minimum of 10 systolic blood pressure and heart rate measurements were recorded on the third day. All sessions were performed at the same time of day, between 17:30 – 18:30, to control for the natural circadian rhythm of blood pressure.

4.2.3 Basal and stimulated echocardiography

In vivo cardiac function of Animal B was assessed by transthoracic echocardiography (Vevo 3100 system with MX400 probe, Fujifilm Visualsonics) under general anaesthesia at 8 weeks of age, both at baseline and after intravenous (IV) administration of the beta-1-adrenoreceptor agonist dobutamine.

Anaesthesia was induced and the system set up as described in Section 3.2.5. However, when first transferred to the heated platform, the mouse was placed on their side and a cannula inserted into the tail vein. The cannula was secured using surgical tape and sterile PBS infused at a rate of 30 uL/hour to keep the cannula patent while baseline echocardiography measurements were made. The mouse was then put in the supine position for measurements and hair on the chest removed using a depilating cream. ECG and respiration rate were recorded in real-time throughout, and body temperature was monitored using a rectal probe and maintained between 36.5-37.5^{o}C.

As described in Section 3.2.5, mitral valve (MV) flow was recorded using Pulsed Wave (PW) Doppler in the apical four-chamber view (Apic-4), and pulmonary artery (PA) flow was recorded using PW Doppler in both the parasternal short-axis view (PSAX) and Apic-4. Both B-mode and M-mode images were obtained at baseline in the parasternal long-axis view (PSLAX).

Once baseline images were taken in PSLAX, the low-level IV infusion of PBS was halted and a bolus of 0.5 mg/kg body weight dobutamine was injected into the tail vein cannula. Dobutamine hydrochloride was dissolved in distilled water to a concentration of 5 mg/mL, and then diluted to 0.5 mg/mL in sterile PBS. Therefore, the bolus of dobutamine delivered was at a volume of 1 mL/kg body weight. Clips were saved every 30 seconds from the time of injection for a total of 4 minutes and 30 seconds, and then once per minute until up to 10 minutes and 30 seconds had passed in total. These clips were used to analyse the effect of dobutamine injection on heart rate. At the end of the protocol, anaesthetised mice were killed by cervical dislocation. As described previously, data from recorded clips were analysed using VevoLAB software version 5.6.1 (Fujifilm Visualsonics).

4.2.4 Tissue collection

At 8 weeks of age, Animal A was subjected to a 16 hour fast and then killed by exposure to a rising concentration of CO_2. Blood glucose was measured immediately prior to culling using a blood sample obtained from a nick in the tail vein. Tissues were dissected, weighed and snap-frozen on dry ice.

4.2.5 Femoral artery myography

Immediately after culling, femoral arteries were excised from Animal A, mounted on the wire myograph, and subjected to the normalisation protocol and K^+ curve as described in Section 2.3. The vasomotive agents used to generate dose-response curves are described in Table 4.2.

Agent	Doses (M)
Phenylephrine (PE)	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}
Sodium Nitroprusside (SNP)	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}
Acetylcholine (ACh)	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}
Angiotensin II (AngII)	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}
Endothelin-1 (ET-1)	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}

***Table 4.2: Vasomotive agents used in wire myography in young adult offspring cohort**. Drugs and doses used in myography experiments with femoral arteries from 8-week-old offspring of Control and Obese dams.*

4.2.6 Statistical analysis

All data were analysed using Prism 9.0 software (GraphPad) and are presented as mean ± standard error of the mean (SEM). N (numbers) refer to independent litters, with no more than one male and one female offspring used for a given measure per litter. Preliminary statistical testing using mixed effects models taking litter as a covariate was performed to verify that including male and female littermates did not impact the data. Data points were excluded as statistical outliers prior to statistical analysis if they were more than two standard deviations from the mean of the group. Normality of data was assessed by the Shapiro–Wilk test. Dam data were analysed by t-test or Mann-Whitney test as appropriate. Longitudinal offspring body weight and TDNMR data were analysed by sex-specific mixed-effects analyses to test for an effect of age and group. If there was a significant effect of group or a significant interaction between age and group, Sidak's multiple comparisons analysis was used to analyse pairwise differences between Con and Ob offspring at each timepoint.

Offspring organ and fat pad weight data were analysed by two-way ANOVA to test for an effect of maternal group (group) and offspring sex (sex). Data are considered statistically significant if $p<0.05$. If there was a significant effect of both group and sex, or if $p<0.07$ for an interaction between the two factors, male and female data were separated and analysed to identify sex-specific effects of maternal group. A t-test was used if the data were normally distributed, and a Mann-Whitney test used if not.

4.2 | Methods

An interaction was taken into account if $p<0.07$ rather than only if it was significant as the focus of this work was to identify effects of maternal group that may or may not be sex-dependent. A trending effect of an interaction could suggest that a sex-specific effect exists but that the mixed-effects model analysis is underpowered to detect it when offspring sex is also taken into account. This threshold was set prior to any data analyses being run.

Alternatively, if only an effect of maternal group was identified and there was no significant interaction between the factors, male and female data were combined and analysed by t-test or Mann-Whitney test. If only a main effect of sex was found, no further analyses were carried out.

Data analysed by t-test were checked for equal variance using an F test and a Welch correction used if groups did not have equal variances. While maximum response myography data was analysed according to the protocol above, dose-response curves were analysed as described in Section 2.3.3.

4.3 Results and Discussion

The aim of this chapter was to investigate the impact of maternal obesity during pregnancy on cardiovascular function in young adult offspring and to establish if there were sex differences in these effects. Previous studies in our well-established mouse model of maternal diet-induced obesity have identified increases in SBP[298,301] in 8-week-old male offspring of obese dams, but changes in vascular function that may underlie this programmed hypertension have not been assessed. Therefore, this study set out to further investigate the impact of maternal obesity on offspring blood pressure regulation by interrogating changes in vascular function as a possible mechanism of the programming of hypertension by maternal obesity. Increases in heart weight[290,295,298,301] and changes in left ventricular systolic function[298,301] have also been identified in male offspring of obese dams at this age, and this study set out to perform the first analyses of left ventricular diastolic function by echocardiography in offspring in this model. Moreover, evidence of cardiac sympathetic dominance has been identified in isolated hearts from 12-week-old male offspring of obese dams[295], and this study set out to establish a new protocol to investigate cardiac sympathetic reactivity *in vivo*. All of these previous studies only included male offspring, and therefore this study also set out to investigate whether there are sex-specifics effects of maternal obesity on cardiovascular function in young adult offspring.

4.3.1 Breeding success and limited numbers

Breeding success of 'Ob dams was very low in this cohort. Of the 17 'Con females mated for P2, 15 produced litters. Of the remaining two 'Con females, one did not fall pregnant within three mating attempts and one animal was culled due to ill health during pregnancy. However, from the 30 'Ob females mated for P2, only eight produced usable litters. These eight viable litters included five independent sibling pairs of male offspring and seven independent sibling pairs of female offspring. Of the remaining 22 'Ob females, one littered but the offspring had to be culled due to ill health before 8 weeks of age, nine littered but their pups did not survive beyond PND2, and 12 were mated at least three times and never fell pregnant. When examining the body weight of which 'Ob females went on to have a viable litter versus those that either lost their litters or never became pregnant (Figure 4.1), it is clear that the females that did not produce viable litters were heavier than those that did at the point of mating.

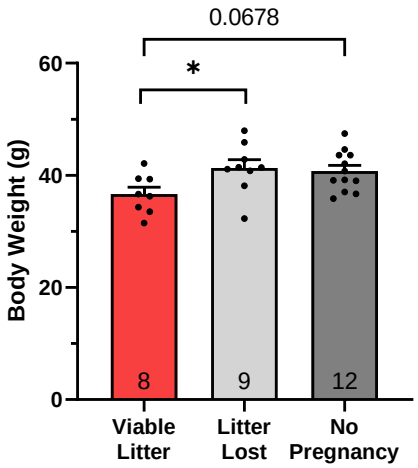


Figure 4.1: Body weight at time of mating and breeding outcome in PreP2 'Ob females. **p<0.05 by Tukey's multiple comparisons after a significant effect of group identified by one-way ANOVA. Numbers presented inside bars.*

In this cohort, 'Ob females were put forward for PreP2 SBP measurement (presented in Section 3.3.6) and then the goal was for them to be mated once they reached a body weight of 35 g. This was a different mating threshold than was used in Chapters 3 and 5. In those chapters, PreP2 females fed the HF-HS obesogenic diet were mated when they reached a pre-determined threshold of obesity of 10 g body fat as measured by TDNMR (Bruker LF90II). This equipment was replaced part way through this PhD with a new TDNMR machine (EchoMRI-100H). At the time that this cohort was established, the same mating threshold used in Chapters 3 and 5 of this thesis could not be used because the Bruker LF90II was in the process of being decommissioned and preliminary studies suggested that the fat mass measurements from the EchoMRI may not match those from the Bruker LF90II. Therefore, a body weight threshold of 35 g was applied to the mating of PreP2 females fed the HF-HS diet ('Ob females). This body weight threshold for the 'Ob females was chosen by examining data across multiple previous cohorts run by the Ozanne group, as it was a body weight at which fat mass as measured by the original TDNMR machine was consistently greater than 10 g. While this body weight threshold is heavier than the average body weight of 'Ob females at mating in Chapter 3, it has been used in previous studies by the Ozanne group[293].

Many 'Ob females were much heavier than 35 g by the time that they were mated. Indeed, the average 'Ob female body weight at mating was 39.8 ± 0.8 g. The average body weight of 'Ob females at mating that went on to have a viable litter was 36.6 ± 1.2 g, while for those that either never plugged or did plug but did not go on to develop an established pregnancy it was 40.7 ± 1.0 g. This is consistent with human data as obese women have a lower chance of conception than non-obese women[100] and being overweight or obese results in it taking longer for a woman to fall pregnant[101]. Therefore, this finding recapitulates the reduction in fertility seen in obese women.

Some of the heavier PreP2 'Ob females did develop an established pregnancy, meaning that the average body weight of the 17 'Ob females who successfully plugged and fell pregnant was 39.1 ± 1.1 g at mating. However, only eight of these dams produced viable litters. In order to avoid potential stress to the dam around delivery and in the first few days after birth, once a pregnant dam is seen to be littering down, we make sure to minimise disturbance to the cage until PND2. Therefore, litters are only recorded as lost on PND2, this means that it cannot be ascertained whether pups were stillborn or died shortly after birth. The average maternal body weight at mating of 'Ob dams who had an established pregnancy but whose litters did not survive beyond PND2 was 41.3 ± 1.5 g. This is also consistent with human data as obese women are more likely to have a miscarriage[104] or deliver a stillborn baby[105], and once delivered, babies of obese women are at a higher risk of perinatal mortality[109], and the rate of neonatal unit admissions correlates with maternal BMI[110].

As a result of the extremely low breeding success in 'Ob females, many of the experiments in this study were underpowered to detect a difference between offspring of 'Con and 'Ob dams. Power calculations (80% power, alpha = 0.05) for the different outcome measures are presented in Table 4.3, alongside the actual numbers of male and female Ob offspring from which data were obtained for each measurement. Data from previous studies of 8-week-old male offspring of 'Con and 'Ob dams from the theses of Drs J. Beeson and J. Schoonejans were used for body weight and fat mass (TDNMR) in 4-week-old offspring, SBP (tail cuff plethysmography) in 8-week-old offspring and heart weight and ejection fraction (M-mode echocardiography) in 8-week-old offspring. Wire myography studies had not previously been performed by the Ozanne group and so preliminary data from this cohort was used to calculate the sample size required to detect a 25% difference in maximal relaxation to ACh.

Measurement	Minimum N	No. Ob Males	No. Ob Females
Body weight (4w)	5	4	6
TDNMR Fat Mass (4w)	4	4	4
SBP (8w)	8	4	6
Heart weight (8w)	6	5	7
Echocardiography (8w)	10	6	3
Myography (8w)	8	5	7

Table 4.3: Power calculations. *SBP = systolic blood pressure. 4,7,8 w = 4-, 7-, or 8-week-old offspring.*

The numbers of Ob offspring generated were only sufficient to investigate the effect of maternal obesity during pregnancy on offspring body and heart weight (in female offspring) and fat mass (both sexes). Insufficient numbers of both male and female Ob offspring were generated to provide sufficient power to address offspring cardiovascular outcomes in relation to maternal obesity, including analysis and interpretation of tail cuff plethysmography, echocardiography, and myography data. Therefore, in this chapter, it was only possible to address the effects of sex on physiological outcomes in Con offspring. Nevertheless, 'Ob dams that did produce viable litters were a similar weight at mating as previous studies in this model[293], meaning that analyses that are sufficiently powered to do so are justified in viewing the effects of maternal obesity during pregnancy in this study in the context of previous findings from our group.

4.3.2 Dam body weight and heart weight

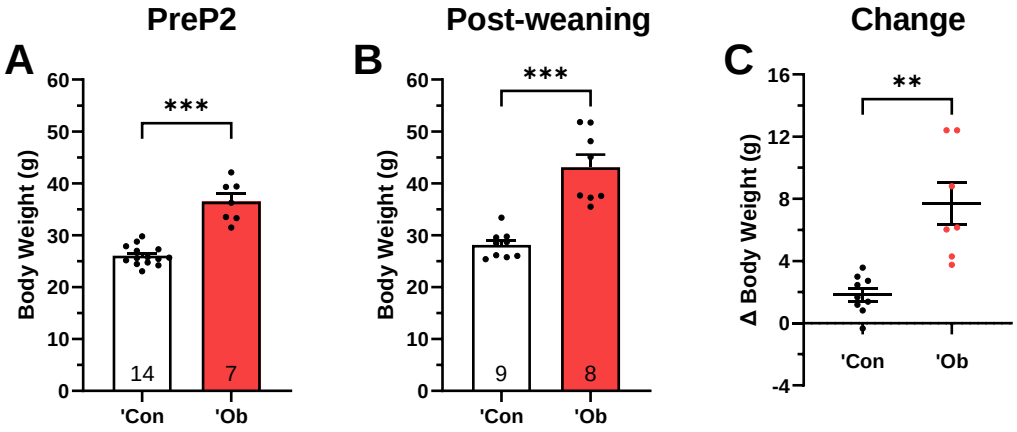


Figure 4.2: Dam body weight from pre-pregnancy to post-weaning. *Dam body weight before pregnancy (PreP2) (A) and after weaning (B), and the change in body weight between the two (C). **p<0.01, ***p<0.001 by student's t-test or Welch's t-test as appropriate. Numbers presented inside bars in (A) and (B), numbers in (C) match those in (B).*

As expected, 'Ob dams were heavier than 'Con dams both before pregnancy and after weaning (Figure 4.2(A,B)). Moreover, 'Ob dams gained more body weight between PreP2 and post-weaning than 'Con (Figure 4.2(C)), resulting in 'Ob dams being significantly heavier after their litters were weaned than before they were pregnant (data not shown, $p<0.001$), whereas no such significant increase in body weight was found in 'Con ($p=0.0759$). This is particularly important as gaining weight between pregnancies, known as interpregnancy weight gain, is associated with an increased risk of poor outcomes in subsequent pregnancies[147], including GDM and hypertensive disorders of pregnancy.

In Section 3.3.5 it was found that while 'Ob dams were heavier and fatter than 'Con both at conception and in late pregnancy, they gained a comparable amount of body weight during pregnancy.

Therefore, the interpregnancy weight gain phenotype in this study is likely due to lean dams losing the extra weight that they put on during gestation, but obese dams failing to do so, rather than the obese dams gaining more weight during gestation.

There was no difference in either absolute or relative heart weight between 'Con and 'Ob post-weaning dams (Table 4.4).

Unit	'Con (n=9)	'Ob (n=8)	p-value
mg	159 ± 9	170 ± 7	0.4088
%BW	0.620 ± 0.066	0.632 ± 0.036	0.8776

Table 4.4: Post-weaning dam heart weight. *P-value calculated by students t-test or Mann-Whitney test as appropriate. %BW = percentage of total body weight.*

4.3.3 Offspring longitudinal body weight and composition

While there was a significant interaction between age and group on male offspring longitudinal body weight ($p=0.0012$) and lean mass ($p<0.0001$) when analysed by mixed-effects analysis, Sidak's multiple comparison testing found no difference in either body weight or lean mass between male Con and Ob offspring at any individual timepoint between 4 and 8 weeks of age (Figure 4.3(A,B)). However, group did have a significant effect on fat mass ($p=0.0002$) and Ob male offspring had an increased fat mass compared to Con as measured by TDNMR at every timepoint (Figure 4.3(C)). Age had a significant effect on all measures.

Mixed-effects analysis found both a significant effect of group ($p=0.0478$) and an interaction between age and group ($p<0.0001$) on female offspring body weight. Sidak's multiple comparisons revealed that Ob female offspring were heavier than Con at 4 weeks of age, and then there was no difference in the body weight between the two groups between 5 and 8 weeks of age (Figure 4.4(A)). There was also a significant effect of group on fat mass ($p=0.0002$) and an interaction between age and group on lean mass ($p<0.0001$) in female offspring. Sidak's multiple comparisons analysis revealed that the increased body weight at 4 weeks of age was associated with a significant increase in fat mass (Figure 4.4(C)) and a non-significant increase in lean mass ($p=0.0866$) (Figure 4.4(B)). Fat mass was also non-significantly greater in Ob female offspring than Con at 5 weeks of age ($p=0.0840$) but not beyond ($p>0.1$). Age had a significant effect on all measures.

