THE INFLUENCE OF PREGNANCY COMPLICATIONS ON FETAL AND NEONATAL CARDIOVASCULAR DEVELOPMENT

By

Christina YL Aye

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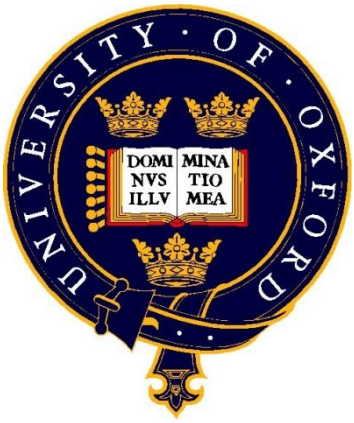
Thesis presented for degree of Doctor of Philosophy in Cardiovascular Medicine

Supervisors: Professor Paul Leeson, Miss Ingrid Granne



St John's College, University of Oxford
Department of Cardiovascular Medicine, Cardiovascular Clinical Research Facility
Nuffield Department of Obstetrics and Gynaecology

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DEDICATIONS

To David, Amelia, Alexander and Isabella who are my world

and

to mum, dad and Kuan without whom none of this would be possible.

With all my love.

ABBREVIATIONS

ASD	……	Atrial septal defect
AUC	……	Area under curve
BMI	……	Body mass index
BP	……	Blood pressure
BPM	……	Beats per minute
BSA	……	Body surface area
CoV	……	Coefficient of variance
DB	……	De Backer Score
DBP	……	Diastolic blood pressure
ECG	……	Electrocardiogram
Echo	……	Echocardiography
EDV	……	End diastolic volume
EF	……	Ejection fraction

EPOCH	……	Effect of Prematurity and hypertensive disorders of pregnancy on Offspring Cardiovascular Health
ESV	……	End systolic volume
HDP	……	Hypertensive disorders of pregnancy
HF	……	High frequency power
HRV	……	Heart rate variability
HTN	……	Hypertensive pregnancy
HUVEC	……	Human umbilical vein endothelial cells
IVS	……	Interventricular septal thickness
LDA	……	Linear discrimination analysis
LF	……	Low frequency power
LV	……	Left ventricle
LVIDd	……	Left ventricular internal diameter in diastole

MAP	……	Mean arterial pressure
NT	……	Normotensive pregnancy
PCA	……	Principal component analysis
PDA	……	Patent ductus arteriosus
PET	……	Preeclampsia
PIH	……	Pregnancy induced hypertension
PlGF	……	Placental growth factor
PPROM	……	Preterm premature rupture of membranes
PT	……	Preterm born offspring
PWd	……	Posterior wall diameter
PWV	……	Pulse wave velocity
RMSSD	……	Root mean squares of successive NN differences
RV	……	Right ventricle

SBP	……	Systolic blood pressure
SD	……	Standard deviation
SDNN	……	Mean of standard deviations of all NN intervals
sENG	……	Soluble endoglin
sFlt-1	……	Soluble fms-like tyrosine kinase-1
SQI	……	Signal Quality Index
SV	……	Stroke volume
T	……	Term born offspring
TDI	……	Tissue Doppler imaging
TVD	……	Total vessel density
USS	……	Ultrasound
VEGF	……	Vascular endothelial growth factor
VLF	……	Very low frequency power

ABSTRACT

THE INFLUENCE OF PREGNANCY COMPLICATIONS ON FETAL AND NEONATAL CARDIOVASCULAR DEVELOPMENT

THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN CARDIOVASCULAR MEDICINE AT THE UNIVERSITY OF OXFORD

Christina YL Aye

St John's College

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It is becoming increasingly clear that exposure to pregnancy complications such as hypertensive disorders of pregnancy and premature birth (birth before 37 weeks gestation) have a long term and specific effect on future cardiovascular disease development and risk in the offspring. Those born to a preeclamptic pregnancy have been shown to have double the risk of stroke in adulthood and studies have consistently shown an increase in blood pressure in these individuals. Higher blood pressure has also been observed in those born preterm in addition to specific cardiac modifications in young adulthood.

Through detailed cardiovascular phenotyping, this thesis investigates the effects of exposure to *in utero* maternal hypertension and/or a preterm birth on offspring cardiovascular development in the antenatal and early postnatal period in a newly established cohort of infants. A stratified recruitment strategy was employed in order to recruit similar numbers of mother and infant dyads from hypertensive and normotensive pregnancies across a range of gestations.

Nomograms for fetal ventricular volume and mass using two dimensional echocardiography were created. These were then used, along with postnatal normative data, to create trajectories of offspring cardiovascular development from 15 weeks postmenstrual age through to three months post birth in order to overlay datasets from babies born preterm. It was demonstrated that premature infants have similar *in utero* cardiac development to controls but post birth they undergo disproportionate cardiac hypertrophy which is associated with a degree of diastolic impairment. Preterm infants at birth had a unique ventricular shape, but these changes had attenuated by three months of age. Exposure to *in utero* maternal hypertension also appeared to have a deleterious effect and on further investigation, postnatal ventricular hypertrophy was also observed when analysis was restricted to term born infants exposed to this pregnancy complication.

Additionally, offspring born to a hypertensive pregnancy demonstrated an increased microvascular density loss over the first three months of life. This was associated with a reduction in vasculogenic potential in their human umbilical vein endothelial cells and also increased levels of maternal anti-angiogenic biomarkers at birth. Again, there was a trend to suggest maternal hypertension and prematurity may have an additive effect. These microvascular changes were not, however, correlated with cardiac hypertrophy in the whole cohort over the same period, but, intriguingly, when subgroup analysis was performed, increased microvascular density loss was correlated with increased left ventricular mass indexed to body size at three months of age in term born infants but not in the preterm group.