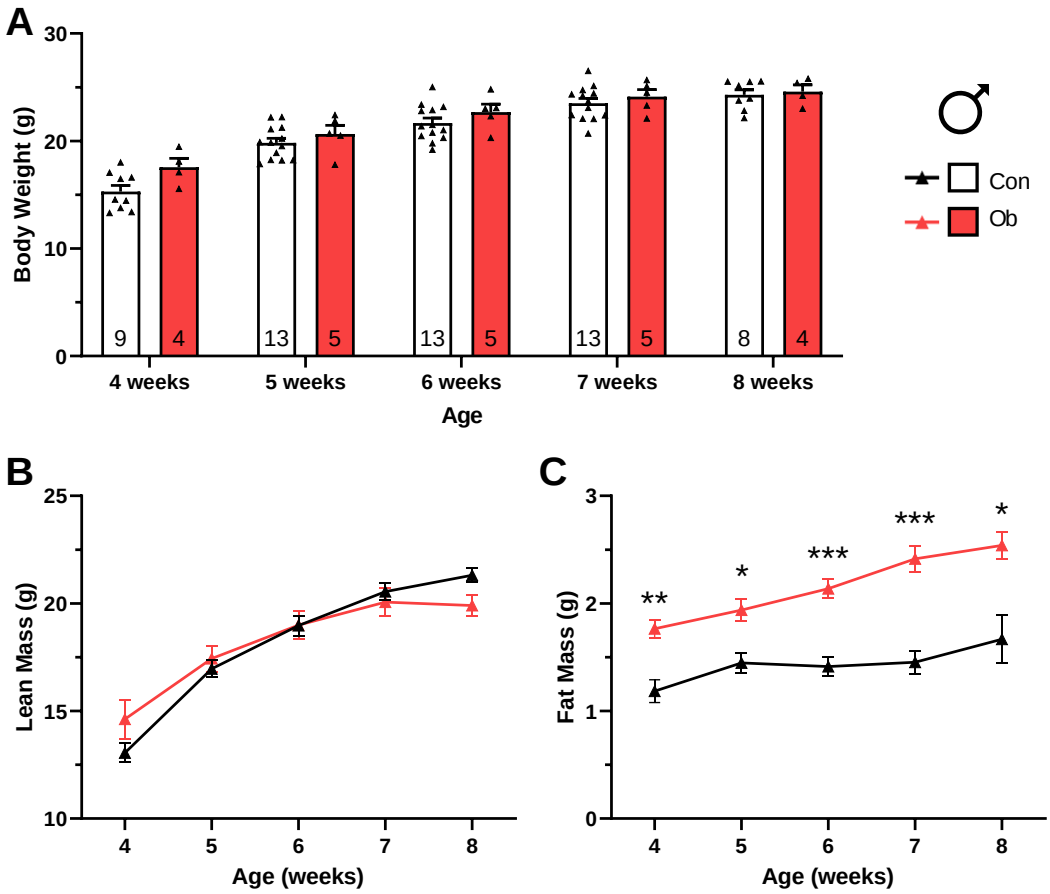


Figure 4.3: Male offspring body weight and composition. *Longitudinal body weight (A), lean mass (B) and fat mass (C) of male offspring of control (Con) and obese (Ob) dams. Fat and lean mass measured by TDNMR. *p<0.05, **p<0.01, ***p<0.001 by Sidak's multiple comparisons test after a significant effect of group found by mixed-effects analysis. Numbers presented in bars in (A) are matched with data in (B) and (C).*

That maternal obesity did not affect body weight of male or female offspring at 8 weeks of age is consistent with previous studies in this model[290,297–299,301,302]. Previously published studies in this model have also found that offspring of obese dams were heavier than those of lean dams after weaning, but that this difference did not persist beyond weaning[297]. This is consistent with the increased body weight of female 4-week-old Ob offspring in this study that was lost by 5 weeks of age. However, previous studies found this transient increase in body weight around weaning was independent of sex, whereas this study found no difference in male body weight at 4 weeks of age. This may be due to lower numbers of 4-week-old Ob male offspring, or perhaps that the male Ob offspring were heavier than Con at weaning but that this difference was already lost by 4 weeks of age.

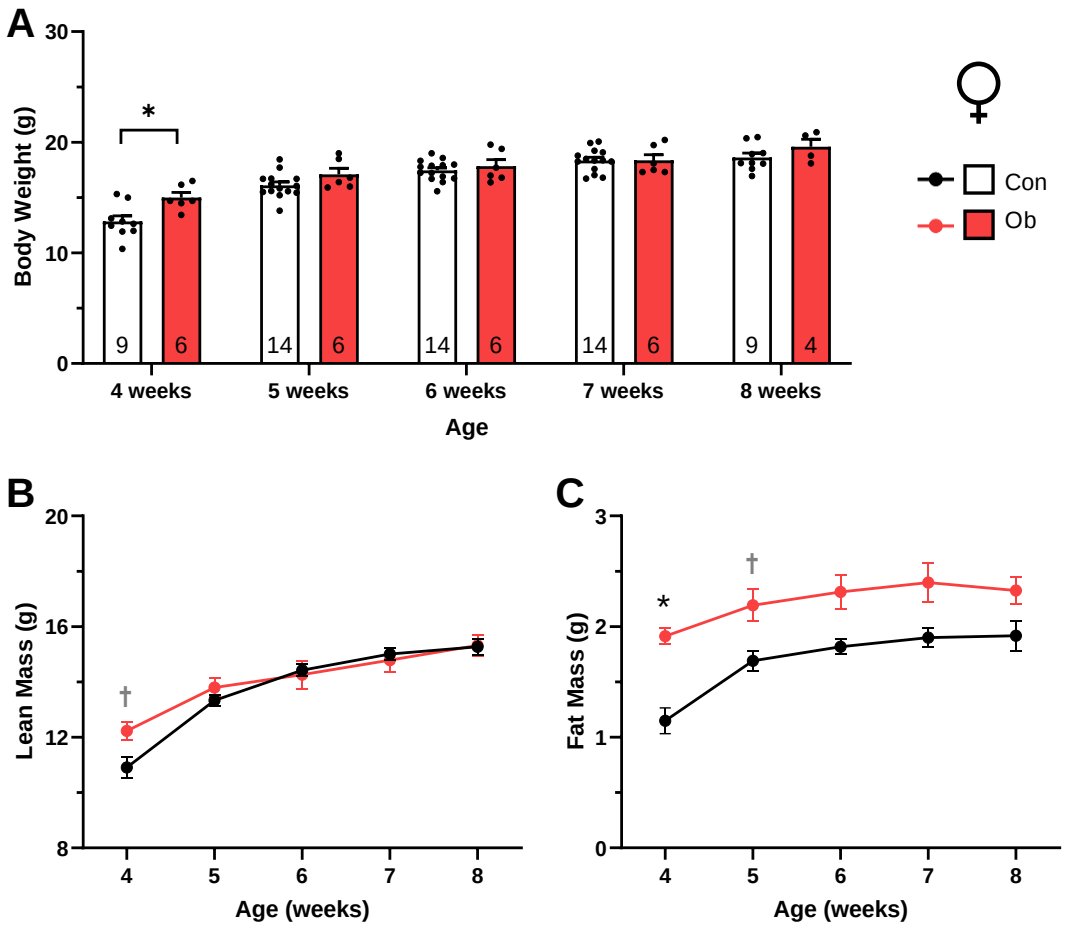


Figure 4.4: Female offspring body weight and composition. *Longitudinal body weight (A), lean mass (B) and fat mass (C) of female offspring of control (Con) and obese (Ob) dams. Fat and lean mass measured by TDNMR. *p<0.05, 0.05<†p<0.10 by Sidak's multiple comparisons test after a significant effect of group found by mixed-effects analysis. Numbers presented in bars in (A) are matched with data in (B) and (C).*

Longitudinal TDNMR data before 8 weeks of age has not previously been published in our model, making these data novel, although increased fat mass as measured by TDNMR has been found in 8-week-old offspring of obese dams[298,301,302]. In this study, male Ob offspring had an increased fat mass compared to Con at every time point between 4 and 8 weeks of age. While mixed-effects analysis also found a significant effect of group on female offspring longitudinal fat mass, unlike in male offspring, Sidak's multiple comparisons found that Ob female offspring were significantly heavier than Con at 4 weeks of age only. This suggests that while exposure to maternal obesity resulted in an increase in fat mass in both male and female offspring between 4 and 8 weeks of age in this study, the phenotype was stronger in male offspring.

4.3 | Results and Discussion

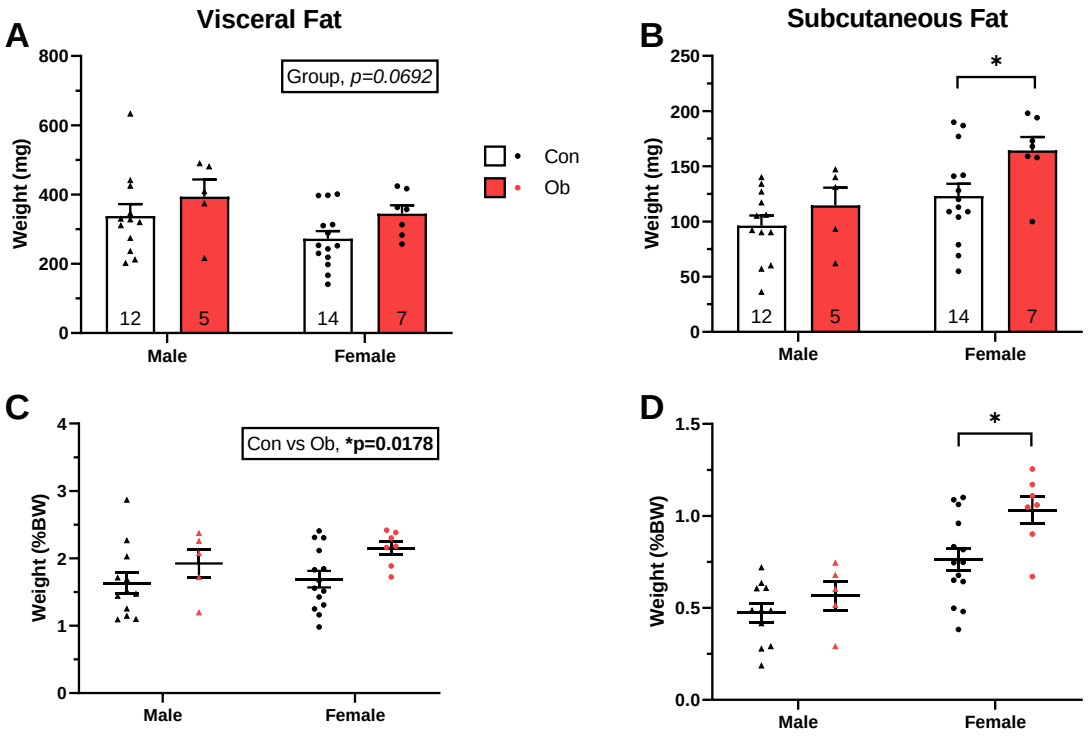
The increased adiposity in 8-week-old offspring of 'Ob dams was associated with an increase in the relative weight of visceral fat of Ob offspring independent of sex (Figure 4.5(C)), suggesting an increase in visceral adiposity. This is of note as increased visceral adiposity is associated with an increased risk of cardiometabolic dysfunction[39,40]. Maternal group and offspring sex had variable effects on the absolute masses of the different visceral depots collected (Figure 4.5(E)).

There was also a sex-specific increase in subcutaneous fat mass relative to body weight in female Ob offspring only (Figure 4.5(D)). When expressed as absolute mass, the sex-specific increase in subcutaneous fat in female offspring persisted (Figure 4.5(B)), but the effect on visceral fat mass no longer reached significance (Figure 4.5(A)). The sex-specific increase in both absolute and relative weight of subcutaneous fat depots in female Ob offspring is in line with human data that shows that women preferentially store fat in their subcutaneous depots[36,37].

Maternal group did not affect 16-hour fasted plasma glucose in 8-week-old offspring (Table 4.5). Fasted glucose was lower in female offspring than male offspring, independent of maternal group.

			Two-Way ANOVA		
Sex	Con (n=11-12)	Ob (n=5-7)	Sex	Group	Inter^{n}
Male	7.3 ± 0.4	7.2 ± 0.4	**0.0374**	0.5294	0.7983
Female	6.5 ± 0.4	6.1 ± 0.3			

Table 4.5: Plasma glucose following a 16-hour fast in 8-week-old offspring. *All units are mmol/L. Right hand columns present results of two-way ANOVA for offspring sex and maternal group.*



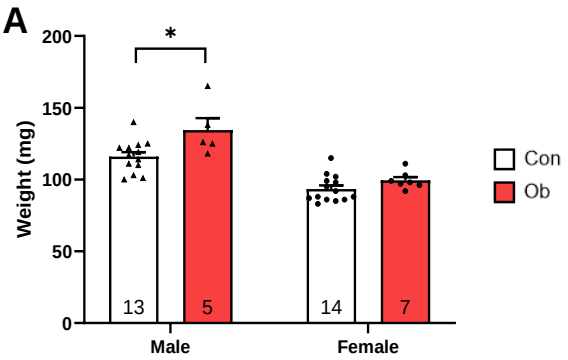
E

mg	Sex	Con (n=11-14)	Ob (n=5-7)	Mixed-Effects Analysis: Sex	Mixed-Effects Analysis: Group	Mixed-Effects Analysis: Inter^n	One-Way Analysis of Group-Effect
Gonadal Fat	Male	184 ± 13	247 ± 34	0.3538	**0.0058**	0.5792	**Combined, p=0.0062**
	Female	177 ± 13	220 ± 16				
IP Fat	Male	113 ± 10	114 ± 11	*0.0707*	0.2926	0.3406	
	Female	80 ± 10	104 ± 8				
RP Fat	Male	23.7 ± 3.0	32.8 ± 5.9	**0.0031**	**0.0234**	0.6311	Male, p=0.1877
	Female	15.1 ± 1.7	21.1 ± 3.1				Female, *p=0.0838*

Figure 4.5: Offspring white adipose tissue (WAT) depots at 8 weeks of age. *Combined mass of visceral fat depots collected expressed as absolute weight (A) and relative to body weight (%BW) (C). Box in (A) presents p-value from mixed-effects analysis of sex and group. Box in (C) presents p-value from Tukey's multiple comparisons test after group effect identified by one-way ANOVA on combined male and female data. Absolute (B) and relative (D) weights of collected subcutaneous fat, *p<0.05 using sex-specific t-test after a significant effect of both sex and group found by two-way ANOVA. Absolute masses of individual visceral fat depots collected presented in the table (E). IP = intraperitoneal, RP = retroperitoneal. Numbers presented in bars in (A) and (B) are matched with data in (B) and (C).*

4.3.4 Offspring organ weights

A



B

mg	Sex	Con (n=13-14)	Ob (n=5-7)	Two-Way ANOVA		
				Sex	Group	Inter[n]
Liver	Male	1014 ± 32	919 ± 60	**<0.0001**	0.1605	0.1937
	Female	811 ± 21	807 ± 23			
Kidney	Male	140 ± 3	158 ± 12	**<0.0001**	*0.0604*	0.1171
	Female	107 ± 3	109 ± 5			

***Figure 4.6: Offspring organ weights at 8 weeks of age**. Absolute weight of offspring heart (A). *p<0.05, using sex-specific t-test after a significant effect of both sex and group found by two-way ANOVA. Numbers presented inside bars. Absolute weight of liver and kidney presented in table (B) with results of two-way ANOVA for sex and group.*

There was an increase in absolute heart weight in male Ob offspring compared to Con (Figure 4.6(A)). Previous studies in this model have consistently found an increase in heart weight in male offspring of obese dams[290,295,298,301], but female heart weight had not been investigated previously. Therefore, as well as replicating the increased heart weight in male Ob offspring, this study found that this effect is sex-specific at this age as there was no difference in heart weight in female Con and Ob offspring.

Absolute liver and kidney weight were also greater in male than female offspring, but maternal group had no effect on liver weight or kidney weight (Figure 4.6(B)).

As discussed in Section 4.3.1, this study was only sufficiently powered to analyse differences in 'Con versus 'Ob dam phenotype and Con versus Ob offspring body composition and organ weights. The rest of this chapter therefore focuses on differences in cardiovascular function between male and female offspring of lean dams only and the establishment of the new methodology to investigate cardiac sympathetic reactivity *in vivo*.

4.3.5 Offspring blood pressure

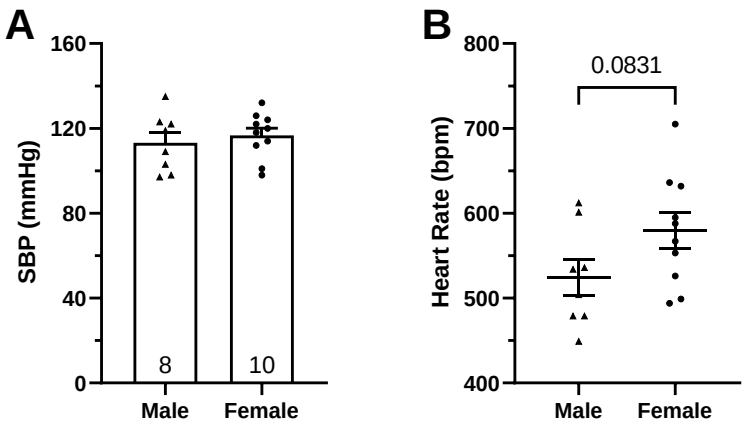


Figure 4.7: Systolic blood pressure (SBP) and heart rate of 7-week-old male and female offspring of lean dams. *SBP (A) and corresponding measurements of heart rate (B). Data from offspring of lean dams only is shown because number of offspring of obese dams was insufficient for analysis. Analysed by student's t-test. Numbers: Male = 8, Female = 10.*

There was no significant difference in either SBP or heart rate between male and female 7-week-old offspring of lean dams (Figure 4.7).

4.3.6 Femoral artery myography

This was the first study by the Ozanne group that used wire myography to investigate the vascular reactivity of isolated vessels from offspring. While it was underpowered to assess the effect of exposure to maternal obesity whilst *in utero* on vascular reactivity, sufficient numbers of control offspring were generated to investigate possible sex-differences. These data can also be used to inform the design of future wire myography experiments, such as by offering insight to the optimal range of doses of vasomotive agents to expose the isolated vessels to.

Response to depolarisation

Femoral arteries from female offspring had a greater contractile response than those from males to exposure to Krebs with an increasing concentration of K^+ (Figure 4.8). While it is relatively standard procedure to perform K^+ curves in myography experiments, results are not typically reported. Rather, K^+ curves are usually run such that vasoconstrictor responses can be normalised against them.

Exposing isolated vessels to an increasing concentration of K^+ stimulates vessel constriction by depolarising VSMC membranes[482], resulting in the activation of voltage-gated Ca^{2+} channels and subsequent increased intracellular Ca^{2+}, which stimulates smooth muscle contraction. Therefore, the contraction elicited in response to exposure to an increasing concentration of K^+ is a property of the VSMCs of that vessel independent of the expression of receptors for specific vasomotive agents.

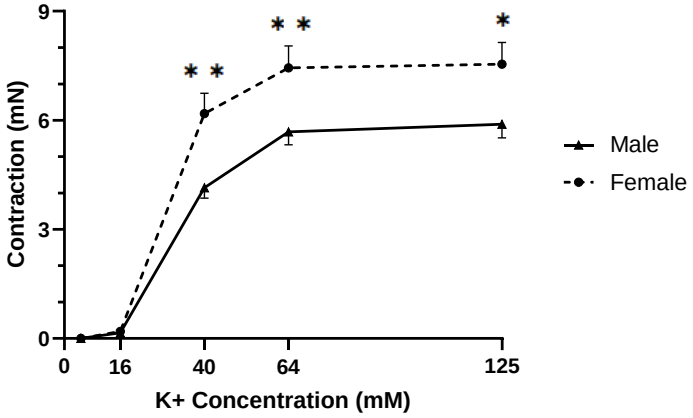


Figure 4.8: Femoral vascular response to increasing concentration of K^+. *Response curves of femoral arteries from male and female 8-week-old offspring of lean dams to Krebs with an increasing concentration of K^+. Data from offspring of lean dams only is shown because number of offspring of obese dams was insufficient for analysis. *p<0.05, **p<0.01 by Sidak's multiple comparisons after a significant effect of sex identified by mixed-effects analysis. Numbers: Male = 11. Female = 13.*

The greater contractile response to VSMC depolarisation in femoral arteries of female offspring compared to males could be due to increased hypertrophy of the smooth muscle cells of the tunica media, resulting in an increased total contractile capacity of the vessel, or a change in the electrophysiology of the VSMCs, such that they contract more forcefully in response to depolarisation.