It was then shown that preterm offspring had reduced heart rate variability measures at birth suggesting autonomic dysfunction. This was, however, not correlated with any previously demonstrated cardiovascular developmental changes in the early postnatal period. Offspring exposed to maternal hypertension and those born small for gestational age did not demonstrate any difference in heart rate variability at birth compared to controls.

The results from my thesis point towards the perinatal and early postnatal period as being a critical window for cardiovascular development. As up to 8% of pregnancies are affected by hypertensive disorders of pregnancy and approximately 10% of all births are preterm, understanding the mechanisms behind these findings and their relevance to long term cardiovascular disease in this population is of great public health interest. Modification of clinical care and discovery of novel targets for disease prevention during this potentially tractable period will be of future interest.

LIST OF PUBLICATIONS

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Aye C, Davis E, Upton R, Lewandowski AJ, Leeson P
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Davis E, Lewandowski AJ, **Aye C**, Williamson W, Boardman H, Huang RC, Mori TA, Newnham J, Beilin L, Leeson P
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Aortic stiffness and blood pressure variability in young people: A multimodal investigation of central and peripheral vasculature.
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Preterm cardiac development: Fetal and neonatal echocardiography reveals that differences in cardiac ventricular mass and function develop early in postnatal life
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Greater left ventricular mass after hypertensive pregnancy independent of blood pressure
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Greater left ventricular mass after hypertensive pregnancy independent of blood pressure
British Hypertension Society Annual Meeting, Dublin, Sept 2016

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Aortic stiffness and blood pressure variability in young people: A multimodal investigation of central and peripheral vasculature.
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Evidence of early postnatal loss of microvascular density in offspring of hypertensive pregnancies: association with maternal angiogenic profile at birth.
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Evaluating the set-up of a dedicated preterm birth clinic in a district general hospital – our one year experience
Accepted for presentation at the British Maternal and Fetal Medicine Society Annual Conference, Amsterdam, March 2017

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1: INTRODUCTION

1.1 Cardiovascular disease and the perinatal period

Cardiovascular disease continues to be a huge public health burden. It remains the number one cause of death worldwide, with an estimated 17.5 million people dying in 2012, accounting for almost one in three global deaths.[1] Although in the United Kingdom mortality from this disease is decreasing due to early identification, treatment and management of risk factors, in 2014 it was still the second largest cause of death behind cancer, responsible for more than a quarter of all deaths.[2] Morbidity is also high, with almost 1.7 million episodes related to cardiovascular disease in NHS hospitals in the same year.[2] The financial cost is substantial, with an estimated £4.3 billion spent on treating cardiovascular disease through Clinical Commissioning Groups within the NHS in England in 2013-2014.[2]

Traditionally, the cause of cardiovascular disease has been thought to be a combination of an interaction between lifestyle and genetic predisposition, with prevention and treatment focussing on modifying these classical risk factors. However,

in recent years, there is a growing body of evidence that exposures and interventions in the perinatal period can result in long term cardiovascular sequelae in individuals.

There has been interest in how cardiovascular development during early life impacts on later risk of disease since the 1980s. An interesting historical example was the suggestion that low birth weight infants had a higher risk of coronary heart disease in later life.[3, 4] Research trying to understand the developmental origins of cardiovascular disease is important for two reasons. Firstly, it will help to identify populations who are at higher risk of developing cardiovascular disease in later life. This will enable targeted education schemes and primary prevention strategies in addition to rigorous monitoring pathways which will result in earlier diagnosis and treatment. Secondly, it raises the possibility of an early critical period of cardiovascular development which may serve as a novel target for prevention of cardiovascular disease. However, the underlying mechanisms and pathophysiological pathways leading to disease firstly need to be fully understood.

Two common pregnancy complications that have been identified as being relevant to long term cardiovascular health are preterm birth and hypertensive disorders of pregnancy which includes preeclampsia. They are also the two major causes of perinatal mortality, morbidity and long-range neurological disability.[5] Maternal hypertension and preterm delivery are often linked, with preeclampsia being identified as the primary pregnancy complication responsible for medically indicated preterm births at all gestations[6, 7] and also recognised as an antecedent factor for 20% of all preterm deliveries.[6, 8]

1.2 Preterm birth

The World Health Organisation defines preterm birth as any birth before 37 completed weeks of gestation or fewer than 259 days since the first day of the woman's last menstrual period.[9] Although this definition is arbitrary[10] as infants born prior to 39 weeks have higher risks of morbidity and mortality than those born after this gestation,[11-13] it the most widely accepted definition in the literature and the one that will be used for the remainder of the thesis. In 2010, it was estimated that just under 15 million babies (11.1% of all births) were born preterm and despite the introduction of public health initiatives and medical interventions,[14] the number is continually rising.[15]

Birth rates vary widely depending on geographical region with a rate of 5% in several northern European countries compared to 18% in Malawi in 2010.[15] Globally, it is responsible for 35% of the world's 3.1 million annual neonatal deaths,[16] and the second most common cause of death in under 5s behind pneumonia.[16] In almost all high and middle-income countries it is the leading cause of child death[16] and also contributes to other causes of mortality such as infection.[17] In 2012, premature birth was also the single biggest cause of neonatal mortality and morbidity in the UK and accounted for 7.3% of live births in this year.[18] Although there has been a recent improved survival in infants that are born extremely preterm, the rates of disability have remained static[19] carrying huge economic and social costs.