Response to increasing concentration of PE

Femoral arteries from female 8-week-old offspring contracted more forcefully in response to PE than those from male offspring (mixed-effects analysis: sex, p=0.0494) (Figure 4.9(A)). Therefore, femoral arteries from female offspring were hyperreactive to alpha-adrenergic vasoconstriction compared to those from males. This may seem to be the opposite of what one would expect given that in pre-menopausal women, noradrenaline administration does not cause significant vasoconstriction, whereas it does in men[468]. However, *in vivo*, enhanced beta-adrenergic vasodilation acts to offset the vasoconstrictive effects of alpha-adrenergic vasoconstriction in women. The same effect would not be seen in the current study as unlike noradrenaline, PE is a selective α1R agonist. The response did not plateau in either male or female vessels within the range of doses given and so it is not known whether this difference would increase or be lost in response to higher PE concentrations.

Response to increasing concentration of AngII

No effect of sex on femoral artery reactivity to AngII was identified by mixed-effects analysis (Figure 4.9(B)). This was unexpected because sex differences in AngII signalling in the vasculature have been identified in both humans[477] and rats. Isolated aortic rings[476] and mesenteric arteries[483] from young female rats have been found to have a reduced contractile response to AngII *ex vivo* than those from males, although no data on femoral artery responses were identified in the literature.

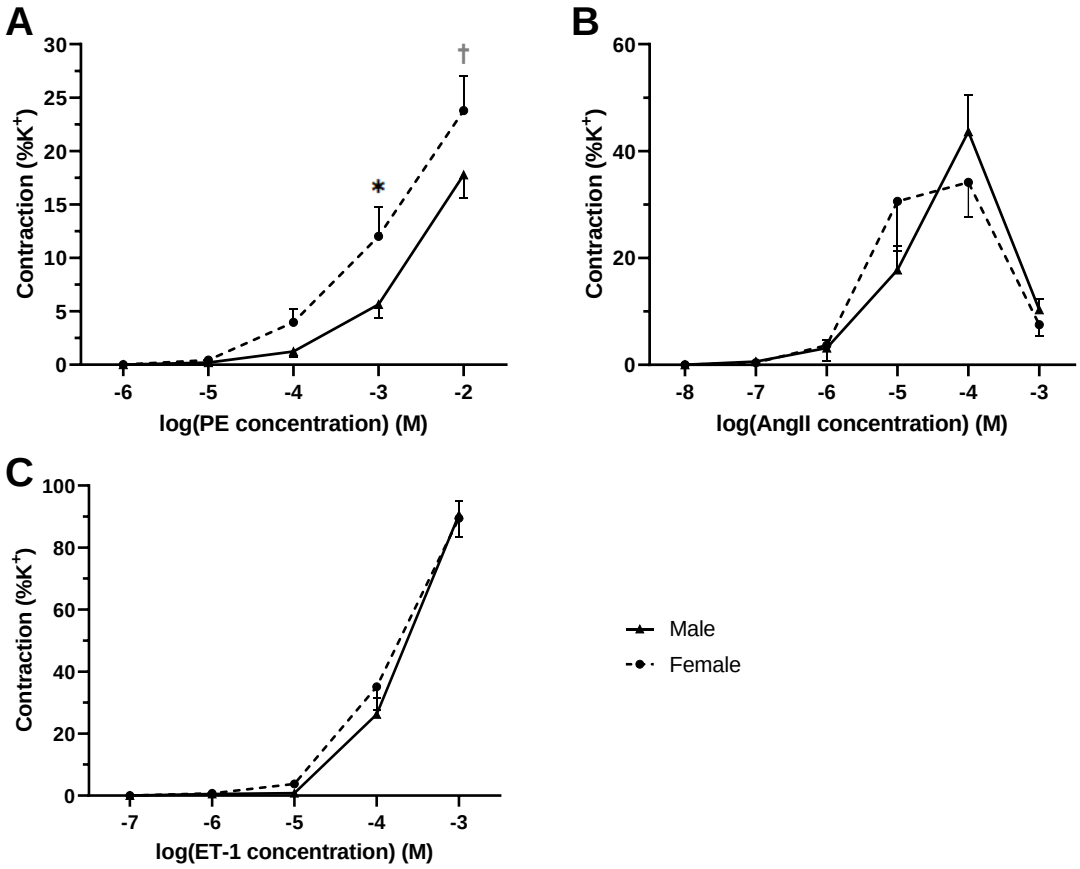


Figure 4.9: Femoral vascular response to vasoconstrictors. *Dose-response curves of femoral arteries from 8-week-old male and female offspring of lean dams to phenylephrine (PE) (A), angiotensin II (AngII) (B), and endothelin-1 (ET-1) (C), all normalised to maximal K^+-induced contraction (%K^+). Data from offspring of lean dams only is shown because number of offspring of obese dams was insufficient for analysis. *p<0.05, 0.05<†p<0.06 by Sidak's multiple comparisons after a significant effect of sex identified by mixed-effects analysis. Numbers: Male = 10. Female = 12.*

In this study, the response of femoral arteries from both male and female offspring to AngII peaked in response to 10^{-4}M AngII and the response to 10^{-3}M AngII was comparatively lower. This has not been seen in the dose-response curves of any other vasomotive agents over the course of this PhD, but was extremely consistent with AngII curves.

Response to increasing concentration of ET-1

No differences in response to ET-1 were identified between femoral arteries from male and female offspring (Figure 4.9(C)). This was unexpected as sex differences in ET-1 signalling in the vasculature have been identified between young men and women[481] and studies using isolated saphenous veins have found greater vascular reactivity to ET-1 in young men than in women[480]. Conversely, one study in rats found that coronary arteries from males had reduced constriction in response to ET-1 then those from to females. No studies demonstrating sex differences in vascular reactivity to ET-1 in mesenteric or femoral arteries from rodents were identified in literature searches.

Moreover, while no difference in femoral artery response to ET-1 between male and female offspring was identified in this study, the response to ET-1 did not plateau within the range of doses given in vessels from either male or female offspring, and therefore sex differences in reactivity may not have been detectable as they may only become apparent at higher concentrations of ET-1. This is supported by the above study using human saphenous veins, as no significant difference in contractility between female and male vessels was identified at low concentrations of ET-1. However, at high concentrations, the contractile response in vessels from men was twice that in women[480]. While not relevant to the discussion of these myography findings, anyone intending to perform myography experiments using ET-1 in the future should note that an ET-1 curve should always be the final dose-response curve performed on a given vessel as it takes a hugely longer period of time to wash out the ET-1 and for the vessel to relax and return to close to its baseline tension than any of the other vasomotive agents used in this PhD.

Response to increasing concentrations of SNP and ACh

Mixed-effects analysis of SNP dose-response curves found a significant interaction between SNP concentration and sex on % relaxation ($p=0.0313$), but no pairwise differences between vessels from male and female offspring were identified by Sidak's multiple comparisons (Figure 4.10(A)). There was no difference in maximal relaxation in response to SNP (Figure 4.10(B)). Offspring sex did not affect femoral artery response to ACh (Figure 4.10(C,D)).

As discussed in Section 4.1.2, oestrogen acts both directly to increase NO production by activating eNOS via ERα and indirectly to increase NO bioavailability by reducing ROS. This contributes to the commonly observed phenomenon that the female vasculature dilates more in response to vasodilatory stimuli in humans[484]. In line with this, increased endothelium-dependent vasodilation (in response to ACh) has been found in mesenteric arteries from 5-month-old female mice compared to those from males[485]. Therefore, it was unexpected that no difference in femoral artery relaxation in response to ACh was identified in this study. Comparing these studies is complicated by the use of vessels from different regions of the body. Studies usually only assess the responses of one vessel type, typically aorta, femoral or mesenteric arteries, as representative of systemic vascular reactivity. However, Zelinskaya et al.[486] found that the induction of a diabetic phenotype by combined high-fat feeding and streptozotocin injection in the rat caused opposite effects on the reactivity of arteries from different regions *ex vivo*. They found that reactivity of mesenteric arteries to ACh was reduced, and that of femoral arteries was increased in diabetic rats, due to differences in downstream signalling pathways[486]. Therefore, that no differences in vascular reactivity to ACh were identified in femoral arteries in this study does not preclude that sex differences in endothelium-dependent vasodilation may be present in other vascular beds.

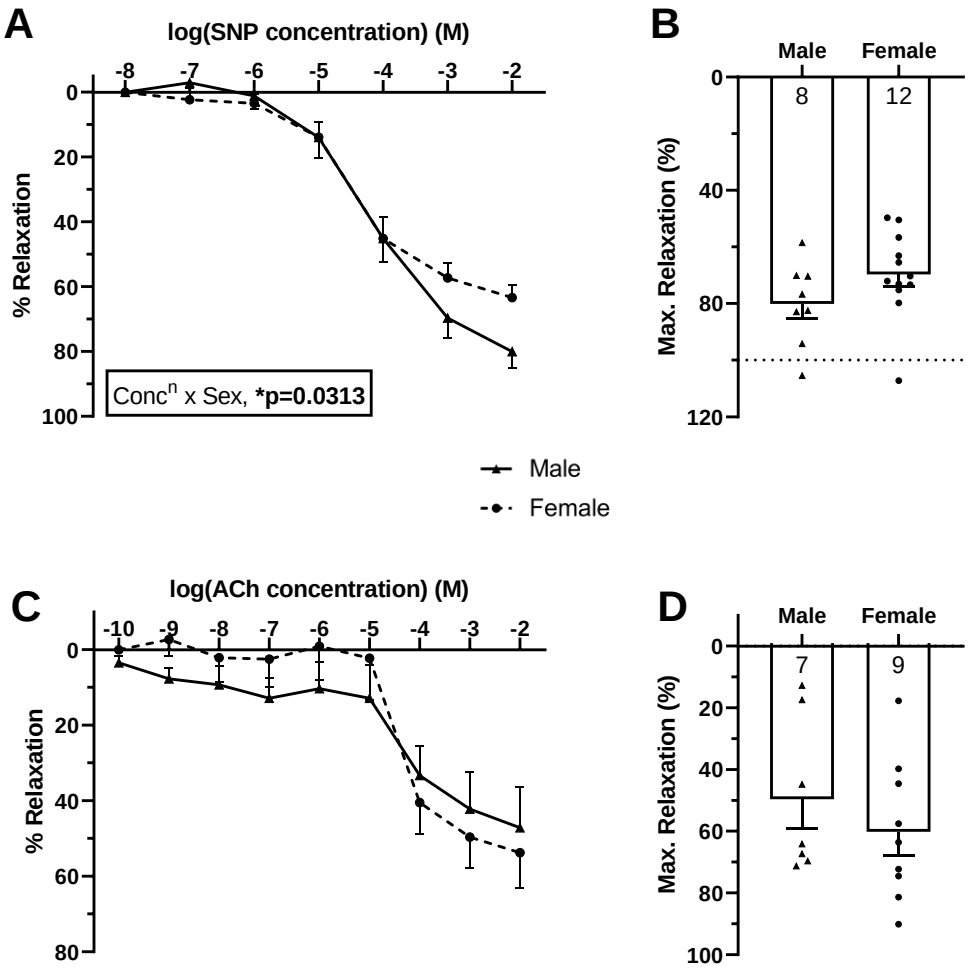


Figure 4.10: Femoral vascular response to vasodilators. *Dose-response curves of femoral arteries from 8-week-old male and female offspring of lean dams to sodium nitroprusside (SNP) (A), and acetylcholine (ACh) (C), expressed as %Relaxation after pre-constriction by exposure to 10^{-2}M noradrenaline. Corresponding maximal relaxation presented in (B) and (D). Data from offspring of lean dams only is shown because number of offspring of obese dams was insufficient for analysis. Box in (A) presents significant interaction term between SNP concentration and sex calculated by mixed-effects analysis. Numbers in (A) and (B): Male = 8, Female = 12, numbers in (C) and (D): Male = 7, Female = 9.*

4.3.7 Offspring left ventricular systolic function

	Male (n=10-11)	Female (n=12-13)	p-value
Heart Rate (bpm)	516 ± 17	551 ± 10	*0.0930*
Diastolic Area (mm^2)	17.9 ± 0.9	18.1 ± 0.7	0.8885
Systolic Area (mm^2)	8.83 ± 0.51	9.80 ± 0.52	0.2196
Diastolic Volume (uL)	39.4 ± 3.2	40.4 ± 2.7	0.8203
Systolic Volume (uL)	11.2 ± 1.2	14.0 ± 1.3	0.1634
Stroke Volume (uL)	28.2 ± 2.1	26.5 ± 1.7	0.5203
Cardiac Output (mL/min)	15.1 ± 0.9	14.3 ± 0.9	0.5494
Ejection Fraction (%)	74.0 ± 2.1	73.2 ± 1.5	0.7595
FS (radial) (%)	42.4 ± 1.9	41.3 ± 1.3	0.6378
LVEnL (diastole) (mm)	7.10 ± 0.14	7.01 ± 0.13	0.6640
LVEnL (systole) (mm)	6.30 ± 0.11	6.17 ± 0.11	0.4318
FS (longitudinal) (%)	11.2 ± 1.1	12.0 ± 1.0	0.6342

Table 4.6: Echocardiographic indices of left ventricular systolic function in 8-week-old offspring. *Right hand column presents p-value calculated by student's t-test or Mann-Whitney test as appropriate. FS = fractional shortening, LVEnL = left ventricle endocardial length. Data from offspring of lean dams only is shown because number of offspring of obese dams was insufficient for analysis.*

No differences in left ventricular systolic function (Table 4.6) or wall widths (Figure 4.11) were identified between male and female 8-week-old offspring of lean dams by echocardiography.

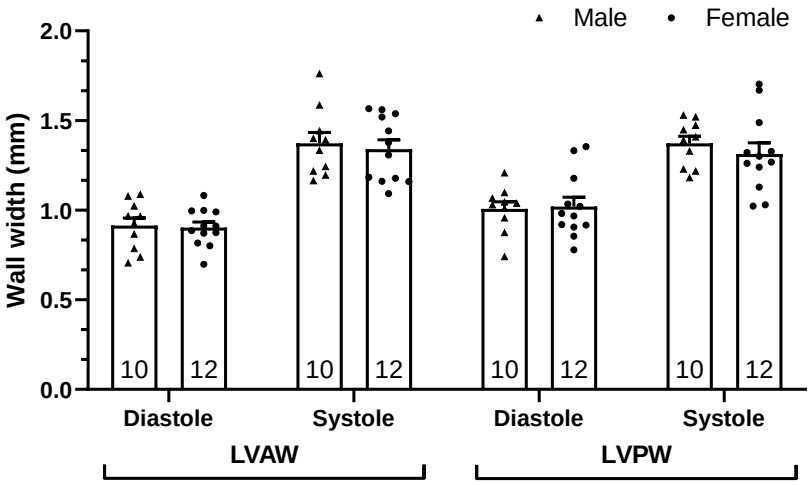


Figure 4.11: Left ventricle (LV) wall widths of 8-week-old offspring of lean dams. *Width of LV anterior wall (LVAW) and LV posterior wall (LVPW) in diastole and systole, as measured by echocardiography. Numbers presented inside bars.*

4.3.8 Offspring left ventricular diastolic function

	Male (n=10)	Female (n=7)	p-value
E wave (mm/s)	749 ± 31	725 ± 19	0.5767
A wave (mm/s)	554 ± 29	570 ± 23	0.7022
E:A	1.35 ± 0.05	1.28 ± 0.05	0.3804
IVCT (ms)	10.5 ± 0.9	10.9 ± 0.8	0.7084
Aortic ejection time (ms)	43.7 ± 1.8	44.1 ± 1.2	0.8558
IVRT (ms)	12.1 ± 1.1	11.5 ± 0.7	0.8874
Non-filling time (ms)	65.9 ± 1.8	66.3 ± 1.8	0.8763
MPI	0.529 ± 0.059	0.515 ± 0.028	0.8409
Decelerⁿ rate (x1000) (mm/s²)	-28.6 ± 4.2	-32.5 ± 4.8	0.5694
Decelerⁿ time (ms)	28.9 ± 2.3	24.9 ± 3.1	0.3061

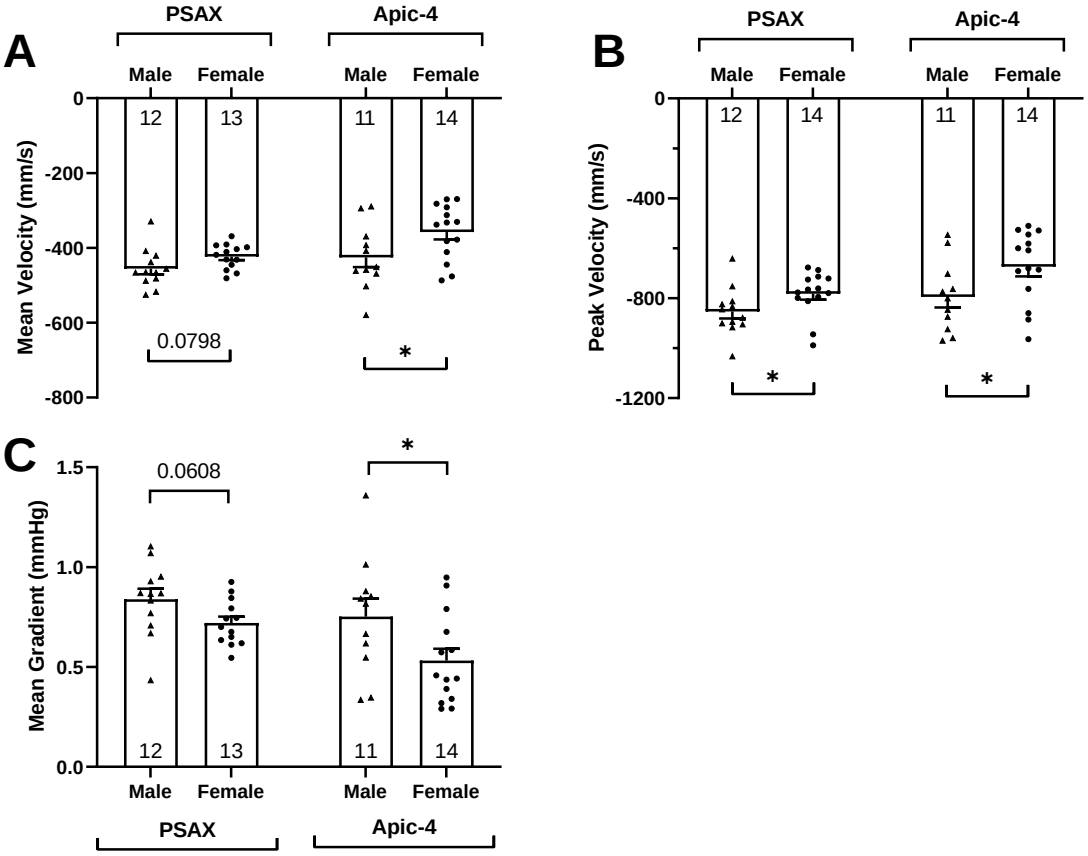
Table 4.7: Echocardiographic indices of left ventricular diastolic function in 8-week-old offspring. *Right hand column presents p-value calculated by student's t-test or Mann-Whitney test as appropriate. IVCT = isovolumetric contraction time, IVRT = isovolumetric relaxation time, MPI = myocardial performance index, Decelerⁿ = deceleration. Data from offspring of lean dams only is shown because number of offspring of obese dams was insufficient for analysis.*

No differences in left ventricular diastolic function (Table 4.7) were identified between male and female 8-week-old offspring of lean dams by echocardiography.

4.3.9 Offspring pulmonary artery flow

When analysed by two-way ANOVA for the effect of view (PSAX or Apic-4) and sex, view had a significant effect on every measure of PA flow other than PAT (Figure 4.12). There was also no effect of sex on PAT. Both sex and view had a significant effect on mean velocity, peak velocity, and mean gradient. These measures were therefore analysed by view-specific student's t-test or Mann-Whitney test as appropriate. Mean (Figure 4.12(A)) and peak velocity (Figure 4.12(B)), as well as mean gradient (Figure 4.12(C)), of flow through the pulmonary artery were reduced in female offspring compared to males. The effect of sex on these measures of PA flow was stronger when PA flow was recorded in Apic-4 than in PSAX.

There was no effect of sex on either PET or PAT:PET (Figure 4.12(D)).



D

				Two-way ANOVA		
	View	Male (n=11-12)	Female (n=14)	View	Sex	Inter^{n}
PAT (ms)	Apic-4	20.6 ± 1.3	20.6 ± 0.9	0.4315	0.9551	0.9892
	PSAX	19.9 ± 0.9	20.0 ± 0.7			
PET (ms)	Apic-4	51.4 ± 1.1	53.7 ± 1.2	**<0.0001**	0.5308	0.3157
	PSAX	60.3 ± 1.2	60.0 ± 1.4			
PAT/PET	Apic-4	0.385 ± 0.020	0.384 ± 0.014	**0.0004**	0.9441	0.8556
	PSAX	0.330 ± 0.012	0.334 ± 0.010			

Figure 4.12: Echocardiographic measures of pulmonary artery (PA) flow in male and female 8-week-old offspring of lean dams*. Mean (A) and peak (B) velocity, and mean gradient (C) of flow through the PA, all analysed by view-specific student's t-test or Mann Whitney U test after a significant effect of both view and sex was found by two-way ANOVA. Table (D) shows PAT, PET and PAT:PET data. Right-hand columns present the results of two-way ANOVA for the effect of view (Apic-4 versus PSAX), sex, and the interaction between the two (inter^{n}). PAT = PA acceleration time. PET = PA ejection time. Numbers presented inside bars in (A)-(C).*

4.3.10 *In vivo* sympathetic stimulation of the heart

Sympathetic dominance is a marker of heart failure. Samuelsson *et al*. first found evidence of increased sympathetic activity in a rat model of maternal diet-induced obesity that uses the same basic protocol and diet as the Ozanne group. Using radiotelemetry, they identified changes in heart rate variability in 12-week-old male offspring of obese dams which were indicative of increased sympathetic activity[283]. Blackmore et al.[295] then found evidence of cardiac sympathetic dominance *ex vivo* in isolated hearts from 12-week-old offspring of obese dams using the Langendorff preparation.

The work in this thesis established a new protocol in order to further probe this potential programming of sympathetic dominance in offspring of obese dams. In this new protocol, a bolus of the selective β1R-agonist dobutamine was administered intravenously to anaesthetised mice at the end of a full echocardiography protocol, without the mouse needing to be moved. In this study the change in heart rate, the chronotropic response, to this cardiac-specific sympathetic stimulation was recorded in real-time. IV administration of the dobutamine into the tail vein of the anaesthetised mouse effectively stimulated a positive chronotropic response in both male and female offspring (Figure 4.13). This response was slightly greater in male than female offspring.

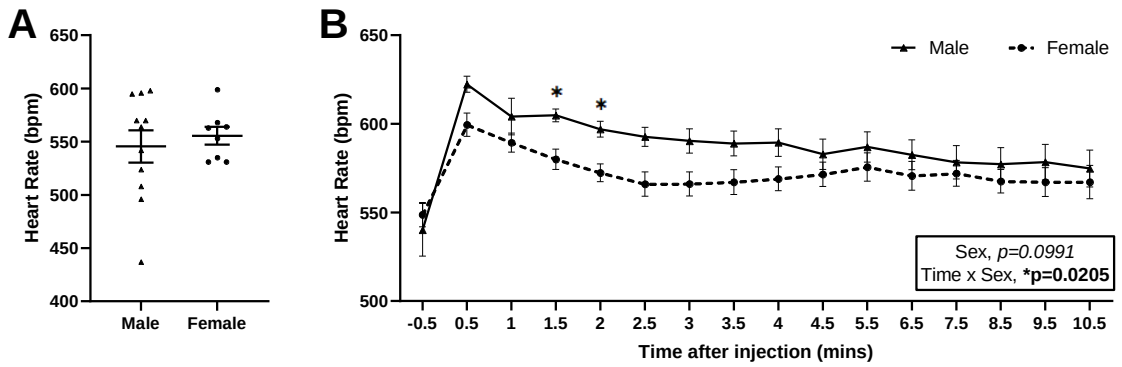


Figure 4.13: Chronotropic response to IV dobutamine in 8-week-old offspring. *Baseline heart rate immediately before (A) and heart rate time course after intravenous administration of 0.5 mg/kg dobutamine in an anaesthetised mouse (B). Box in (B) presents results of mixed-effects analysis of the time course for the effect of time after injection and offspring sex. Data from offspring of lean dams only is shown because number of offspring of obese dams was insufficient for analysis. Numbers: Male = 11, Female = 8.*

This new protocol offers a quick, effective, and repeatable method of introducing cardiotropic agents to a mouse under anaesthesia, while also allowing real-time assessment of cardiac response and comparison with baseline cardiac function. This is due to this technique being specifically designed to be used as an additional protocol on top of standard, baseline echocardiographic measurements such as those made in our lab previously and in Chapters 3 and 5 of this thesis.

This technique can also be adapted to obtain more complete data on the cardiac response to exogenous cardiotropic agents *in vivo*. For example, recording M-mode images throughout would allow for any changes in cardiac contraction to be measured, allowing for the analysis of the inotropic response over time in addition to the chronotropic response assessed here.

Using this technique to IV administer a selective β1R-agonist, such as the dobutamine used in this study, allows specific sympathetic stimulation of the heart, without confounding effects of simultaneous sympathetic stimulation of the peripheral vasculature. Alternatively, a vasomotive agent such as ET-1, AngII or a selective α1R agonist like PE could be administered. The measured cardiac response to these agents would depend on both any direct effects on the heart, and indirect effects on cardiac function due to changes in peripheral vascular resistance. This would be an especially powerful technique when paired with wire myography, as the cardiac response to the exogenous agent *in vivo* can then be considered in the context of the reactivity of isolated blood vessels from the same animal to the same agent *ex vivo*. Moreover, sex- or maternal diet-dependent differences in NO signalling could be assessed *in vivo* using this technique by administering L-NAME, which inhibits *de novo* NO synthesis. This would be particularly interesting given that differences in NO bioavailability are implicated in the sex-specific differences in CVD morbidity and mortality[487].

There are advantages to this technique over existing protocols used to assess cardiac responses to cardiotropic agents. Previously, Blackmore et al.[295] used the isolated Langendorff technique to assess reactivity in the hearts of 12-week-old mice. The main advantage of the protocol established in this thesis over using the Langendorff technique is that findings from this technique will be more physiologically relevant, as the animal is alive and intact. Additionally, and somewhat anecdotally, experience from Dr H. Blackmore, myself, and some collaborators has indicated that the Langendorff technique is likely only feasible if the mice used in the study are at least 12 weeks old, as hearts from younger mice seem to be extremely fragile and consistently do not survive once they are mounted on the apparatus. Conversely, the work in this thesis has clearly demonstrated that this technique using IV administration can be effectively applied to 8-week-old mice.

While this technique was being developed, I spoke with someone using a similar technique to assess cardiac reactivity to exogenous agents during echocardiography. Rather than IV administration of the cardiotropic agent, that protocol used intraperitoneal administration. Variation in the specific site of injection and the rate of absorption from the peritoneal space can result in the dose and time-course of the agent in the blood being less consistent between animals than using IV administration. This would introduce greater inter-animal variation in the data and may impair accurate interpretation, therefore making this technique less powerful than the technique established in this thesis.

4.4 Key Findings

1) Severe obesity in female mice resulted in a decrease in fertility and an increased risk of offspring perinatal mortality.
2) Body weight changes consistent with an increased risk of interpregnancy weight gain were found in obese compared to lean dams.
3) Maternal obesity programmed increases in fat mass in young adult offspring, without any change in body weight.
4) Exposure to maternal obesity *in utero* resulted in a sex-specific increase in heart weight in male offspring at this age.
5) There are sex differences in vascular reactivity between male and female offspring, including increased alpha-adrenergic vasoconstriction in female offspring.
6) A new protocol was developed which allows effective and reproducible assessment of cardiac reactivity to exogenous agents *in vivo.*

4.5 Future work

As in Chapter 3, while the data presented in this chapter are primarily physiological *in vivo* and functional data, both fixed and frozen tissues were collected from animals upon culling. These tissues would once again be available for molecular and histological analyses to investigate the underlying mechanisms and structural changes associated with the functional differences identified in this thesis. However, the low numbers in this study and the resulting limited ability to assess the impact of exposure to maternal diet *in utero* on offspring cardiovascular function means that it is unlikely that carrying out these additional experiments in tissues from this cohort of animals is the best use of laboratory resources. Instead, I would hope that this study is more considered a pilot study and that the novel findings, new protocol, and lessons learned from the poor breeding outcomes are used to design and run a new cohort of young adult offspring of obese dams which generates more data.

4.5.1 Body weight at point of mating

Compared to Chapter 3, PreP2 'Ob females were heavier in this study. The average 'Ob female body weight at mating was 39.8 ± 0.8 g in this study, with the average body weight of 'Ob females that went on to have a viable litter being 36.6 ± 1.2 g and those that didn't being 40.7 ± 1.0 g. In Chapter 3, on the other hand, the average 'Ob dam body weight on the day of plug was 33.3 ± 0.7 g. The findings in this thesis therefore highlight the careful balance required in the study of developmental programming related to maternal diet-induced obesity. While the 'Ob dams in Chapter 3 didn't have as strong an obese phenotype as previous studies, which likely contributed to the lack of replication of the increased PI observed by Hufnagel *et al*., in this study the 'Ob females were too heavy and, as a result, did not reliably produce viable offspring for study. These findings should be used to inform the body weight thresholds used in future cohorts.

4.5.2 Myography

Although this study was not sufficiently powered to assess the impact of maternal obesity on offspring vascular reactivity in this thesis, sex difference is in the response of isolated vessels from offspring of lean dams were identified, and dose-response curves were performed using vasomotive agents that had not been previously used by the Ozanne lab. This can provide context for future experiments and be used in the design of subsequent myography experiments, particularly in the order that vasomotive agents are applied to the vessels and the range of doses that are used.

The finding that femoral arteries from female 8-week-old offspring contracted more forcefully in response to PE than those from male offspring went against expectation, as noradrenaline administration causes significant vasoconstriction in men, but not in pre-menopausal women[468]. This is most likely because, *in vivo*, the vasoconstrictive effects of alpha-adrenergic vasoconstriction in women are offset by enhanced beta-adrenergic vasodilation, but PE is a selective α1R agonist. It may therefore be interesting to include both PE and a non-selective adrenoreceptor agonist in future myography experiments.

Chapter 5: Vascular Function in Aged Offspring

5.1 Introduction

5.1.1 Animal models of metformin intervention during pregnancy

As introduced in Section 1.7.3, metformin is the most widely used glucose-lowering medicine in the world. It is commonly administered in many settings during pregnancy, including to treat PCOS and either pre-existing or gestational diabetes, and is also being trialled in non-diabetic obese pregnant women[352,354]. Given the increasing prevalence of gestational diabetes[131], which is now thought to affect one in seven pregnancies globally, this means that an increasing number of babies are being exposed to metformin *in utero*. While it has been found to have numerous positive effects on the mother, metformin freely crosses the placenta[355], rapidly reaching equivalent concentrations in the maternal and fetal circulations[488], and thus may exert direct effects on the developing fetus that are independent of its effects on maternal physiology.

Longitudinal follow-up of human data is limited by the current age of the children (see Section 1.7.4) and so researchers turn to animal models in order to investigate how metformin exposure *in utero* affects offspring health in adulthood. Several studies investigating the effects of metformin administration during pregnancy on offspring cardiometabolic health have been published in rodents.

In some studies[489–492], metformin is administered in solution once per day by oral gavage, which allows for highly controlled dosing. However, oral gavage can be a stressful procedure for the dam[493]. Given that it is established that maternal stress can programme the cardiometabolic health of adult offspring[90–92], oral gavage is likely not the most suitable method of administering metformin in pregnancy during programming studies. Moreover, in humans, metformin tablets are typically taken two to three times per day with meals and metformin has a half-life in the serum of between four and nine hours[494], meaning that women taking metformin during pregnancy have metformin in their system throughout their waking hours. Therefore, a single dose of metformin per day, especially during the daytime, which is the inactive phase in mice and rats, is less clinically relevant to the dosing procedure used in humans. Dissolving metformin in the drinking water[495–497] represents a stress-free method of ensuring that mice are continuously exposed to metformin throughout their active phase. Whilst this method reduces stress and more closely mimics human metformin consumption, it is more challenging to control for dosage as the amount of metformin dissolved in the water must be regularly adjusted according to water intake in order to control dosage. At the time that this study was set-up, it was not possible to accurately measure water intake in our animal facility. Therefore, metformin was administered to the dams by dissolving it in the sweetened condensed milk, with dosage adjusted according to milk intake (see Sections 5.1.3 and 5.2.1).

5.1 | Introduction

Several studies investigating the effects of *in utero* exposure to metformin on offspring health only include dams fed a control chow diet, which is not clinically relevant as it would not normally be given to a lean healthy mother. Studies that have introduced some form of maternal metabolic challenge alongside metformin administration during pregnancy have done so in a variety of ways.

One strategy has been to investigate the effect of metformin in rats given fructose in their drinking water during pregnancy. Western diets are typically high in fructose and high fructose consumption is associated with obesity and the metabolic syndrome[498]. In non-pregnant female rats, three weeks of high-fructose consumption was not found to alter body weight but resulted in increased adiposity, hyperleptinaemia, and increased plasma triglycerides and FFAs[499]. When given from the day of plug onwards, fructose administration has been found to result in increased maternal insulin resistance in mid-gestation and increased offspring birth weight[495]. Male offspring of fructose-fed dams fed a control diet themselves from weaning have elevated MAP at 12 weeks of age compared to controls, and this hypertension is exacerbated if the offspring are fed a HFD from weaning[489]. Maternal metformin treatment during gestation prevents the increases in mid-gestation insulin resistance and offspring birth weight[495], and fully corrects the increase in MAP seen in offspring fed a HFD from weaning[489]. However, whether *in utero* metformin-exposure corrects the hypertensive phenotype in 12-week-old male offspring of fructose-fed dams fed a control diet from weaning was not investigated[489].

Given that metformin is most commonly prescribed to treat maternal diabetes[340], other studies have investigated the effect of metformin administration in pregnant rodents that have been injected with streptozotocin in order to induce diabetes[490,500]. However, the diabetic phenotype that develops after streptozotocin injection reflects a loss of insulin secretion, akin to type 1 diabetes, rather than the dysregulation of glucose-insulin signalling involving both the pancreas and increased peripheral insulin resistance that occurs in T2D and GDM. Therefore, findings from such studies are unlikely to be clinically relevant to women taking metformin during pregnancy to treat either GDM or pre-existing T2D. Animal models that are most clinically relevant to the use of metformin during obese and diabetic pregnancy are therefore those of maternal diet-induced obesity with impaired glucose tolerance during pregnancy.

5.1.2 Metformin in animal models of maternal diet-induced obesity

Studies of the effect of gestational metformin in obese pregnancy have been published in which maternal obesity is induced by feeding of a HFD for four[497], six[501] or eight[496] weeks before mating and throughout pregnancy and lactation. However, results from these studies are not consistent, especially when considering the effects of maternal obesity and metformin on fetal growth and offspring birth weight. For example, two different studies, one in mice[496] and one in rats[497], found that maternal obesity resulted in increased offspring birth weight compared to controls. Metformin treatment had no effect on birth weight in the mouse study[496], but further increased birth weight in the rat study[497]. On the other hand, a different study in mice found that offspring of obese dams were growth restricted in late gestation, and that this was corrected by maternal metformin treatment[501], and a further study in rats found that neither maternal obesity nor metformin had any effect on fetal growth[492]. While somewhat variable, these studies combine to suggest that maternal metformin treatment during obese pregnancy either does not affect[492,496] or increases[497,501] offspring birth weight. However, Salomäki et al.[491] found that offspring of HFD-fed mice given metformin throughout pregnancy were born growth restricted compared to those of dams who were not given metformin. The discrepancy between these studies is not fully explained, although differences in species and strain (e.g. C57Bl/6J[496] versus CD-1[501] mice) or the diet used (e.g. HFD with between 45%[496] and 62%[501] of calories coming from fat) may contribute. Regardless, inconsistent findings on the effect of maternal obesity on fetal growth and birth weight are somewhat expected. This is in line with human data as babies born to overweight or obese mums are at an increased risk of being born either large[104,111,112] or small[104,112] for gestational age (see Section 1.4). Nevertheless, meta-analysis of human data suggests that gestational metformin treatment in diabetic pregnancy is at least associated with decreased birth weight compared to insulin treatment[324].

The only study not from the Ozanne group in which both male and female offspring of HFD-fed rodents plus a metformin intervention were studied beyond birth found in literature searches was published by Salomäki et al.[491], although it only included offspring of HFD-fed mice with or without gestational metformin. It did not include a control group of offspring of lean dams. In this study, all offspring were first weaned onto a healthy chow diet, which was then switched to a 60% HFD at 10 weeks of age. Exposure to metformin *in utero* had no effect on either male or female body weight up to 10 weeks of age when fed a healthy chow diet. However, maternal metformin administration during pregnancy resulted in offspring gaining less weight when fed a HFD. This effect was stronger in female offspring and resulted in female offspring of metformin-treated dams having reduced adiposity compared to those of HFD-fed dams after six weeks of high-fat feeding. This suggests that *in utero* metformin exposure confers protection against diet-induced obesity in young offspring of obese dams.

Moreover, both male and female 16-week-old offspring of obese dams had reduced glucose tolerance after six weeks of high-fat feeding, and this was not seen in metformin-exposed offspring. Therefore, this study supports the idea that that metformin administration during obese pregnancy is beneficial and confers protection against diet-induced obesity and metabolic dysfunction in young adult offspring. However, there are two key limitations to this study. Firstly, while dams in this study were fed a 60% HFD for four weeks prior to mating, this did not result in an increase in maternal body weight at mating compared to dams fed a healthy chow diet. Moreover, while dams were fed a 60% HFD throughout pregnancy, this was removed at delivery and lactating dams were fed a healthy chow diet until their offspring were weaned. Therefore, while this study does demonstrate changes in offspring obesity and metabolic health with metformin administration to HFD-fed pregnant females, it cannot be easily translated to a human clinical setting of metformin intervention during obese pregnancy.