1.2.1 Aetiology of preterm birth

Preterm birth is a syndrome and is defined by time and not a clinical phenotype. The causes of preterm birth are multifactorial and many risk factors have been identified (Table 1.1). [7, 20-44]

Table 1.1: Major risk factors for preterm birth

Maternal demographics	Ethnicity
	Low or high maternal age
	Low or high BMI
Social history	Low socioeconomic & educational status
	Single marital status
	Occupation
	Nutritional intake
	Smoking, heavy alcohol intake or illicit drug use
Obstetric/medical history	Previous preterm birth
	Thyroid disease
	Asthma
	Diabetes
	Hypertension
	Depression/stress
	Cervical trauma/surgery/abnormalities
Pregnancy factors	Assisted reproduction
	Short inter-pregnancy interval
	Infection/inflammation
	Multiple pregnancy
	Oligo or polyhydramnios
	Vaginal bleeding
	Fetal anomaly
	Fetal growth restriction
Genetics	Family history
	Fetal and maternal genotype

It can be subdivided in a number of different ways. The first is by classification based on gestational age: extremely preterm (<28 weeks gestation), very preterm (28 to <32 weeks gestation) and moderate or late preterm (32 to <37 weeks gestation).[9] Approximately 5.2% of preterm births are extremely preterm with the vast majority (84.3%) being born after 32 weeks gestation.[15] Increasing gestational age is associated with improved morbidity and mortality and therefore this classification is useful when counselling parents. It may also give some indication as to the cause as early preterm births are more frequently associated with infection or inflammation.[45-47]

Another method classically used is division based on clinical presentation: spontaneous preterm labour with intact membranes, preterm premature rupture of the membranes (PPROM) and iatrogenic or indicated preterm birth.[48] Approximately 40-45% of preterm deliveries follow spontaneous preterm labour and 25-30% are the result of PPROM. Together these constitute "spontaneous" preterm births.[49] The remaining 30-35% are medically indicated,[49] a category which is aetiologically and prognostically heterogenous.[7] There have been large differences found between demographic and pregnancy characteristics and gestational age at delivery between the two groups.[6] The recent increase in medically indicated preterm births has been seen as a major factor in the continuing rise in preterm birth rate[7] as intervention in obstetric care becomes more common.[50-52] However, there have been associated decreases in perinatal mortality in this cohort, suggesting that intervention may be beneficial.[7] Increasing medically indicated preterm deliveries also prevents study of the natural phenotype surrounding the pregnancy which is likely to complicate research in this field in future. Interestingly, studies looking at the risk of recurrence after a preterm

deliveries are higher than controls regardless of the type i.e. women who have a medically indicated preterm delivery are at higher risk of another medically indicated or a spontaneous preterm delivery next time and vice versa.[7] In addition, the most common conditions prompting an iatrogenic preterm birth such as preeclampsia, placental abruption, intrauterine growth restriction and fetal distress also predispose to spontaneous preterm labour.[53-55] These observations point to an overlap between these two subtypes of preterm birth perhaps through a common pathophysiological mechanism.[56, 57]

A novel method for the classification of preterm birth has also been proposed following the Global Alliance to Prevent Prematurity and Stillbirth Meeting in 2009.[58] As the cause of any preterm birth is rarely known,[59] the authors aimed to identify aetiologic factors and phenotypic characteristics that may coexist and be independent of each other. Components of this classification system included significant maternal, fetal and placental conditions, signs of initiation of parturition and pathway to delivery (caregiver initiated or spontaneous).[58] In this way, the majority of preterm births could be accurately and consistently be described. However, due to the large number of subdivisions, there would be major implications for sample size and statistical power which the authors acknowledged.[58]

1.2.2 Cardiovascular risk in preterm-born infants

The link between preterm birth, irrespective of their birthweight, and increased risk of cardiovascular disease later in life has emerged in recent decades due to the improving survival of these infants and their progression into adulthood. Infants born at 24, 28

and 32 weeks now have a 62%, 94% and almost 100% chance of survival to hospital discharge respectively.[60, 61] It is estimated that in Europe alone, there are 9 million children and adolescents that are survivors of preterm birth.[62]

There is growing evidence for a link between hypertension and preterm birth. However, studies in pre-pubertal infants have so far been inconsistent showing either no difference or a mildly elevated systolic blood pressure (0-4mmHg).[63-66] Follow up studies in adolescents up to the age of 19 years have suggested a more demonstrable increase in blood pressure of around 2 to 11mmHg[67-72] with only two failing to find a difference in preterm-born offspring.[73, 74] The largest study was in a Swedish birth cohort comprising of 329,477 men. An increase in peripheral systolic blood pressure of between 2 and 3mmHg was observed in preterm born offspring at 18 years old which was inversely related to gestational age at birth and independent of fetal growth restriction.[67] A more worrying finding was in another cohort from the Netherlands which reported a 10.5% prevalence of clinical hypertension and 45.9% of prehypertension in 19 year old adults born preterm.[70] The lack of consistency is not a surprise as adolescence and puberty is a time of huge growth and development which does not always occur at the same time or at the same rate in individuals.