Studies investigating the effect of metformin during obese pregnancy on offspring health in which HFD-fed dams were heavier than controls at mating have been published by Tong et al.[496] in male offspring only, and in Álvarez et al.[497] in female offspring. Álvarez et al.[497] found that female 8-week-old offspring of obese dams fed a healthy chow diet from weaning were comparably heavier than offspring of obese dams irrespective of maternal metformin treatment. Tong et al.[496] found that maternal metformin treatment limited the increased body weight of male 8-week-old offspring of obese dams fed a HFD from weaning compared to offspring of lean dams. These findings are in line with those found by Salomäki et al.[491]. However, Tong et al.[496] found that 8-week-old offspring of obese dams were glucose intolerant compared to offspring of lean dams, and that this was not corrected by maternal metformin treatment, despite it reducing offspring body weight and adiposity. These studies suggest that maternal metformin treatment does not prevent the programming of increased body weight in offspring of obese dams compared to offspring of lean dams. However, it may be beneficial in limiting the risk of diet-induced obesity in young adult offspring if they consume an obesogenic diet themselves. Therefore, these studies suggest that maternal metformin intervention during obese pregnancy is not detrimental to the metabolic health of young adult offspring and may in fact be beneficial. However, no studies were identified in literature searches in which the effect of metformin during obese pregnancy on offspring cardiovascular health was investigated.

5.1.3 Metformin in our model of maternal diet-induced obesity

The Ozanne group has published numerous papers using our well-established mouse model of diet-induced obesity to investigate the programming of offspring cardiometabolic health by maternal obesity during pregnancy. As described in Section 1.6, wild-type females fed a HF-HS diet from weaning are heavier and fatter than controls at conception[249,290,291], as well as hyperleptinaemic and hyperinsulinaemic[289]. These characteristics persist through pregnancy[217] and they also become glucose intolerant by late gestation[292,293]. This glucose intolerance is then resolved by weaning[294]. Therefore, this is a reasonable mouse model of maternal diet-induced obesity during pregnancy with a GDM-like phenotype. Our model is thus appropriate for metformin intervention studies.

As introduced in Section 5.1.1, we administer metformin to our obese dams by dissolving it in the sweetened condensed milk that forms the "high simple carbohydrate" component of our HF-HS obesogenic diet. The amount of metformin dissolved in the milk is adjusted twice weekly to achieve a dosage of 300 mg/kg body weight per day. Most rodent studies of metformin intervention during pregnancy use approximately this dosage[491,500,502–507] as it equates to a comparable dosage given to pregnant women[508] and thus results are clinically relevant. Indeed, in our model metformin concentration in the maternal serum in late gestation is comparable to that previously reported in pregnant women taking metformin to treat GDM[293]. While most studies begin metformin administration at conception[491,492,495,496,501,505–507], we introduce our dams to the metformin from one week prior to mating. This is because a dietary change in early pregnancy may be stressful to the dam and may influence her food intake behaviours independent of any direct pharmacological effect of the metformin and therefore have confounding effects on the offspring. Providing the metformin for one week prior to mating ensures that the dams have acclimated to having the metformin dissolved in the condensed milk and thus avoids confounding effects of a dietary change in early pregnancy. Some rodent studies, although none yet in obese dams, have begun metformin administration in mid-gestation[490,500,504] as this more closely resembles the developmental time point at which fetuses of mothers with GDM would be diagnosed and therefore begin to be exposed to metformin *in utero*, as GDM is typically diagnosed in women between 24 and 28 weeks of gestation. In our model, the metformin is removed at E19, shortly before delivery, and not administered to the lactating dam as this reflects the human situation where women with GDM stop taking metformin after birth. The metformin is consistently removed at E19 as this ensures that a fresh milk pot without metformin is provided to the dam shortly before birth such that the cage does not have to be re-opened again until PND2, thereby removing potential added stress to the new dam.

Previous studies by the Ozanne group have found that this metformin intervention prevents the development of maternal glucose intolerance in late gestation[293], thereby showing that metformin effectively improves maternal glycaemic control in this model. Metformin also reduces fat mass in mid[297] and late gestation[293] in our model. This is consistent with human data as metformin administration reduces GWG in diabetic pregnancy[341]. Moreover, Hufnagel et al. found that maternal obesity increased uterine artery PI in late gestation, and this was also corrected by metformin[293]. Given that an increased PI in humans is associated with an increased risk of pre-eclampsia[440], this may contribute to the observed reduction in pre-eclampsia risk with gestational metformin treatment[341]. While metformin rescues maternal glycaemic control and improves uteroplacental function in obese dams in our model, it does not correct the reduction in body weight in offspring of obese dams in late gestation[293] or at birth[297]. This may in part be due to metformin not correcting structural defects in the placentas of obese dams[293], including a decrease in the size of the exchange region of the placenta[446], the labyrinth zone, and an increase in placental calcification[293] .

As described previously[297–299], when fed a healthy chow diet from weaning, there is no difference in the body weight of 8-week-old offspring of lean and obese dams. This is unchanged if obese dams are given metformin during pregnancy[297], consistent with the findings from other studies described in Section 5.1.2[491,497]. However, in a longitudinal body weight and adiposity study by Schoonejans et al.[306], offspring of obese dams fed a healthy chow diet from weaning had an increased fat mass, as measured by TDNMR, than controls from five to six months of age onwards, and this adiposity phenotype emerged one month earlier in offspring exposed to metformin *in utero*. This opposes what the studies described in Section 5.1.2 inferred from their findings in younger offspring[491,496,497] and suggests that metformin may detrimental to long-term offspring metabolic health. It also highlights the need for long-term follow-up in developmental programming studies as poor cardiometabolic health is associated with advancing age.

5.1.4 The impact of age on cardiovascular disease

In humans, the risk of an individual developing CVD increases as they get older. While this is partially explained by the increase in other risk factors for CVD with age, including obesity and metabolic dysfunction, it is also related to age-associated structural and functional changes in the heart and vasculature. Heart weight increases with age[509], and this is associated with increased cardiac wall width[510,511]. There is progressive deposition of collagen in the cardiac interstitial and perivascular space[512], resulting in reduced myocardial compliance and subsequent diastolic dysfunction.

Cardiomyocytes in the ageing heart are also more susceptible to oxidative stress and this is associated with increased apoptosis and necrosis[511]. Moreover, the number of pacemaker cells in the sinoatrial node also decreases with age, which is associated with conduction abnormalities[511] and thus an increased risk of arrythmia.

While age-related changes in the heart increase the risk of heart failure, changes in the vasculature that impact cardiac function directly, such as by impeding blood flow through the coronary arteries, and that increase the risk of hypertension also increase the risk of CVD with age. As described previously, factors including increased sympathetic nervous system activity, changes in peripheral vascular reactivity and vascular remodelling have all been implicated in the pathogenesis of hypertension[60]. There are well established effects of ageing on all of these factors. Firstly, the activity of sympathetic vasoconstrictor nerves increases with age[469,513]. Secondly, age is associated with reduced endothelium-dependent vasodilation[514] and endothelial dysfunction. As discussed in Chapter 3, a major component of endothelium-dependent vasodilation is NO-dependent vasodilation and the endothelial dysfunction in ageing is associated with decreased production and bioavailability of NO[515]. Increased oxidative stress and ROS production in ageing blood vessels contribute to the reduced NO bioavailabilty[515] (see Section 4.1.1). Ageing in the vasculature is also associated with increased arterial stiffness, which is due to both endothelial dysfunction and vascular remodelling, including arterial wall thickening[509,516] and the deposition of collagen in the vascular wall[510].

As introduced in Section 1.2 and expanded upon in Chapter 4, while CVD is an ageing-related disease[11], pre-menopausal women are relatively protected against CVD, meaning that men usually develop CVD at an earlier age than women. However, after menopause, the incidence of new onset CVD in women increases rapidly such that it is quickly greater than that seen in men[517] (as seen in Figure 1.3). While hypertension is more common in men until approximately 40 years of age[464–466], the prevalence is then similar between men and women until the age of around 65[464,465], and is greater in women beyond this age[464,466]. The increased risk of CVD and hypertension in postmenopausal women is associated with the loss of the differences in cardiovascular function between young men and women discussed in Section 4.1.2. For example, SVR does not correlate with MSNA in young women[471], as it does in men[470], due to enhanced beta-adrenergic vasodilation acting to offset the sympathetic vasoconstrictor nerve activity. However, SVR does positively correlate with MSNA in postmenopausal women[468]. The significant reduction in circulating oestrogen after menopause also reduces NO bioavailability. In premenopausal women, oestrogen signalling through ERα activates eNOS[467].

However, in postmenopausal women the decrease in circulating oestrogen is associated with reduced expression of both ERα and eNOS in the peripheral vasculature[487]. Moreover, the antioxidant qualities of oestrogen action discussed in Section 4.1.1 are lost at menopause, resulting in increased ROS production in the vasculature and subsequent reduced NO bioavailability. Therefore, changes in cardiovascular structure and function contribute to an increased risk of developing CVD and hypertension with age. Premenopausal women are relatively protected against some of these changes compared to age-matched men, and much of this protection is attributed to the actions of oestrogen. However, the protective effects of oestrogen are lost at menopause. This results in rapid increase in the prevalence of CVD and hypertension in postmenopausal women.

5.1.5 Ageing and programmed phenotypes

The prevalence of obesity and cardiometabolic disease increases with age independently of the influence of developmental programming. Therefore, it is unsurprising that programmed phenotypes of cardiometabolic dysfunction worsen with age. This was discussed in the context of the development of obesity[217,306], adipocyte dysfunction[306], and glucose intolerance[303] with advancing age in offspring of obese dams in our model of maternal diet-induced obesity in Section 1.6.4. It has also been proposed that accelerated ageing itself can be programmed by exposure to a suboptimal fetal environment. In a rat model of maternal protein restriction during pregnancy, both male[518] and female[519] offspring that were born growth restricted, but had rapid postnatal catch-up growth, displayed signs of accelerated cellular ageing in adulthood, including increased telomere shortening and oxidative stress. Findings indicative of programming accelerated ageing have also been identified in female offspring in models of maternal high-fat feeding[520,521].

Only two studies have investigated the effect of exposure to maternal high-fat feeding *in utero* on blood pressure in offspring older than 6 months of age. Elahi et al.[279] found that 8-month-old offspring of obese dams had elevated SBP compared to controls independent of sex. Conversely, in the previously described rat model in which female rats are fed a HFD for 10 days prior to mating and throughout pregnancy and lactation, both 6- and 12-month-old female offspring had elevated SBP compared to controls, but male offspring did not[281]. While Khan et al.[281] did investigate vascular reactivity in six-month-old offspring in their model, no studies have been published in which vascular reactivity to vasomotive agents has been investigated in offspring of obese dams beyond 6 months of age. Moreover, no studies have been published assessing the effect of metformin intervention during obese pregnancy on vascular reactivity in the offspring.

Given that we have shown previously that maternal obesity programmes both metabolic[249,290,296,303] and cardiovascular[295,298,301] dysfunction in young adult offspring, that the metabolic dysfunction worsens with age[217,306], and is exacerbated by metformin[306], and that cardiovascular function is known to deteriorate with age, it is paramount that the long-term effects of metformin exposure during obese pregnancy on offspring vascular function are investigated.

5.1.6 Chapter aims

1 To investigate whether the hypertensive phenotype observed in young adult offspring of obese dams persists or changes with advancing age.

2 To assess systemic vascular reactivity and structure in aged offspring of obese dams.

3 To establish if there are sex differences in the programming of blood pressure and vascular function in aged offspring.

4 To determine whether exposure to metformin *in utero* improves or exacerbates any changes in blood pressure and vascular function in aged offspring of obese dams.

5.2 Methods

5.2.1 Animal model

Within this chapter three groups of 12-month-old offspring were studied, those born to 'Con dams (Con), those born to 'Ob dams (Ob) and those born to obese-metformin dams (Ob-Met). This cohort was established before I started my PhD, so my work on this cohort started when the animals had been aged and just prior to them reaching 12 months of age. All maternal data and that on the young offspring is therefore not reported in this thesis (and is part of the PhD thesis of Dr J. Schoonejans). My work on the 12 months animals therefore maximised the output gained from the cohort in line with The Three Rs principle.

As described previously, PreP2 females fed a control diet from weaning became 'Con dams and were mated for P2 for up to three attempts from one week after weaning of their first litter. Once PreP2 females fed the high fat obesogenic diet reached a pre-determined threshold of obesity of 10 g body fat as measured by TDNMR (Bruker LF90II), they were randomly assigned to become either 'Ob dams or obese-metformin dams ('Ob-Met). 'Ob dams were then mated for P2 for up to three attempts.

Before mating for P2, 'Ob-Met dams were single housed and treated orally with metformin-hydrochloride supplemented in the condensed milk for one week and were then mated for P2. The metformin dose aimed for was 300 mg/kg/day, and this was achieved by adjusting the amount of metformin dissolved in the milk twice weekly according to the animal's current body weight and their average milk intake over the preceding days. Metformin dosing was performed by Dr J. Schoonejans. The condensed milk, and therefore the metformin, was removed while the dams were paired with wild-type stock males and returned once a copulatory plug was noted and the mating pairs were separated. If 'Ob-Met dams did not plug on the first mating attempt, they were removed from the study to avoid confounding effects of different periods of exposure to metformin on the PreP2 dam.

'Ob-Met dams continued to receive metformin until E18.5, at which point the metformin-supplemented milk was removed, and the dams instead received the standard condensed milk through littering and lactation. Data from these dams has been published by Dr J. Schoonejans[297]. Litter sizes were standardised to six pups at PND2 in order to standardise milk supply and maternal attention, and then pups were left with their mother to suckle naturally. Offspring of all groups were weaned at PND21. Animals were housed in same-sex littermate pairs, given *ad libitum* access to standard RM1 laboratory chow and maintained until one year of age.

5.2.2 Non-invasive blood pressure measurement

The protocol of blood pressure measurement and analysis is described in Section 2.2. Following two days of training, conscious heart rate and SBP were measured at 50 weeks of age both in the evening at the beginning of the active phase, between 17:00-18:30, and in the morning at the beginning of the inactive phase, between 08:00-09:00.

5.2.3 Femoral artery myography

At one year of age, mice were fasted overnight for 16 hours and then culled by exposure to a rising concentration of CO_2[1]. Immediately after culling, femoral arteries were excised and mounted on the wire myograph and were subjected to the normalisation protocol and K^+ curve as described in Section 2.3. The vasomotive agents used in this cohort, and their dosing, are described in Table 5.1.

Agent	Doses (M)
Phenylephrine (PE)	10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}
Sodium Nitroprusside (SNP)	10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}
Acetylcholine (ACh)	10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}
ACh + 10^{-2}M L-NAME	As above after 10 minute incubation with L-NAME

***Table 5.1: Vasomotive agents used in wire myography in aged adult offspring cohort**. Drugs and doses used in myography experiments with femoral arteries from 12-month-old offspring of Control, Obese, and Obese-Metformin dams.*

5.2.4 Aorta histology

A section of the abdominal aorta was excised[2] from a same-sex littermate that was housed with the fasted animal from weaning onwards and killed in the fed state by cervical dislocation at one year of age. Aortic segments were immediately fixed in 4% formalin. Formalin-fixed aortas were then processed, embedded, and sectioned. Sections were stained with Pico Sirus Red as this enabled clear distinction between the tunica media and adventitia. Whole stained sections were scanned using the Axioscan digital slide scanner (Zeiss, Germany)[3] (Figure 5.1).

[1,2] Animal culling, dissection (not including femoral arteries) and aorta collection by Dr J. Schoonejans
[3] Processing, embedding, sectioning, staining, and imaging performed by T. Ashmore

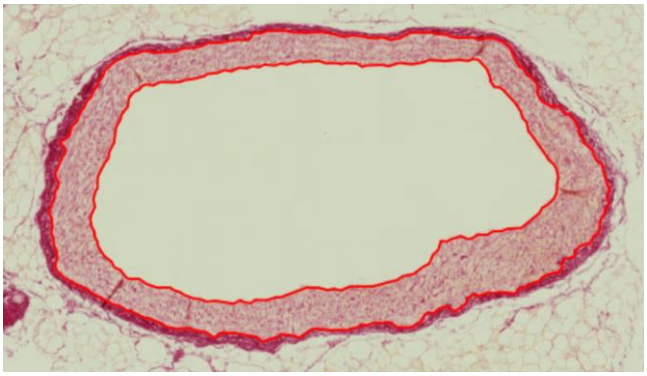


Figure 5.1: Histological analysis of aorta. *Image of thoracic aorta cross-section stained with Pico Sirius Red. Outer and inner boundaries of the tunica media, the smooth muscle layer, shown in red.*

Images were analysed using QuPath[522], and average thickness of the tunica media was calculated.

$$\textbf{Average thickness} = \frac{\textbf{2 x (Cross} - \textbf{sectional area of layer)}}{\textbf{(Outer perimeter of layer)} + \textbf{(Inner perimeter of layer)}}$$

5.2.5 Isolated Langendorff technique

The isolated Langendorff technique, or Langendorff preparation, is an *ex vivo* technique that allows the investigation of inotropic and chronotropic responses of the heart to exogenous stimulation without any confounding effects of wider full-body physiology in an intact animal. This technique has previously been used by the Ozanne group to assess sympathetic dominance in 12-week-old male offspring of obese dams[295]. This study set out to repeat these experiments in male and female 12-month-old offspring in order to investigate how this phenotype changed with advancing age. However, drug-response data was only successfully obtained from 21 of the 68 hearts taken for Langendorff. Of the remaining 47 experimental procedures, 11 were unsuccessful due to equipment or software failures, while 36 failed due to the heart either not surviving or never stabilising. These numbers were insufficient for data analysis and so no Langendorff data is included in this thesis.

5.2.6 Statistical analysis

All data were analysed using Prism 9.0 software (GraphPad). Unless stated otherwise, all data are presented as mean ± standard error of the mean (SEM). N (numbers) refer to independent litters, with no more than one male and one female offspring used for a given measure per litter. Preliminary statistical testing using mixed effects models taking litter as a covariate was performed to verify that including male and female littermates did not impact the data. Data points were excluded as statistical outliers prior to statistical analysis if they were more than two standard deviations from the mean of the group. Normality of data was assessed by the Shapiro–Wilk test.

All data were analysed by mixed effects models to test for an effect of maternal group (Group) and offspring sex (Sex). Data were considered statistically significant if $p<0.05$.

If there was a significant effect of both maternal group and offspring sex, or if $p<0.07$ for an interaction between the two factors, male and female data were separated, and sex-specific one-way ANOVAs or Kruskal-Wallis tests were run to identify any sex-specific effects of maternal group. An interaction was taken into account if $p<0.07$ rather than only if it was significant as the focus of this work was to identify effects of maternal group that may or may not be sex dependent. A trending effect of an interaction could suggest that a sex-specific effect exists but that the mixed-effects model analysis is underpowered to detect it when offspring sex is also taken into account. This threshold was set prior to any data analyses being run.

Alternatively, if only an effect of maternal group was identified and there was no significant or trend for an interaction between the factors, male and female data were combined and analysed by one-way ANOVA or Kruskal-Wallis test. When data were analysed by ANOVA, the Brown-Forsythe test was used to verify if groups had equal variances. If groups did not have equal variances, a Welch ANOVA was used. If a significant effect of maternal group was identified in either combined or sex-specific analyses, Tukey's multiple comparison test was used to identify pairwise differences between groups. If only a main effect of offspring sex was found, no further analyses were carried out. While maximal response femoral artery wire myography data was analysed according to this protocol, dose-response curves were analysed as described in Section 2.3.3.