Studies in young adults up to the age of 30 years have reported increases in systolic blood pressure of between 2.4-15mmHg.[75-82] These differences may be of direct clinical relevance in later life as blood pressure is known to track throughout life.[83] In addition, a modest increase of 5mmHg in peripheral systolic blood pressure confers a 21% increase in cardiovascular death and 34% increased incidence of stroke at a

population level.[84] However, there is evidence to suggest that even in young adulthood, prevalence of clinical hypertension is increased in preterm born individuals. In a Swedish national cohort study of over 630,000 individuals, those born preterm (28,220) had an increased relative rate of requiring antihypertensive medication.[85] This was inversely associated with decreasing gestational age at birth and was independent of fetal growth. Those born between 23 and 27 weeks were more than twice as likely to be prescribed one or more antihypertensive medications per year and even those born after 35 weeks gestation had an increased risk.[85]

There is limited long term epidemiological data on the effect of preterm birth on offspring who have reached middle or old age. Preterm birth has in the past been associated with increased risk of stroke in two birth cohorts,[86, 87] although a relationship with later hypertension or ischaemic heart disease is not always consistently shown.[88-90] However, there have been two large birth cohort studies which have been published in the last few years which seem to provide stronger evidence of a link between preterm birth and long term disease. In a birth cohort from Helsinki comprising of 19,015 subjects born between 1924 and 1944, preterm women, but not men, were found to have a 1.98 fold increase in lifetime risk of coronary heart disease if born before 34 weeks gestation.[91] The other study, this time from Sweden, which included all 1,306,943 young adults aged between 18 and 30 years, demonstrated a two-fold increase in cerebrovascular disease in those born prior to 32 weeks.[92] It is important to note, however, that results from older birth cohorts are not necessarily translatable to those from the modern times due to huge advances in perinatal care and more accurate records on gestational age at birth. Some evidence is

also emerging of increased insulin resistance in those born preterm[75, 76, 78, 79, 93] which may be a precursor to later development of the metabolic syndrome[94] or type 2 diabetes,[93] both of which will further influence cardiovascular risk.

1.2.3 Cardiovascular changes in preterm-born infants

It has been postulated that cardiovascular changes occurring as a result of preterm birth may lead to long term cardiovascular sequelae. The last trimester is a critical time for cardiovascular development and it may therefore be that early exposure to the *ex utero* environment results in pathological changes to the cardiovascular phenotype of the individual predisposing to disease later in life.

Cardiac development

Two significant events occur at the time of preterm birth that may have long term implications on cardiac development. At birth, there is a switch in the myocardial cells from a fetal hyperplastic phenotype to one of hypertrophy which is completed after the first few weeks of postnatal life.[95] Animal models have demonstrated that this switch also happens at the time of preterm birth.[96, 97] In addition, significant flow changes take place in the fetal circulation during its transition into the *ex utero* environment going from a low resistance placental circulation to a high resistance arterial system[98] which is relatively hyperoxic.[99] The immature preterm heart is not able to easily deal with the increased preload that occurs after birth[100] or the higher oxygen concentrations which result in increased oxidative stress and related damage[101, 102] through the generation of free reactive oxygen species.[103] These all result in cardiac remodelling which has been demonstrated in animal models.

Bensley et al. demonstrated that preterm born sheep examined at term equivalent age had a six to seven fold increase in collagen deposition and cardiomyocytic hypertrophy in both ventricles and the interventricular septum. In addition, there was a greater proportion of mononucleated cells which showed an increase in ploidy, suggesting abnormal maturation.[96] This is important as polyploidy is associated with irreversible changes in DNA and is linked to cardiac dysfunction.[104-106] There were localised areas of lymphocyte and mast cell infiltration in some of the preterm hearts[96] suggesting inflammation and injury and similar findings have been shown in human heart failure patients.[107] A preterm rat model exposed to transient neonatal high O_2 to replicate preterm birth related complications showed similar cardiac remodelling. At 16 weeks old these rats showed left ventricular hypertrophy and enhanced fibrosis but more worryingly, after exposure to pressure overload using an angiotensin II infusion, developed severe heart failure.[97]

Recent human studies in our group have also demonstrated a hypertrophic pattern in young adults born preterm using cardiovascular magnetic resonance imaging.[108, 109] Those born preterm had an increased left ventricular mass which was inversely related to gestational age at birth.[109] The average increase in left ventricular mass was equivalent to a 9kg/m^2 increase in body mass index[110] and which would also confer a greater than 50% increased risk of cardiovascular clinical events in later life based on longitudinal studies.[111, 112] Using computational modelling in order to create a statistical cardiac atlas, Lewandowski et al. reported that these individuals had a distinct left ventricular shape with shorter ventricles, small internal diameters and a displaced apex.[109] Proportionately greater differences were also demonstrated in the

right ventricle[108] with preterm born young adults also showing left and right systolic and diastolic dysfunction.[108, 109] Left ventricular diastolic dysfunction displayed a similar pattern to that seen in a cohort of individuals a decade older, suggesting premature ageing of the myocardium.[109] A coexisting diagnosis of preeclampsia in the pregnancy had an additional deleterious effect on left ventricular systolic function.[109]

Macrovascular development

The third trimester is an important time for arterial development with 60% of aortic growth occurring within this period.[113] In animal models, the loss of the placental circulation results in a reduction in abdominal aortic blood velocity which is associated with a reduction in aortic growth in the postnatal period.[114] In addition, the placenta produces vascular growth factors such as IGF-1 which is required for normal vascular development.[115] Therefore, the exposure of the immature macrovascular system to the *ex utero* environment may have long lasting effects on the arterial tree.

In support of this hypothesis, Schubert et al. demonstrated an aortic and carotid artery growth arrest in preterm infants at term equivalent age and three months later compared to controls.[113] Several studies have also reported that large arteries are significantly smaller in children and adolescents born preterm,[68, 116-118] and our group have also recently demonstrated that adults born preterm had 20% smaller thoracic and abdominal aortic lumens.[119] These findings suggest that any changes seen may track into later life.