5.3 Results and Discussion

Hypertension is an age-related disease and numerous studies have found sex- and age-dependent differences in the programming of offspring hypertension and vascular dysfunction by maternal obesity. Increased SBP has been identified in 8-week-old offspring of obese dams in our model of maternal diet-induced obesity[298,301], but whether this phenotype persists or changes with advancing age has not previously been investigated. The aim of this chapter was to determine whether exposure to maternal obesity *in utero* programmed changes in blood pressure in aged offspring, and to investigate differences in vascular function and structure that could underlie any differences. Moreover, it set out to assess how metformin intervention during obese pregnancy affected these factors given that there is currently little consensus on the effects of metformin on long-term cardiovascular health and it is difficult to address this in humans.

5.3.1 Fasted body weight and fat mass

There was no difference in fasted body weight between 12-month-old Con and Ob offspring independent of offspring sex (Figure 5.2(A)). Indeed, there was no effect of either maternal obesity or gestational metformin on body weight in male offspring. However, male Ob offspring had an increased absolute fat mass compared to Con (Figure 5.2(B)), resulting in an increased fat mass relative to body weight (Figure 5.2(C)). There was no significant difference in fat mass between female Con and Ob offspring (Figure 5.2(B,C)). A similar pattern of male-specific adiposity has been found previously in 12-month-old offspring of obese dams in this model in the fed state[306]. In that study, the increase in adiposity in male offspring was associated with adipocyte dysfunction and hepatic lipid accumulation, which was not seen in female offspring of obese dams[306]. This suggests that female offspring of obese dams may be more protected against the development of obesity in later life than males.

Metformin administration during obese pregnancy partially corrected the increase in adiposity in male offspring compared to Con (Figure 5.2(B,C)), although there was also no significant difference in adiposity between Ob and Ob-Met offspring. Taken in isolation, this may suggest that gestational metformin is beneficial and prevents the sex-specific programming of increased adiposity of male offspring of obese dams at this age. However, Schoonejans et al.[306] have shown that despite 12-month-old male offspring of obese-metformin dams having similar fed body weight and fat mass to offspring of lean dams, their white adipose tissue is dysfunctional, with increased adipocyte hypertrophy and a highly inflammatory phenotype. This increased adipose tissue dysfunction was associated with more severe hepatic steatosis in male offspring of obese-metformin dams than was seen in those not exposed to metformin *in utero*.

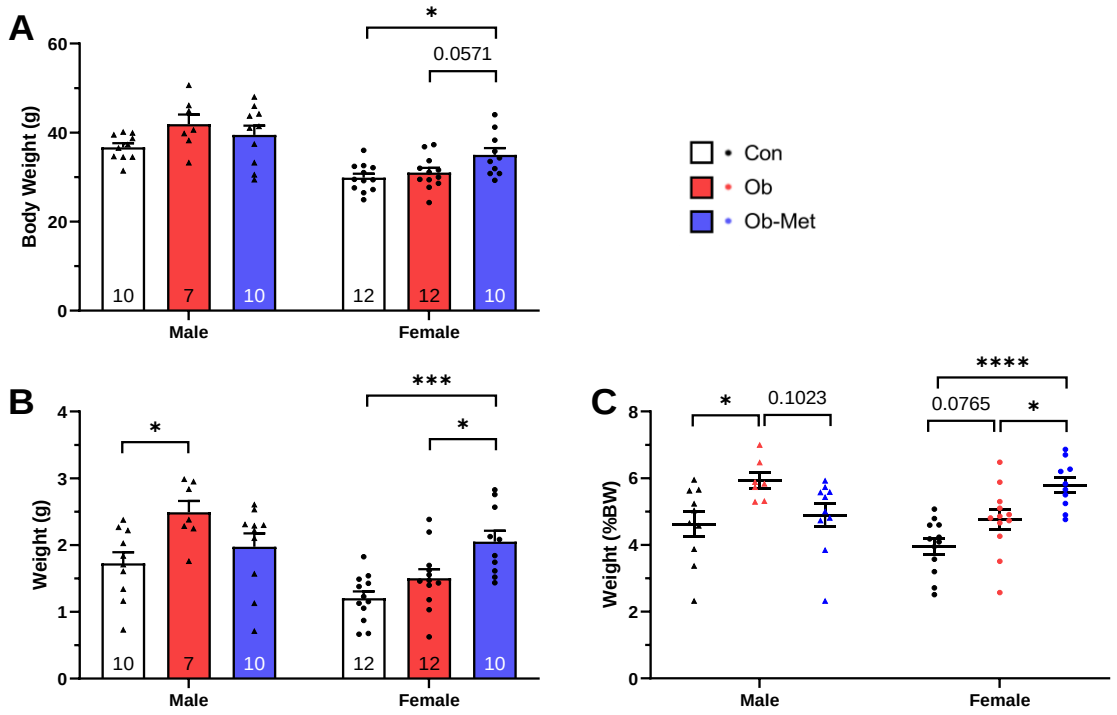


Figure 5.2: Fasted body weight and gonadal fat pad mass of 12-month-old offspring.
*Body weight (A), and absolute (B) and relative (C) gonadal fat pad mass of male and female offspring after a 16 hour fast. *p<0.05, ***p<0.001, ****p<0.0001 using Tukey's multiple comparison test after a significant effect of maternal group found by sex-specific one-way ANOVA. Numbers presented in bars in (A) and (B) are matched with data in (C).*

The detrimental effects of gestational metformin on fasted body weight and adiposity were more striking in 12-month-old female Ob-Met offspring (figure 5.2). While there was no significant difference in fasted body weight or fat mass between Con and Ob offspring, combined exposure to maternal obesity and metformin *in utero* resulted in female Ob-Met offspring being both heavier and fatter than Con. Ob-Met female offspring were also non-significantly heavier than Ob (p=0.0571), which was associated with them having increased absolute and relative gonadal fat pad weights. Once again, this increase in body weight and adiposity in 12-month-old female Ob-Met offspring is consistent with previous studies in fed offspring at the same age[306] in which this increased fat mass was associated with increased adipose tissue inflammation and hepatic steatosis.

Therefore, there were sex-specific effects of *in utero* exposure to both maternal obesity and metformin on offspring body weight and adiposity following a 16-hour overnight fast at 12 months of age. Taken together, these data suggest that maternal metformin treatment during pregnancy not only does not improve adiposity and metabolic health in aged offspring of obese dams, but it worsens these phenotypes, especially in aged female offspring.

5.3.2 Systolic blood pressure and heart rate

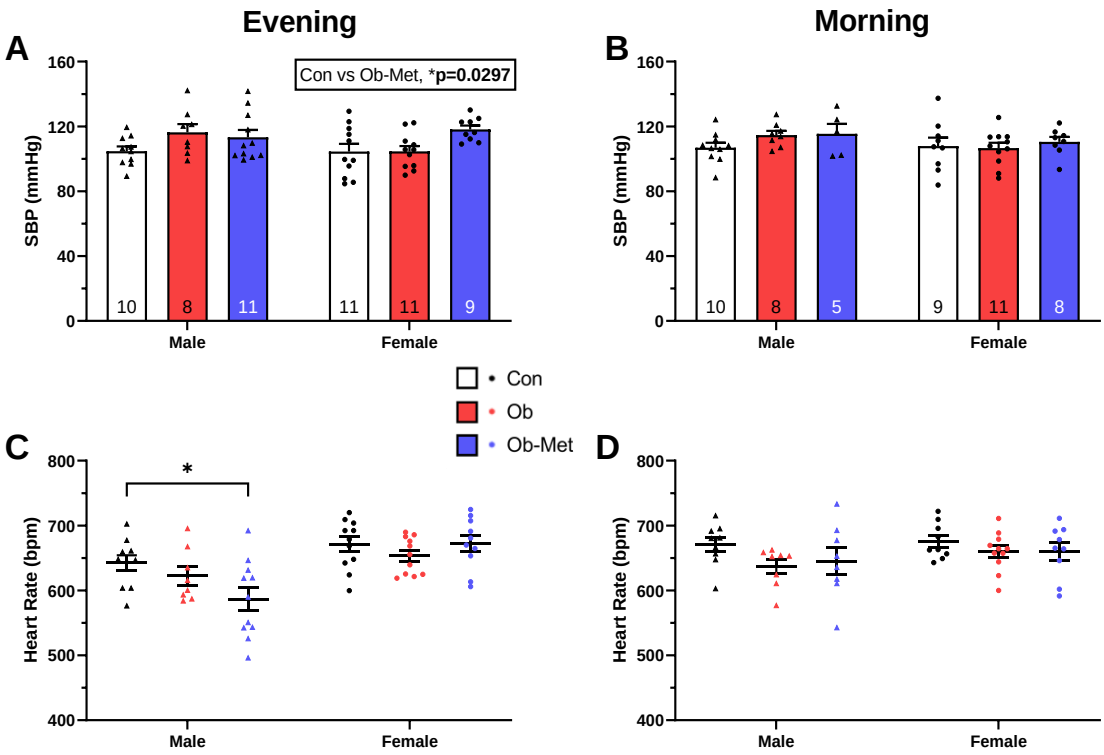


Figure 5.3: Blood pressure and heart rate in 12-month-old offspring.
*Systolic blood pressure (SBP) measured by tail cuff plethysmography in the evening (A) and morning (B), with corresponding measurements of heart rate (C,D). Box in (A) describes difference identified by Tukey's multiple comparisons following significant effect of group identified by one-way ANOVA of combined male and female SBP data. In (C), *p<0.05 using Tukey's multiple comparison test after a significant effect of maternal group found by sex-specific one-way ANOVA. Numbers presented in bars in (A) and (B) are matched with data in (B) and (C) respectively.*

When analysed by two-way ANOVA for effect of sex and group, there was a significant effect of group on evening SBP (p=0.0237) but no effect of sex (p=0.4616). Male and female data were therefore combined and analysed by one-way ANOVA. SBP measured in the evening, at the start of the active phase, was increased in 12-month-old Ob-Met offspring compared to Con (Figure 5.3(A)). At this time, there was also a reduction in heart rate in male Ob-Met offspring compared to Con (Figure 5.3(C)), as identified by sex-specific one-way ANOVA after a significant effect of sex (p<0.0001) and a trending interaction between sex and maternal group (p=0.0562) was identified. There was no effect of group on female heart rate (p=0.4028). No differences in either SBP or heart rate were found when measurements were taken in the morning, at the beginning of the inactive phase (Figure 5.3(B,D)).

Exposure to maternal obesity *in utero* thus did not result in changes in SBP as measured by tail cuff plethysmography in 12-month-old male or female offspring in either the morning or evening in this study. While exposure to maternal obesity alone did not result in detectable changes in SBP, combined exposure to maternal obesity and metformin resulted in increased SBP in the evening independent of offspring sex. That this increase in SBP is only seen when blood pressure was measured in the evening and not in the morning further exemplifies how the time of day that blood pressure is measured is so critical to the identification of a hypertensive phenotype.

The increase in SBP was associated with a decrease in heart rate in male Ob-Met offspring, while no decrease in heart rate was seen in female Ob-Met offspring. An increase in SBP with a corresponding decrease in heart rate is indicative of an intact baroreflex response. The baroreflex response is a critical part of sympathetic regulation of blood pressure. Increases in arterial blood pressure are detected by arterial baroreceptors in the aortic arch and carotid sinus, which then stimulate cardiac regulatory centres in the brainstem. The result of this stimulation is a decrease in sympathetic and increase in parasympathetic outputs to the heart resulting in negative chronotropy and inotropy in order to reduce cardiac output and therefore restore arterial blood pressure. Therefore, in male Ob-Met offspring, that SBP is raised and heart rate is lowered compared to controls is indicative of an intact baroreceptor reflex. However, while female Ob-Met offspring also had an increased SBP compared to Con, their heart rates were comparable. This suggest that in these female offspring, the baroreceptor reflex had been reset and is no longer acting to reduce heart rate and attempt to restore SBP. Resetting of the baroreceptor reflex is commonly seen in hypertension[523].

That no differences were found between Con and Ob offspring does not rule out that differences may be found during the night if blood pressure was measured by radiotelemetry. Indeed, increased SBP in six-month-old offspring of obese dams using our model of maternal diet-induced obesity has only been identified previously during the night using radiotelemetry[217]. However, this study clearly shows that exposure to metformin *in utero* programmes hypertension in aged offspring of obese dams. Therefore, gestational metformin is worsening long-term cardiovascular health in these offspring. This highlights the importance of long-term follow-up in developmental programming studies and thus the power of using animal models with far shorter lifespans than humans, as current human follow-up of cardiovascular outcomes only extends to children who are five years old[365], and the most recent data from the MOP trial suggests that metformin may be cardioprotective in four-year-old children[353].

5.3.3 Femoral artery myography

As has been discussed earlier in this thesis, changes in peripheral vascular reactivity and vascular remodelling have been implicated in the pathogenesis of hypertension[60]. Therefore, femoral artery myography was used to investigate the reactivity of the systemic vasculature to different vasomotive agents to establish if changes in vascular reactivity contributed to the hypertension identified in Ob-Met offspring.

Response to depolarisation

There was no effect of maternal group on the contractile response of femoral arteries from male 12-month-old offspring to VSMC depolarisation by exposure to Krebs with an increasing concentration of K^+ (Figure 5.4(A)). However, femoral arteries from both female Ob and Ob-Met offspring contracted more forcefully in response to an increasing concentration of K^+ than female Con offspring (Figure 5.4(B)). Therefore, there was a sex-specific increase in the contractile capacity of femoral arteries from offspring of obese dams that was not altered by *in utero* metformin exposure. This is discussed further in Section 5.3.4.

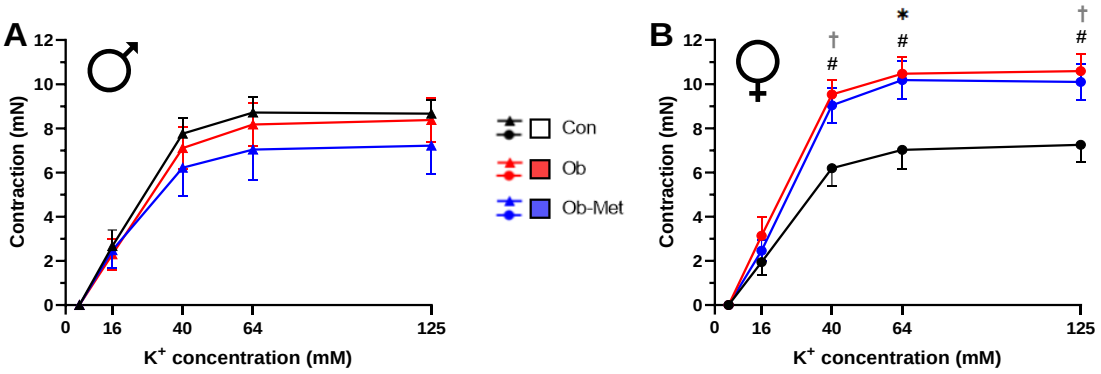


Figure 5.4: Femoral vascular response to increasing concentration of K^+.
*Response curves of femoral arteries from male (A) and female (B) 12-month-old offspring to Krebs with an increasing concentration of K^+. Curves analysed by mixed model analysis followed by post hoc Tukey's multiple comparison test if a significant effect of maternal group identified. Con vs Ob-Met: *p<0.05, 0.05<†p<0.06 and Con vs Ob: $^{\#}$p<0.05. Numbers in (A): Con = 9, Ob = 7, Ob-Met = 9. Numbers in (B): Con = 11, Ob = 9, Ob-Met = 11.*

Response to increasing concentration of PE

There was a significant effect of maternal group on PE dose-response curves in male offspring only, whether expressed as absolute change in tension (p=0.0075) (Figure 5.5(A)) or relative to K^+ (p=0.0001) (Figure 5.5(C)). This was driven by an increased PE reactivity in femoral arteries from Ob-Met offspring compared to Con.

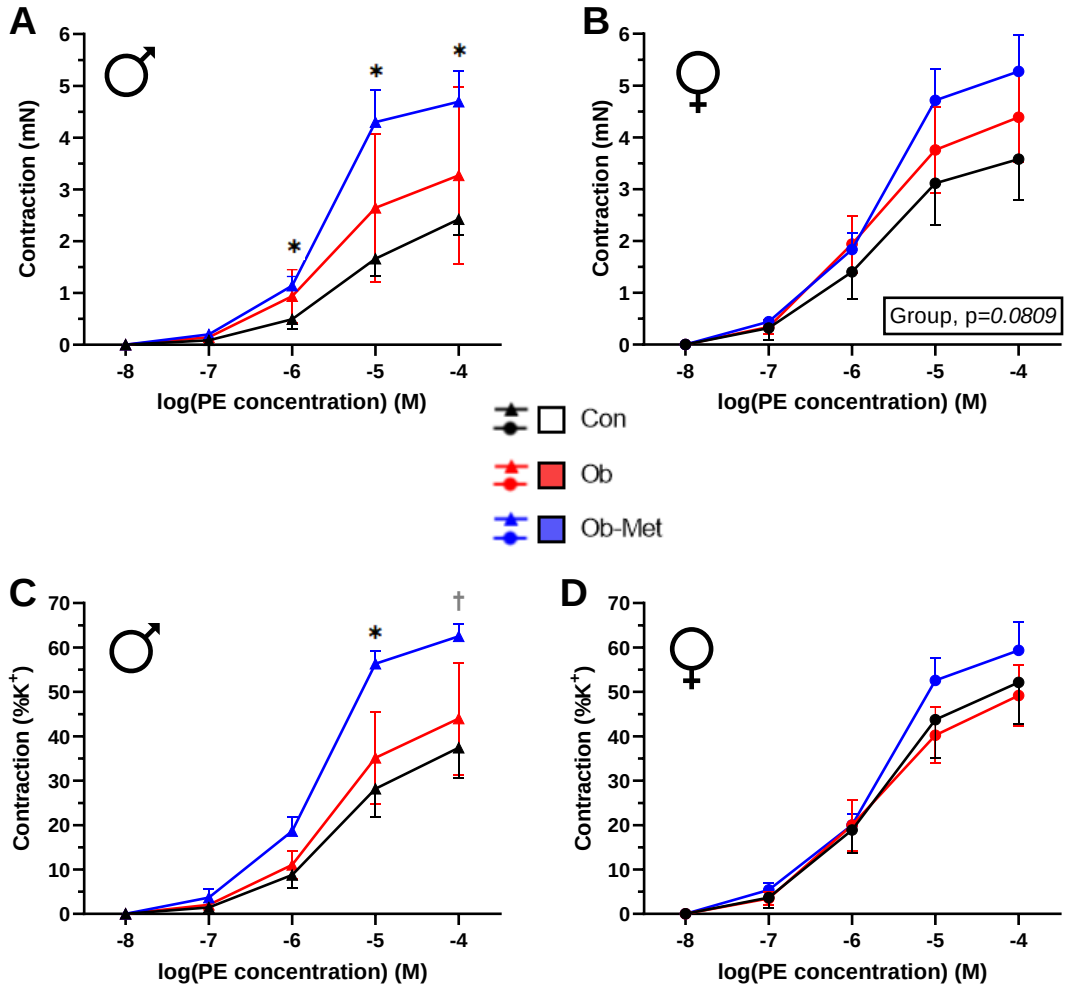


Figure 5.5: Femoral vascular response to increasing concentration of phenylephrine (PE).
*Femoral artery dose-response curves to PE from male (A) and female (B) 12-month-old offspring expressed as absolute change in tension normalised to 10^{-8}M PE. Data from these dose-response curves is then transformed according to the vessel's maximal contraction to elevated K^+ (%K^+) (C,D). Curves analysed using mixed models followed by post hoc Tukey's multiple comparison test if a significant effect of maternal group found. Con vs Ob-Met: *p<0.05, 0.05<†p<0.06. Box in (B) shows p value for effect of Group from mixed-effects analysis. Numbers in (A) and (C): Con = 7, Ob = 5, Ob-Met = 7. Numbers in (B) and (D): Con = 8, Ob = 10, Ob-Met = 9.*

There was a trending effect of maternal group on PE dose-response curves in female offspring expressed as absolute change in tension from baseline (p=0.0809) (Figure 5.5(B)), but this was lost when normalised to K^+-response (p=0.2122) (Figure 5.5(D)). This suggests that any subtle differences in the response to PE in female vessels were most likely due to differences in the contractile or electrophysiological properties of the VSMCs, rather than any difference in the specific reactivity to alpha-adrenergic stimulation.