Preterm birth may have an additional impact on large vessel function. The third trimester is also when the rates of elastin synthesis are the greatest, falling rapidly after the perinatal period.[120] Elastin, together with collagen, is a structural matrix

protein which is essential to the mechanical properties of the extracellular matrix in vessel walls, acting as an elastic reservoir.[121-123] In animal models, accumulation of elastin has been shown to be sensitive to changes in blood flow during the perinatal period,[124-126] and therefore it is plausible that the haemodynamic insult on the poorly developed vascular tree may result to changes in its functional ability.

A few previous studies have shown increased arterial stiffness in in children born before 34 weeks gestation[127-131] although this is not always consistent.[132] Some studies have also demonstrated that preterm individuals as young adults display higher levels of arterial stiffness,[133-135] although our group have found only small differences which are unlikely to be of clinical relevance.[119] In other populations, increased arterial stiffness is a predictor of cardiovascular events[136, 137] with increased arterial stiffness being associated with atherosclerosis,[138] diabetes,[139, 140] obesity,[35-37] smoking,[141, 142] hyperlipidaemia[143] and the metabolic syndrome.[144] However, the links between macrovascular development and later cardiovascular disease in preterm individuals is yet to be clarified.

Microvascular development

Abnormalities in the microvascular system have been demonstrated with a reduction in cutaneous capillary[63, 145] and retinal vascular[146-148] beds in individuals born preterm having been previously described. Capillary rarefaction is thought to play an important role in the increased peripheral vascular resistance found in patients with hypertension[149-151] and it has been hypothesized to play a causative role in this disease as it is also observed in normotensive at-risk populations.[152] In addition, arteriolar narrowing in the retina has been shown to be an accurate predictor of hypertension.[153]

Several mechanisms have been put forward including the hypothesis that preterm birth causes a shift towards a more anti-angiogenic profile in the offspring which may then have an effect on microvascular development. Cord sera from preterm infants has been shown to impair vasculogenesis *in vitro*[154] and the genetic profile of endothelial colony-forming cells isolated from cord blood demonstrates an increased expression of anti-angiogenic functions.[155] In addition, young adults born preterm exhibited an enhanced anti-angiogenic state with elevations in soluble endoglin (sENG) and soluble fms-like tyrosine kinase-1 (sFlt-1) and it was found that there were associations with elevations in blood pressure mediated through capillary rarefaction in this cohort.[145] Endothelial colony-forming cells from preterm babies were also found to be more sensitive to hyperoxic stress,[156, 157] which is known to occur in the postnatal environment. This may be another pathophysiological pathway through which capillary rarefaction develops in these individuals.

1.2.4 Other factors contributing to cardiovascular risk

Perinatal interventions

There are, of course, a huge number of perinatal exposures and interventions that the preterm infant is exposed to which may have an effect on long term health, not all of which will be discussed here.

Nutrition in early life has been shown to play an important role in cardiovascular development. Intravenous lipids, which, in the past, were widely given in the neonatal period to preterm infants have been shown to increase aortic stiffness in early adulthood and cause subclinical systolic dysfunction, potentially through elevations in neonatal cholesterol levels.[158] In addition, a previous randomised control feeding trial

in preterm infants found on average a 4mmHg lower mean arterial pressure in those that were breastfed compared to those given formula in adolescence.[159] A further follow up of a subset of this cohort demonstrated a beneficial association between breast milk and cardiac morphology and function in adult life.[160] Rate of weight gain or postnatal catch-up growth has also been implicated in long term cardiovascular disease risk in preterm offspring[72, 161] and may underlie the relationship between body mass index and blood pressure in this group.[70-72, 77, 80, 162]

Antenatal glucocorticoids have been shown to prevent neonatal respiratory distress in the preterm infant[163] and are widely given as standard practice to mothers at risk of preterm delivery.[164] Our group have previously demonstrated that exposure to glucocorticoids in the antenatal period is associated with increased aortic arch stiffness equivalent to that of term adults a decade older. Changes in glucose metabolism were also noted.[165]

Mechanical ventilation is often required to support the respiratory system in the preterm neonate.[166] Whilst the benefit to survival is unquestionable, changes to pulmonary physiology may result in detrimental effects on cardiac function. This may, in turn, result in cardiac remodelling with Lewandowski et al. showing that increases in right ventricular mass in preterm born young adults were partly accounted for by postnatal ventilation.[108]

Perinatal exposures

Various conditions that are commonly seen in cases of preterm birth may also have a negative effect on cardiovascular development. Infection is one of the most common risk factors for preterm birth and is present in most cases when delivery occurs before

30 weeks gestation.[47] In a sheep model of chorioamnionitis, exposed lambs showed marked differences in cardiac function and growth.[167]

Intrauterine growth restriction (IUGR), another major risk factor for preterm birth,[168] has also been shown to have a relationship with long term cardiovascular risk.[169, 170] Animal models have demonstrated a significant reduction in cardiomyocyte number[171] with the number being proportional to weight at birth.[172] As in preterm sheep models,[96] the cardiomyocytes were in a more immature form,[173, 174] in addition to there being a heightened vasoconstrictor response in the coronary arteries.[173] Furthermore, IUGR has also been show to result in fetal cardiac remodelling and associated systolic and diastolic dysfunction[175, 176] which persists into infancy and childhood.[177-179]

Hypertensive disorders of pregnancy are also often linked to preterm birth as previously described earlier in the chapter and will be discussed in detail in the following section.