That there was no difference in femoral artery reactivity to PE in male or female Ob offspring compared to Con shows that maternal obesity alone did not affect vascular reactivity to alpha-adrenergic stimulation in offspring at this age. Nevertheless, *in utero* exposure to metformin programmed a sex-specific increase in reactivity to alpha-adrenergic stimulation in male offspring of obese dams. This therefore represents a sex-specific effect of combined maternal obesity and metformin-exposure on vascular function in aged offspring.

Response to increasing concentration of SNP

Neither maternal group nor offspring sex had any effect on vessel relaxation in response to the NO donor SNP (Figure 5.6). The mean maximal response to SNP was greater than 85% in all groups.

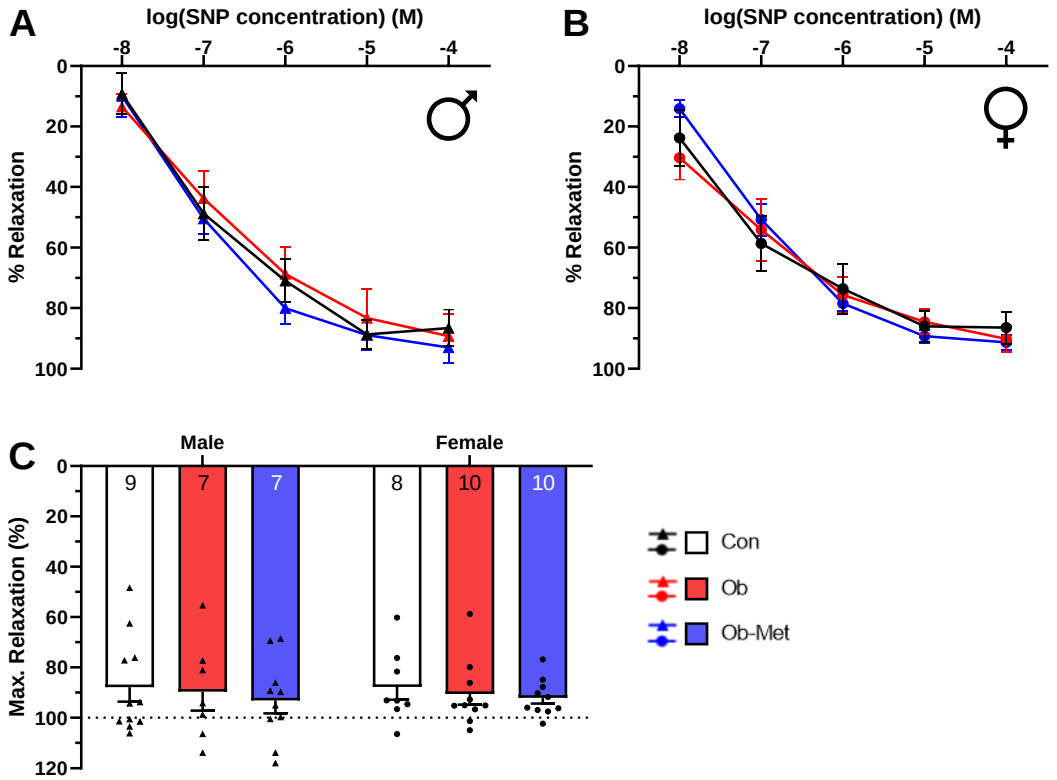


Figure 5.6: Femoral vascular response to increasing concentration of sodium nitroprusside (SNP). *Dose-response curves to an increasing concentration of SNP by femoral arteries from male (A) and female (B) 12-month-old offspring, expressed as %Relaxation following pre-constriction by exposure to noradrenaline. Corresponding values of maximal %Relaxation presented (C). Numbers presented in bars in (C) are matched with data in (A) and (B).*

Response to increasing concentration of ACh, with or without *de novo* NO synthesis

While there was a trending effect of maternal group on maximal relaxation in reponse to ACh ($p=0.0709$), no significant effect of either maternal group or offspring sex was found (Figure 5.7(A-C)). When *de novo* NO synthesis was inhibited by incubation with L-NAME, vessels relaxed in response to ACh up to a concentration of 10^{-6}M, after which point vessels tended to contract (Figure 5.7(D,E)).

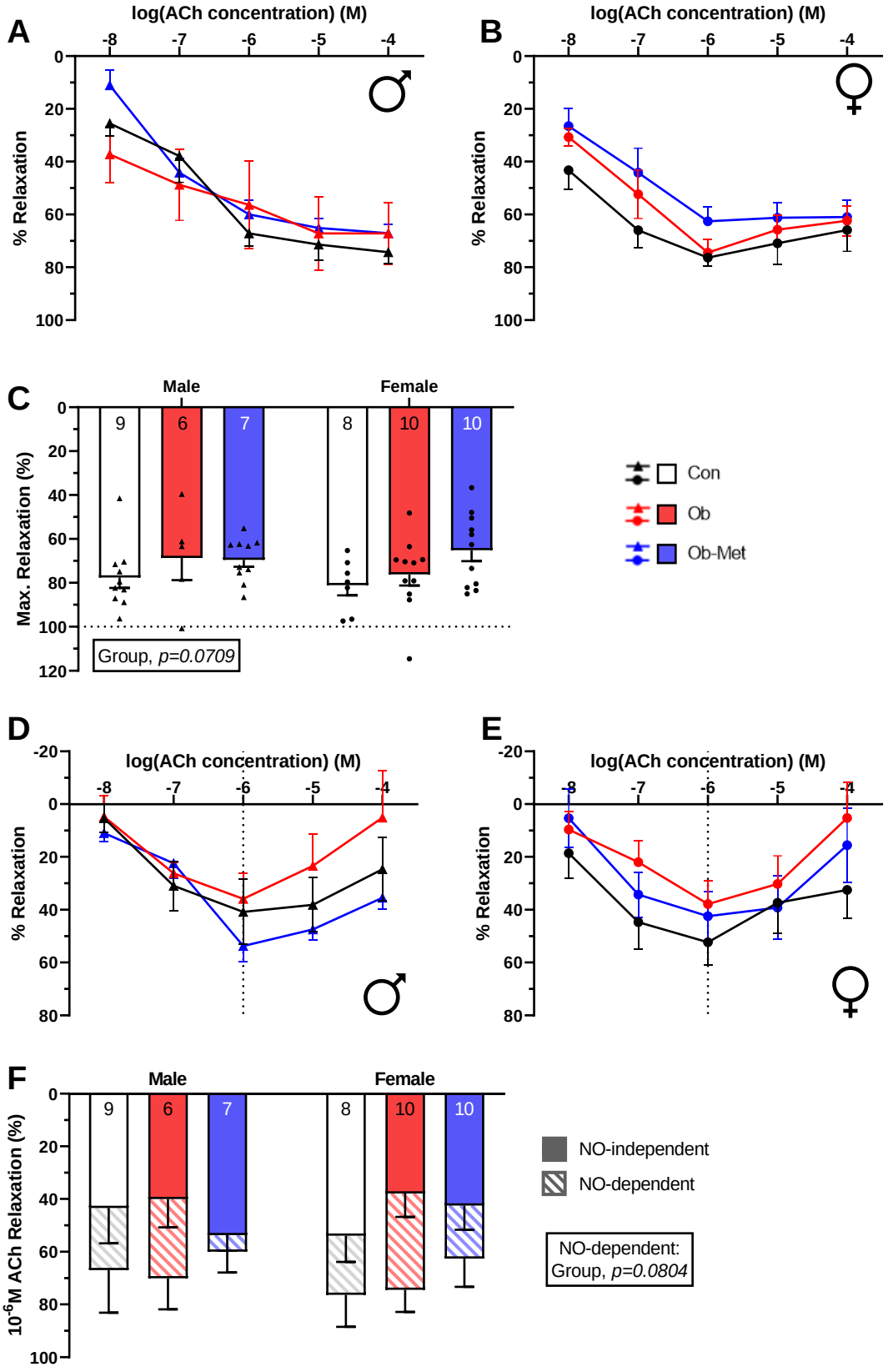


Figure 5.7: Femoral vascular response to increasing concentration of acetylcholine (ACh).
Caption overleaf

Figure 5.7: Femoral vascular response to increasing concentration of acetylcholine (ACh).
Dose-response curves to ACh by femoral arteries from male (A) and female (B) 12-month-old offspring, expressed as %Relaxation following pre-constriction by exposure to noradrenaline. Corresponding values of maximal %Relaxation presented (C), with trending effect of maternal group when analysed by two-way ANOVA. ACh dose-response curves from the same vessels following 10-minute incubation with L-NAME to inhibit de novo *NO synthesis (D,E). Relative contribution of NO-independent and NO-dependent relaxation to response to 10^{-6}M ACh derived from dose-response curves (F) with trending effect of maternal group on NO-dependent component of ACh-response when analysed by two-way ANOVA. Numbers presented in bars in (C) are matched with data in (A) and (B). Numbers presented in bars in (F) are matched with data in (D) and (E).*

Therefore, the relative contributions of NO-dependent and NO-independent relaxation in response to ACh were assessed at 10^{-6}M ACh. While there was no significant effect of either maternal group or offspring sex on the magnitude of response to 10^{-6}M ACh with or without *de novo* NO synthesis, there was a trend towards an effect of maternal group on NO-dependent relaxation to ACh (p=0.0804), the component lost when vessels are incubated with L-NAME (Figure 5.7(F)).

5.3.4 Aortic tunica media thickness

In Section 5.3.3 it was found that there was a sex-specific increase in the contractile capacity of femoral arteries from female offspring of obese dams with or without exposure to metformin during gestation. As discussed in Section 4.3.6, an increase in the contractile response of femoral arteries to VSMC depolarisation could be due to hypertrophy of the smooth muscle cells of the tunica media, resulting in an increased total contractile capacity of the vessel, or a change in the electrophysiology of the VSMCs, such that they contract more forcefully in response to depolarisation. In this study, femoral arteries were not collected for histological analyses as both arteries in each animal were taken for myography, although in hindsight, a second, successive segment of each artery could be collected for further analyses. However, segments of thoracic aorta were collected and fixed in this study.

When analysed by two-way ANOVA for effect of sex and group, there was a significant effect of group on the thickness of the aortic smooth muscle layer, the tunica media, (p=0.0341) but no effect of sex (p=0.4425) (Figure 5.8(A)). Male and female data were therefore combined and analysed by one-way ANOVA. Thoracic aorta tunica media thickness was increased in Ob offspring compared to Con independent of sex (Figure 5.8(A)). This supports that maternal obesity during pregnancy is associated with VSMC hypertrophy in vessels from 12-month-old offspring. However, this increase in aortic tunica media thickness was independent of offspring sex, despite the increased contractility in response to VSMC depolarisation only being present in female offspring of obese dams. Moreover, while K^+-response was increased in both Ob and Ob-Met female offspring, aortic tunica media thickness was only increased relative to Con in Ob offspring. Therefore, there is no simple relationship between aortic tunica media thickness, femoral artery contractile response to K^+ and offspring blood pressure.

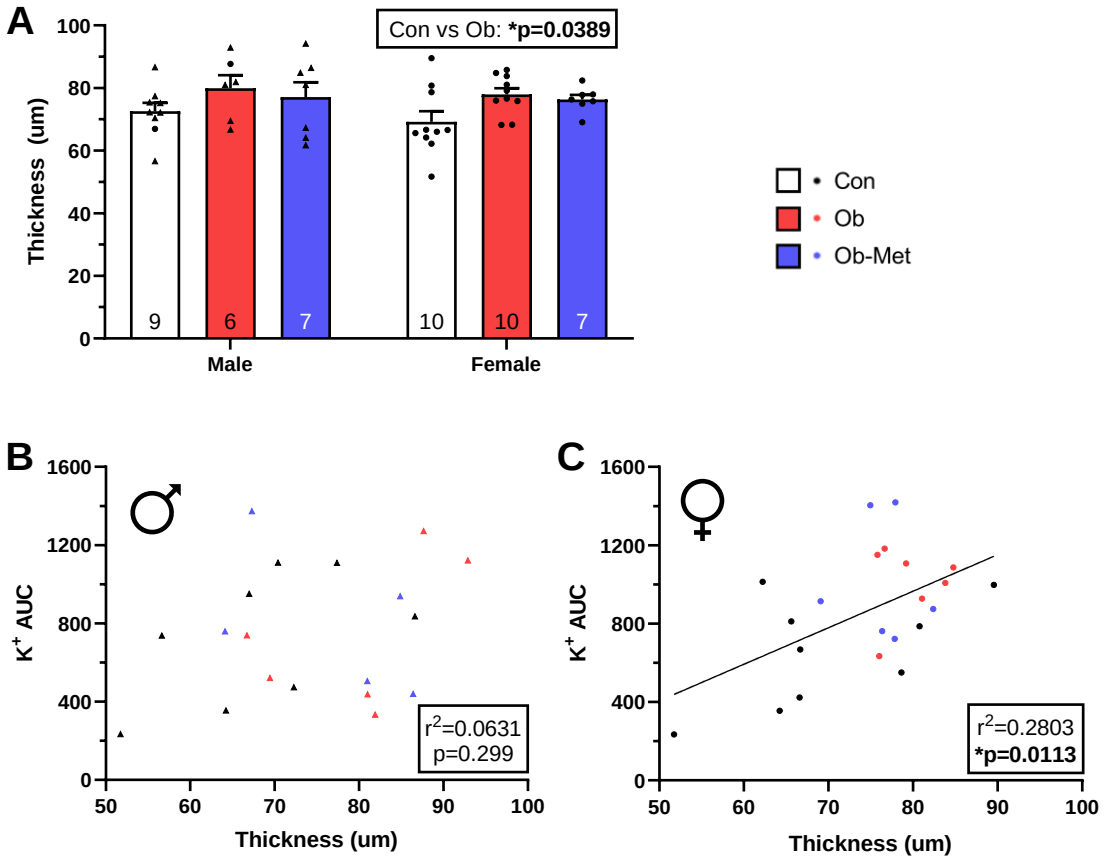


Figure 5.8: Aortic tunica media thickness. *Average thickness of the smooth muscle layer of the thoracic aorta (A), and how this relates to area under the curve (AUC) from K^+ curves in male (B) and female (C) offspring, with r^2 and p-values from simple linear regression analyses. Numbers presented in bars in (A) are matched with data in (B) and (C).*

Interestingly, aortic tunica media thickness correlated with K^+ AUC in femoral arteries from female, but not male, offspring (Figure 5.8(B,C)). This suggests while maternal obesity may promote VSMC hypertrophy independent of offspring sex, there may be sex-dependent differences in the electrophysiology of these vessels.

5.4 Key Findings

1) Maternal obesity alone resulted in increased adiposity in male offspring only, whereas combined exposure to both maternal obesity and metformin resulted in increased adiposity in female offspring.
2) No detectable increase in SBP was found in aged offspring of obese dams in this study. However, combined exposure to maternal obesity and metformin increased SBP in both male and female offspring.
3) Combined exposure to both maternal obesity and metformin *in utero* increased alpha-adrenergic vasoconstriction in male offspring only.
4) Aortic tunica media thickness was increased in 12-month-old offspring independent of sex, revealing that maternal obesity programmed changes in vascular structure.
5) This change in vascular structure was associated with increased VSMC contraction in response to depolarisation in female offspring only, indicating sex differences in the electrophysiology of VSMCs in these offspring.
6) Combined exposure to maternal obesity and metformin resulted in offspring hypertension, and this was associated with sex-specific changes in vascular function.

5.5 Future work

5.5.1 Further characterisation of vascular structure and function

This study identified two sex-specific programmed changes in vascular function in femoral arteries isolated from 12-month-old offspring.

The first was an increase in the contractile capacity of femoral arteries from female offspring of obese dams irrespective of metformin exposure. While maternal obesity was associated with VSMC hypertrophy in collected aortas, the lack of clear relationship between aortic tunica media thickness and femoral K^+ response indicates that there may be sex-dependent differences in the electrophysiology of the VSMCs in these vessels, or that maternal obesity and metformin are having different, but related, effects on femoral artery and aortic function. In addition to collecting fixed femoral arteries in a future study such that dose-response curves and structural analyses can be performed on matching, if not the same, vessel, programmed changes in the electrophysiology of VSMCs could be investigated in future studies using whole-cell voltage-clamps of VSMCs isolated from femoral arteries.

The second was that combined exposure to maternal obesity and metformin programmed hyperreactivity to alpha-adrenergic stimulation in male offspring. In future studies, whether this is associated with changes in VSMC expression of α_1Rs could be investigated using immunohistochemical analysis of fixed femoral arteries, or molecular analyses of receptor expression using frozen tissue.

Chapter 6: General Discussion

Children whose mothers are overweight or obese while they are pregnant with them are at an increased risk of developing obesity[168–173], hypertension[192–196] and cardiovascular disease[174,185] in later life. While children sharing both genetic traits and a postnatal obesogenic environment with their parents may contribute to this inheritance of poor cardiometabolic health, increasing evidence shows that part of this increased risk is developmentally programmed *in utero*. This forms part of the Developmental Origins of Health and Disease (DOHaD) hypothesis. Today, the majority of people worldwide are overweight or obese[1], and the leading cause of death globally is cardiovascular disease[11,52,53]. Due to the ongoing obesity epidemic[2], an increasing number of women are entering pregnancy overweight or obese[102,103]. Therefore, it is paramount that we understand how and why maternal obesity programmes poor offspring cardiometabolic health and develop effective intervention strategies to counter this. If this is not achieved, we are currently set to experience an ever-increasing prevalence of obesity and cardiovascular disease worldwide.

In addition to programming poor cardiometabolic health in the offspring, maternal obesity is associated with an increased risk of disorders of pregnancy such as gestational diabetes (GDM)[118] and pre-eclampsia[119] in the mother. The increased risk of these conditions in obese mothers may reflect a reduced capacity of obese women to physiologically adapt to pregnancy. These conditions not only impact maternal health during pregnancy, but they are also associated with the development of type 2 diabetes (T2D) (in the case of GDM)[135–137], and hypertension[150] and cardiovascular disease (CVD)[151] (in the case of pre-eclampsia) in the years after delivery. This further adds to the cardiometabolic health burden of obesity during pregnancy.

This thesis used a well-established mouse model of maternal diet-induced obesity. Previous studies in this model have characterised the metabolic phenotype of these females before, during, and after pregnancy and established that this is a reasonable model of maternal diet-induced obesity with a GDM-like phenotype[217,249,289–294]. Hufnagel *et al*.[293] found an increase in uterine artery PI in late gestation in obese dams in this model, which is consistent with human data that shows that pregnancy-associated reductions in PI are impaired in obese women[25,426]. This indicates that cardiovascular adaptation to pregnancy may be impaired in obese dams in this model. However, the cardiovascular phenotype of these dams has not previously been characterised. The first outcome of this thesis was therefore to establish the impact of obesity on maternal cardiovascular adaptation to pregnancy in our model. Given that we have shown that our obese dams have a GDM-like phenotype, our mouse model is also appropriate for assessing the efficacy and impact of interventions that are used to treat GDM in humans. In this thesis, two such interventions were assessed at different time points, the first of which was the effect of a maternal exercise intervention implemented during pregnancy on the cardiovascular function of obese dams.

Changes in cardiac structure[290,295,298,301] and systolic function[298,301], and an increased risk of developing hypertension[298,301] have been identified in 8-week-old male offspring of obese dams in this model. However, whether these programmed effects are altered by advancing offspring age or present whatsoever in female offspring of obese dams has not been established. Moreover, no studies have assessed either diastolic cardiac function or vascular reactivity in offspring of obese dams in our model. The second outcome of this thesis was therefore to investigate the effect of maternal obesity during pregnancy on blood pressure and vascular reactivity in both male and female young and aged offspring of obese dams. In young male and female offspring only, systolic and diastolic cardiac function were also investigated. Unfortunately, poor breeding success led to limited numbers of young adult offspring being generated, which restricted data interpretation. Sufficient numbers of animals were generated to investigate the impact of exposure to maternal obesity during pregnancy on blood pressure and vascular reactivity in aged offspring.

The second intervention that is used to treat GDM in humans that was assessed in this thesis was the glucose-lowering agent metformin. While numerous positive effects of gestational metformin treatment on maternal health have been reported[325,341,343], there are concerns that metformin may directly impact fetal development. In line with this, exposure to metformin *in utero* may be associated with an increased risk of childhood obesity[357]. Follow-up data from human trials is limited and almost exclusively considers the impact on offspring metabolic health, rather than cardiovascular function. The final outcome of this thesis was therefore to assess the effect of metformin intervention during obese pregnancy on blood pressure and vascular reactivity in aged offspring.

6.1 Limitations of rodent models

There are numerous challenges to investigating developmental programming in humans. This includes the confounding effects of genetic variation and postnatal environment, that cardiovascular disease does not typically develop until middle age or beyond, and that it is typically impossible, unethical, or immoral to manipulate the conditions around pregnancy, which is required both to establish causal mechanisms and assess potential therapies. Therefore, many researchers turn to animal models.

The vast majority of animal models used in DOHaD research, including the one used in this thesis, are established using rodents. There are numerous advantages to using rodents in DOHaD research over other animals, including their short gestation period and lifespan, that, like in humans, their placentas are haemochorial[524], and that they have multiple fetuses in a single litter.

Having multiple fetuses in a litter allows sex-dependent programming effects to be investigated using littermates exposed to identical conditions *in utero*. C57Bl/6J mice, the strain used in our mouse model, are particularly useful in studying the impact of maternal diet-induced obesity on cardiometabolic health because they effectively put on weight in response to feeding with an obesogenic diet and are susceptible to the development of obesity-associated hypertension and cardiac dysfunction.

There are also numerous limitations to the use of rodent models in DOHaD research, that must be taken into consideration when interpreting the clinical relevance of any findings. For example, while having multiple fetuses in a single litter is experimentally useful, it does mean that resource allocation between the mother and each individual fetus is not comparable to human pregnancy. Moreover, while both the human and mouse placenta are haemochorial, there are significant differences in their morphology which may alter nutrient transport between the maternal and fetal circulations[524]. Furthermore, several maturational changes that occur during gestation in humans occur postnatally in rodents.

Two major limitations of rodent models in the study of developmental programming of cardiometabolic health are the existence and importance of the neonatal leptin surge[525] and the resistance of wild-type rodents to the development of atherosclerosis. In rodents, there is a substantial increase in plasma leptin that occurs within the first two weeks of life, known as the neonatal leptin surge[242]. This is critical to the normal development of hypothalamic feeding circuits[243], and maternal obesity is associated with changes in the neonatal leptin surge that programme offspring obesity[244,247]. An equivalent neonatal leptin surge does not happen in humans. Atherosclerosis is a major cause of CVD in humans[526]. However, wild-type rodents are not susceptible to the development of atherosclerosis. One study in which ten different inbred mouse strains were fed an atherogenic diet did find that C57Bl/6J mice were the most susceptible to the development hypercholesterolaemia and the formation of fatty streak lesions[527], but even this extreme dietary challenge was not sufficient to induce atherosclerotic lesions comparable to those seen in humans.

Nevertheless, the data generated in thesis does have translational impact.

6.2 Impaired cardiovascular adaptation to pregnancy in obese mothers

In Chapter 3 it was found that while heart rate increased between conception and mid-gestation in lean dams as expected, this was abolished in obese dams. This was due to the obese dams entering pregnancy with an elevated heart rate compared to lean dams, and then their heart rate failing to increase further as pregnancy progressed. This is consistent with human data that shows that obese women typically enter pregnancy with an elevated heart rate[398,399], and have a reduced capacity to raise their heart rate with advancing gestation to the same extent as lean women[401]. This is indicative of impaired cardiovascular adaptation to pregnancy in the obese dams.

However, any such impairment did not result in detectable differences in cardiac function or vascular reactivity in late gestation. This suggests that any impact of obesity on cardiovascular function during pregnancy in this model may be relatively subtle.

Nevertheless, impaired cardiovascular adaptation may contribute to the increased risk of pre-eclampsia in obese pregnant women[119,144,145]. A key limitation of the study in Chapter 3 is that maternal blood pressure was only measured up until mid-gestation, whereas the increased blood pressure seen in pre-eclampsia only develops in late gestation[139]. This timepoint was chosen to establish whether the pregnancy-associated decrease in arterial blood pressure was detectable in lean dams using tail cuff plethysmography, and to see whether this was altered by obesity, because decreases in both SBP and DBP that reached their nadir around E9 have been identified in pregnant mice using radiotelemetry[431]. The next step would be to measure maternal blood pressure at a later timepoint in order to assess whether obese dams in our mouse model are hypertensive in late gestation, which would be indicative of a pre-eclampsia-like phenotype, albeit perhaps in a cohort with a more severe obesity phenotype.

6.3 Exercise intervention in obese pregnancy

Although individual human RCTs of exercise interventions during pregnancy report conflicting outcomes on GDM incidence[308,310,311,313,315], a large systematic review and meta-analysis of 106 studies, including more than 270,000 women, found that exercise during pregnancy reduces the incidence of both GDM and pre-eclampsia[416]. The decreased GDM incidence is consistent with previous studies from our group that found that the exercise intervention used in this thesis improves insulin sensitivity in late gestation in obese dams[292]. Overall, maternal exercise was found to have minimal effect on cardiovascular function in obese dams in Chapter 3, although this may have been in part due to the limited detectable effects of obesity alone on cardiovascular function.

In the future, echocardiography could be used once again to assess the effect of exercise on maternal cardiovascular function in late pregnancy in a cohort of animals with a more severe obesity phenotype.

An interesting consideration when comparing the exercise intervention used in this study with those assessed in obese human pregnancy is the timing of the intervention. The exercise interventions in the humans RCTs discussed in Chapter 1 of this thesis were initiated between 10 and 20 weeks of gestation, whereas the exercise intervention in this thesis was initiated one week before the mice were mated. The importance of the timing of the exercise intervention during pregnancy warrants further study. This may affect the optimal design of future intervention studies, or even highlight that promoting public awareness of the value of exercise before and during pregnancy would be valuable in improving the cardiometabolic health of both obese mothers and their children.

6.4 Metformin administration during pregnancy

In Chapter 5, combined exposure to maternal obesity and metformin *in utero* resulted in an increase in systolic blood pressure compared to controls in 12-month-old offspring, independent of sex. This adds to growing evidence from animal models that metformin exposure *in utero* has negative effects on long-term offspring health, with research from our group identifying the programming of obesity, metabolic dysfunction, adipose dysfunction[297,306], and now hypertension. Current findings from human studies suggest that metformin exposure during fetal development may be neutral[360,365], if not beneficial[353,361], to offspring cardiometabolic health. However, diabetes, hypertension, and CVD are age-related disorders and these human longitudinal follow-up studies are limited by the age of the children involved.

While metformin administration during pregnancy is associated with numerous positive effects on maternal health, including reduced GWG[325,341], it likely has negative effects on offspring health in the long term. However, this does not necessarily mean that metformin use during pregnancy should be halted altogether. Metformin treatment has been associated with a reduced risk of pre-eclampsia[341], and could be an effective treatment to prolong gestation in women with pre-term pre-eclampsia[343]. In pregnancies that are at a high risk of pre-eclampsia, the beneficial effects of metformin in the short-term may well outweigh the risks to long term offspring health. This is especially true in developing countries, were pre-eclampsia is highly prevalent and mortality from pre-eclampsia is high[142,143]. Further research is needed in order to inform best practice use of metformin during pregnancy.

In the context of GDM, metformin fails to restore maternal glycaemic control in approximately one third of patients who require pharmacological therapy and the remaining 14-46% require the use an additional treatment, which is typically insulin[324]. This situation provides two opportunities for optimising metformin use during pregnancy. Firstly, it could be made standard practice to stop metformin treatment entirely if it fails and switch to another therapy, such as insulin, rather than prescribing the two treatments concomitantly. Secondly, studies can be set up with the goal to establish how to identify which diabetic women have an increased likelihood of not being treated successfully with metformin as this could be used to inform prescribing decisions. These two changes would reduce or prevent unnecessary exposure of the fetus to metformin.

6.5 Concluding remarks

This thesis assessed cardiovascular function in both mothers and their offspring using a well-established mouse model of maternal diet-induced obesity with a GDM-like phenotype. It explored how maternal exercise intervention and metformin treatment, both of which are current clinical interventions used in obese and glucose-intolerant pregnancy, impacted maternal and offspring cardiovascular function respectively. Moreover, a new *in vivo* methodology to assess cardiac responses to exogenous agents was developed.

As well as adding to the characterisation of this mouse model and establishing a new protocol for investigating cardiovascular function, data presented in this thesis has important clinical implications.

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Imaging Mode	Secondary Measurements	VevoLAB Calculation
B-Mode LV Trace	LV volume (systole) (LV Vol;s)	$\text{LV Vol; s} = \left(\frac{7.0}{2.4 + \text{LVID; s}}\right) \text{x LVID; s}^3$
	LV volume (diastole) (LV Vol;d)	$\text{LV Vol; d} = \left(\frac{7.0}{2.4 + \text{LVID; d}}\right) \text{x LVID; d}^3$
	Stroke volume (SV)	$\text{SV} = \text{LV Vol;d} - \text{LV Vol;s}$
	Ejection fraction (EF)	$\text{EF} = 100 \text{ x } \left(\frac{\text{LV Vol;d} - \text{LV Vol;s}}{\text{LV Vol;d}}\right)$
B-Mode Linear	Fractional shortening (FS) (longitudinal)	$\text{FS (longitudinal)} = 100 \text{ x } \left(\frac{\text{LVEnL; d} - \text{LVEnL; s}}{\text{LVEnL; d}}\right)$
M-Mode LV Trace	Fractional Shortening (FS) (radial)	$\text{FS (radial)} = 100 \text{ x } \left(\frac{\text{LVID; d} - \text{LVID; s}}{\text{LVID; d}}\right)$
Mitral valve (MV) PW Doppler	Myocardial performance index (MPI)	$\frac{\text{NFT} - \text{AET}}{\text{AET}}$

Imaging Mode	Secondary Measurements	Equation
B-Mode LV Trace	Cardiac Output (CO)	$\text{CO} = \text{SV x HR}$
Pulmonary artery (PA) PW Doppler	PAT:PET ratio	$\text{PAT: PET} = \frac{\text{PAT}}{\text{PET}}$
Mitral valve (MV) PW Doppler	E:A ratio	$\text{E: A} = \frac{\text{Peak E wave velocity}}{\text{Peak A wave velocity}}$
Uterine artery (UtA) PW Doppler	UtA pulsatility index (PI)	$\text{UtA PI} = \frac{\text{PSV} - \text{EDV}}{\text{Mean Velocity}}$
	UtA resistive index (RI)	$\text{UtA RI} = \frac{\text{PSV} - \text{EDV}}{\text{PSV}}$

Appendix 3.1: Calculations for secondary measurements in echocardiography and uterine artery PW Doppler*. LV = left ventricle. LVID = LV inner diameter. LVEnL = LV endocardial length. NFT = LV non-filling time. AET = aortic ejection time. HR = heart rate. PAT = PA acceleration time. PET = PA ejection time. PSV = UtA peak systolic velocity. EDV = UtA end diastolic velocity.*

***Appendix 4.1: Baseline echocardiography data from 8-week-old Con and Ob offspring**. Measurements in offspring of obese dams (Ob) were extremely underpowered and thus could not be used for analysis of the effect of maternal obesity on echocardiographic measures, but they are presented here alongside data from offspring of control dams (Con).*

	Con M (n=10-11)	Con F (n=12-13)	Ob M (n=7)	Ob F (n=3)
Heart Rate (bpm)	516 ± 17	551 ± 10	553 ± 11	560 ± 17
Diastolic Area (mm^2)	17.9 ± 0.9	18.1 ± 0.7	19.5 ± 1.0	17.5 ± 1.5
Systolic Area (mm^2)	8.83 ± 0.51	9.80 ± 0.52	9.80 ± 0.94	9.07 ± 0.09
Diastolic Volume (uL)	39.4 ± 3.2	40.4 ± 2.7	45.6 ± 4.1	37.6 ± 5.9
Systolic Volume (uL)	11.2 ± 1.2	14.0 ± 1.3	14.4 ± 2.4	12.0 ± 0.4
Stroke Volume (uL)	28.2 ± 2.1	26.5 ± 1.7	31.2 ± 1.9	25.6 ± 5.5
Cardiac Output (mL/min)	15.1 ± 0.9	14.3 ± 0.9	17.4 ± 1.3	15.0 ± 3.0
Ejection Fraction (%)	74.0 ± 2.1	73.2 ± 1.5	72.3 ± 3.2	70.9 ± 2.3
FS (radial) (%)	42.4 ± 1.9	41.3 ± 1.3	41.0 ± 2.6	39.3 ± 2.0
LVEnL (diastole) (mm)	7.10 ± 0.14	7.01 ± 0.13	7.24 ± 0.12	7.00 ± 0.07
LVEnL (systole) (mm)	6.30 ± 0.11	6.17 ± 0.11	6.30 ± 0.12	6.03 ± 0.30
FS (longitudinal) (%)	11.2 ± 1.1	12.0 ± 1.0	13.0 ± 1.3	13.8 ± 5.1

***Appendix 4.1.1: Echocardiographic indices of left ventricular systolic function* in 8-week-old offspring**. *M = male, F = female. FS = fractional shortening, LVEnL = Left ventricle endocardial length.*

	Con M (n=10)	Con F (n=7)	Ob M (n=4-6)	Ob F (n=2-3)
E wave (mm/s)	749 ± 31	725 ± 19	775 ± 34	862 ± 24
A wave (mm/s)	554 ± 29	573 ± 23	556 ± 21	605 ± 19
E:A	1.35 ± 0.5	1.28 ± 0.05	1.40 ± 0.05	1.43 ± 0.05
IVCT (ms)	10.5 ± 0.9	10.9 ± 0.8	11.0 ± 1.3	12.7 ± 1.2
Aortic ejection time (ms)	43.7 ± 1.8	44.1 ± 1.2	42.7 ± 0.9	45.6 ± 2.5
IVRT (ms)	12.1 ± 1.1	11.5 ± 0.7	10.8 ± 0.8	11.3 ± 0.7
Non-filling time (ms)	65.9 ± 1.8	66.3 ± 1.8	64.5 ± 1.9	69.3 ± 1.7
MPI	0.529 ± 0.059	0.515 ± 0.028	0.508 ± 0.016	0.526 ± 0.047
Deceler^n rate (x1000) (mm/s^2)	-28.6 ± 4.2	-32.5 ± 4.8	-33.1 ± 6.4	-33.7 ± 5.6
Deceler^n time (ms)	28.9 ± 2.3	24.9 ± 3.1	24.9 ± 4.5	26.5 ± 5.5

***Appendix 4.1.2: Echocardiographic indices of left ventricular diastolic function* in 8-week-old offspring**. *M = male, F = female. IVCT = isovolumetric contraction time, IVRT = isovolumetric relaxation time, MPI = myocardial performance index, Deceler^n = deceleration.*

	View	Con M (n=11-12)	Con F (n=14)	Ob M (n=7)	Ob F (n=5)
Mean Velocity (mm/s)	Apic-4	-425 ± 26	-358 ± 20	-352 ± 23	-418 ± 23
	PSAX	-455 ± 15	-423 ± 9	-443 ± 24	-460 ± 15
Peak Velocity (mm/s)	Apic-4	-795 ± 43	-673 ± 39	-655 ± 42	-755 ± 35
	PSAX	-853 ± 28	-781 ± 24	-813 ± 46	-847 ± 39
Mean Gradient (mmHg)	Apic-4	0.752 ± 0.089	0.532 ± 0.060	0.509 ± 0.068	0.706 ± 0.075
	PSAX	0.839 ± 0.052	0.721 ± 0.032	0.799 ± 0.089	0.850 ± 0.055
PAT (ms)	Apic-4	20.6 ± 1.3	20.6 ± 0.9	20.0 ± 1.1	21.3 ± 1.5
	PSAX	19.9 ± 0.9	20.0 ± 0.7	20.5 ± 1.1	21.2 ± 0.9
PET (ms)	Apic-4	51.4 ± 1.1	53.7 ± 1.2	53.1 ± 1.1	59.5 ± 1.3
	PSAX	60.3 ± 1.2	60.0 ± 1.4	60.9 ± 2.0	60.5 ± 1.7
PAT/PET	Apic-4	0.385 ± 0.020	0.384 ± 0.014	0.376 ± 0.019	0.360 ± 0.031
	PSAX	0.330 ± 0.012	0.334 ± 0.010	0.336 ± 0.014	0.351 ± 0.011

Appendix *4.1.3: Echocardiographic measures of pulmonary artery (PA) flow in 8-week-old offspring*.
M = male, F = female. PAT = PA acceleration time. PET = PA ejection time.