1.3 Hypertensive disorders of pregnancy

De novo hypertension in pregnancy, which encompasses preeclampsia and pregnancy induced or gestational hypertension, presents after 20 weeks gestation in up to 8% of pregnancies[180] with 3-5% being affected by preeclampsia alone.[181] There are various definitions in the literature, but the International Society for the Study of Hypertension in Pregnancy (ISSHP) defines preeclampsia as new onset hypertension (brachial systolic blood pressure ≥140mmHg and/or diastolic blood pressure ≥90mmHg) after 20 weeks gestation, returning to normal postnatally in addition to new onset proteinuria

(≥300mg/day or a protein/creatinine ratio of ≥30mg/mmol).[182] Pregnancy induced hypertension refers to isolated new onset hypertension without proteinuria. The difference between preeclampsia and pregnancy induced hypertension is controversial. Some believe pregnancy induced hypertension to be a distinct pathophysiological condition.[183, 184] Others suggest that they both belong to the same disease spectrum with both preeclampsia and gestational hypertension sharing similar risk factors,[185-187] anti-angiogenic associations,[188] genetic factors [189] and cardiovascular changes.[190-192] Pregnancy induced hypertension can also evolve into preeclampsia in 15-20% of cases,[193] and women with previous episodes of preeclampsia or gestational hypertension are at increased risk of both conditions in subsequent pregnancies.[194, 195]

Chronic hypertension which predates the pregnancy and superimposed preeclampsia (the development of preeclampsia on the background of chronic hypertension) can also occur, but their effect on the cardiovascular system of the offspring will not be investigated in this thesis.

Hypertensive disorders of pregnancy are remains a major cause of maternal and perinatal mortality and morbidity worldwide.[8, 196, 197] In Latin America and the Caribbean it is responsible for more than one in four maternal deaths,[196] although in the United Kingdom, mortality rates have fallen to their lowest ever in recent years, with less than one in a million women dying due to improvements in care from the introduction of evidence-based guidelines.[198] Preeclampsia is estimated to account for 20% of all antenatal admissions and, as discussed earlier, is thought to be an antecedent for 20% of those born preterm but also for 12% born small for gestational

age.[8] In its severe form, it is also associated with adverse perinatal outcomes with one in four stillbirths and neonatal deaths in developing countries being associated with this condition.[8] The majority of the work in this field concentrates on preeclampsia rather than gestational hypertension and therefore this will be the focus for the remainder of the chapter.

1.3.1 Aetiology of preeclampsia

Observational studies have identified many risk factors associated with the development of preeclampsia although many also predispose to pregnancy induced hypertension. Table 1.2 summarises the main risk factors that have been previously identified.

Table 1.2: Main risk factors for preeclampsia[199, 200]

Maternal demographics	Ethnicity (black race)
	Increased maternal age
	High BMI
Social history	Low socioeconomic status
Obstetric History	Nulliparity
	Previous preeclampsia
Medical History	Chronic hypertension
	Diabetes/increased insulin resistance
	Inherited thrombophilia
	Renal disease
	Antiphospholipid syndrome/autoimmune disease
Pregnancy factors	Long inter-pregnancy interval
	Multiple pregnancy
	Hydatidiform mole
Genetics	Family history of preeclampsia

1.3.2 Pathophysiology of preeclampsia

The cause of preeclampsia is not yet completely understood, but impaired remodelling of the spiral arteries has traditionally been implicated.[201] It is then thought that this leads to chronic placental ischaemia or intermittent flow resulting in an ischaemia-reperfusion phenomenon.[202] The ischaemic placenta releases reactive oxygen species and inflammatory cytokines in addition to over-expression of anti-angiogenic factors which, in combination, is thought to lead to widespread maternal endothelial dysfunction.[202-204]

However, in recent years some have proposed that there are two distinct categories of preeclampsia with placental disease only featuring in the early-onset phenotype

(presentation before 34 weeks gestation).[56, 205] Late-onset preeclampsia is more common, accounting for 80% of cases,[205] and is usually seen in association with long standing maternal co-morbidities.[206] As placental dysfunction is not thought to be a key factor, preterm delivery and fetal growth restriction are not usually observed.

Most women are asymptomatic at presentation, although preeclampsia can involve multiple organs and systems including the heart, kidney, coagulation system, liver and the central nervous system.[207] Serious complications include HELLP syndrome (Haemolysis, Elevated Liver enzymes and Low Platelets), stroke, cortical blindness, placental abruption, pulmonary oedema, ascites, renal failure, disseminated intravascular coagulation, liver capsule rupture and eclampsia (tonic clonic seizures) which in low and middle income countries, can have a fatality rate can be as high as 3-5%. [208]

1.3.3 Prevention and treatment of preeclampsia

The only interventions that have been shown to reduce the risk of developing preeclampsia are low-dose aspirin, which is associated with a 10% reduction in preeclampsia and preterm birth,[209] and calcium supplementation which halves the relative risk but with no obvious benefit in infant outcome.[210]

The only definitive treatment for preeclampsia is delivery,[56, 196] although this is not always in the best interest of the fetus. Current therapies have not been shown to reverse the underlying disorder.[207] The aims of management are to prolong the pregnancy until such time as the risks to the mother outweigh the risks to the fetus

and to monitor for any complications resulting from the condition.[196, 207] Antihypertensive medication such as methyldopa, labetalol (a mixed alpha and beta blocker) and nifedipine (a calcium channel blocker) are commonly used to control blood pressure in order to minimise the risk of complications and allow prolongation of the pregnancy. There is currently no evidence to suggest that one agent is better than another.[211]

It has been recommended that all women with the condition be considered for delivery before 40 weeks gestation and after 32 to 34 weeks gestation if severe preeclampsia is present.[56, 207] Expectant management may be an option before this time,[56] with a recent systematic review suggesting that prolonging the pregnancy until 34 weeks reduced neonatal complications without significantly increasing the risk to the mother.[212] Delivery at any gestation is indicated if there are worsening of maternal symptoms, development of complications, end-organ dysfunction or deterioration in the fetal condition.[207]

1.3.4 Cardiovascular risk in infants exposed to maternal hypertension

In addition to the increased mortality and morbidity during pregnancy and the puerperium, a preeclamptic pregnancy is an independent risk factor for maternal cardiovascular disease with an increased risk of developing hypertension, coronary artery disease and stroke in the 10 to 15 years following the pregnancy.[213, 214] Of note, a graded relationship between the risk of cardiac disease and the severity of preeclampsia has been demonstrated.[214]

Furthermore, there is also growing evidence that there are long term offspring cardiovascular sequelae from *in utero* exposure to hypertensive disorders of pregnancy which are independent from other co-existing pregnancy complications. Our group have previously conducted a meta-analysis using data from over 45,000 individuals which reported a 2.39mmHg higher systolic and 1.35mmHg higher diastolic blood pressure in children and young adults who had been born to preeclamptic pregnancies.[215] If this difference tracked into adult life, it would be associated with an 8% increased risk of mortality from ischaemic heart disease and a 12% increased risk of stroke.[215]

These findings are supported by our 20 year prospective follow up birth cohort study of 2,868 young adults which reported that clinical incidence of hypertension were increased in those exposed to hypertensive disorders of pregnancy *in utero.* These young adults were 2.5 times more likely to have global lifetime risk (QRISK) scores above the 75th centile and 30% of 20 year olds with hypertensive blood pressures were born following a hypertensive pregnancy. The exposure to *in utero* hypertension in addition to a preterm birth resulted in a threefold greater risk of being clinically hypertensive as a young adult. Intriguingly, the phenotype between offspring exposed to preeclampsia and pregnancy induced hypertension appeared to be different with those born following isolated high blood pressure demonstrating a smaller blood pressure rise but a significantly increased body mass index.[216]

These increases in blood pressure are evident in later life, with one study showing that offspring of preeclamptic pregnancies were more likely to be prescribed

antihypertensive medication by the age of 50 years old.[217] Interestingly, a 60 year follow up of the Helsinki birth cohort also demonstrated that individuals born following severe preeclampsia have a 1.5 relative risk of hypertension although this was not seen in milder cases. However, this study also reported that offspring exposed to preeclampsia had double the risk of stroke in adult life even after adjusting for birthweight or gestational age[218] which could not be explained by increases in blood pressure suggesting that this alone cannot fully explain the cardiovascular risk associated with exposure to this pregnancy complication.

1.3.5 Cardiovascular changes in infants exposed to maternal hypertension

There is growing evidence to suggest that the offspring of preeclamptic pregnancies have a distinct vascular phenotype. Our group have previously shown that preterm born young adults demonstrated impaired flow mediated endothelial responses only if they were exposed to maternal hypertension.[81] An increased intima-media thickness was also found[81] suggesting an early atherogenic phenotype consistent with aortic arterial thickening which has been shown in preeclamptic offspring at birth.[219] Endothelial dysfunction has also been demonstrated in childhood[220] and adolescence,[221] although this is not always consistent.[222]

A recent study has also found that there are differences in offspring cardiac structure in adolescent offspring exposed to hypertensive disorders of pregnancy suggesting cardiac remodelling. Exposure to maternal hypertension was associated with a greater relative wall thickness compared to controls and those exposed to preeclampsia also

demonstrated a reduced left ventricular end-diastolic volume.[223] Whether these changes are present earlier on in life and whether they are of relevance to future cardiovascular disease risk in population will be of interest.

1.4 Thesis Hypotheses and Objectives

The aim of this thesis is to characterise the cardiovascular phenotype of offspring born to pregnancy complications in the early postnatal period. It is predicted that being exposed to maternal hypertension *in utero* and/or being born preterm will result in distinct cardiovascular changes in the first three months of life. More specifically, I hypothesise that:

1. Preterm birth will be associated with disproportionate cardiac hypertrophy and diastolic dysfunction which is proportional to the degree of prematurity in the early postnatal period;

2. Exposure to maternal hypertension will result in capillary rarefaction in the offspring in the early postnatal period which is related to *in vitro* vasculogenic potential and maternal angiogenic profile;

3. Preterm born individuals will demonstrate reduced heart rate variability at birth compared to their term-born counterparts;

4. There will be an interaction between preterm birth and maternal hypertension resulting in an additional deleterious effect on offspring cardiovascular phenotype compared to if the infant was only exposed to one pregnancy complication.

In order to investigate these hypotheses, my objectives are therefore to:

1. Create nomograms of fetal cardiac structure using two dimensional echocardiography in order to create trajectories of cardiac development with which to compare data from preterm infants;

2. Quantify changes in left and right ventricular structure, function and shape between preterm and term born offspring and investigate how perinatal factors including exposure to maternal hypertension relate to these changes;

3. Establish changes in *in vivo* microvascular structure between birth and three months of age in offspring exposed to maternal hypertension and their association with *in vitro* vasculogenic potential, maternal angiogenic profile and preterm birth;

4. Quantify changes in heart rate variability parameters at birth in babies born to pregnancy complications and investigate their relationship with any cardiovascular changes observed in these individuals;

5. Discuss the findings from this thesis including possible mechanisms, implications and future work.

2: STUDY POPULATION

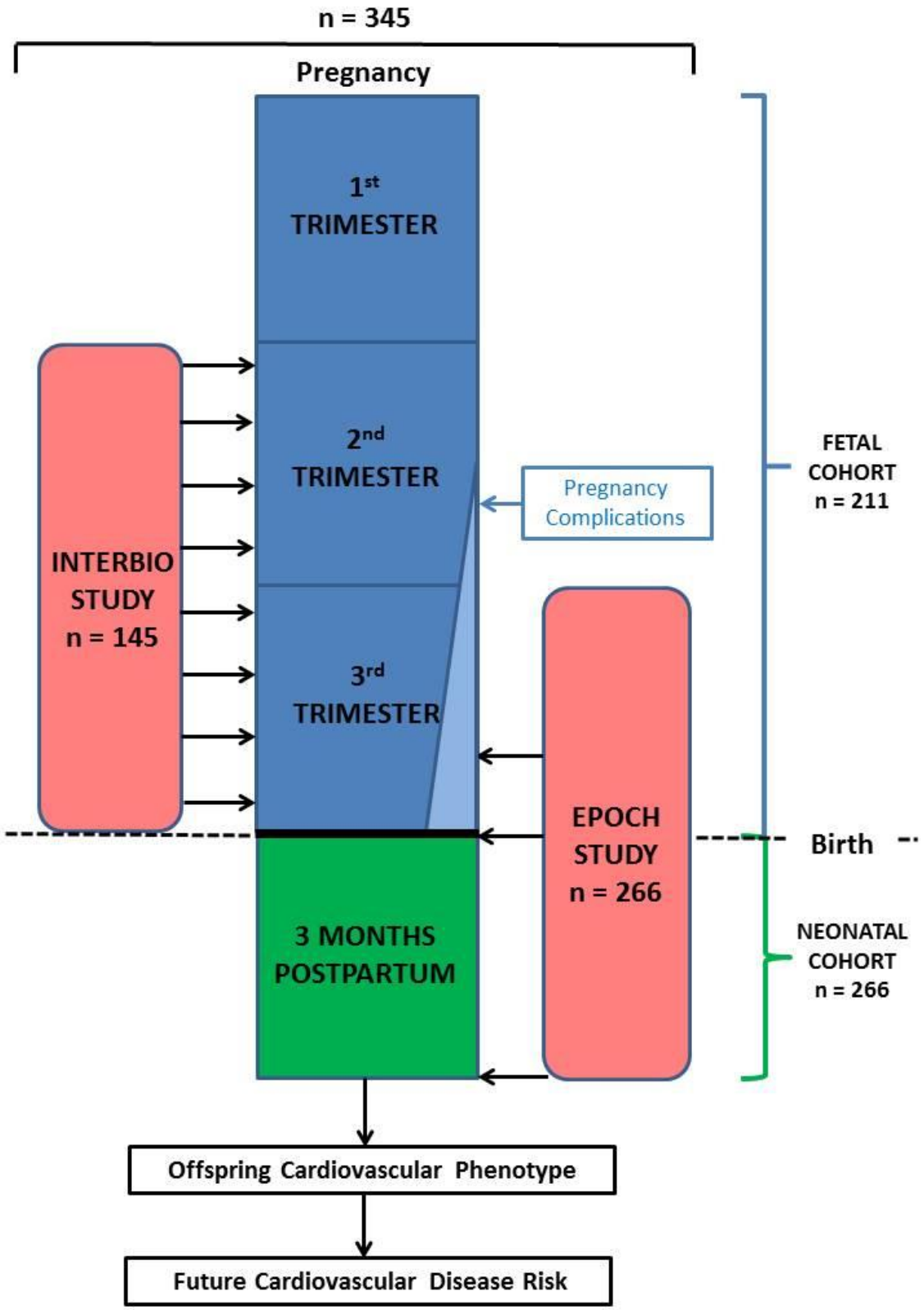
The work in this thesis is based on a study (Effect of Prematurity and hypertensive disorders of pregnancy on Offspring Cardiovascular Health (EPOCH),South Central Berkshire Research Ethics Committee ref. 11/SC/0006, UKCRN/clinical trials ref. NCT01888770), the pilot of which was designed by a previous DPhil candidate (Dr Esther Davis) and one of my supervisors, Professor Paul Leeson. The original study was designed to assess the impact of severe preeclampsia (PET) on cardiovascular development of the offspring by comparing preterm babies who had and had not been exposed to PET at birth and three months postnatal age. It also aimed to assess the feasibility of recruiting in a neonatal intensive care environment.

Professor Leeson and I made several modifications to the original study and incorporated it into a larger programme of work. I continued recruitment and data collection for this “neonatal” cohort and also expanded the selection criteria to include

several groups of term born infants in order to investigate the effect of less severe forms of hypertensive disorders of pregnancy i.e term PET and term pregnancy induced hypertension (PIH). Recruiting term born infants allowed me to investigate the effect of prematurity on infant cardiovascular development and its interaction with maternal hypertension. In addition, novel measures of heart rate variability were undertaken as well as umbilical cord collection in order to compare *in vivo* and *in vitro* microvascular measures.

Furthermore, through antenatal recruitment in a subgroup of mothers, I performed fetal cardiac measures in addition to the postnatal assessments in order to establish a second "fetal" cohort. Normative fetal data for this cohort was supplemented by using cardiac datasets that were taken from women from the same population who were taking part in a longitudinal fetal surveillance study called INTERBIO-21st (South Central – Oxford C Research Ethics Committee ref. 08/H0606/139) using the same protocol for image acquisition. The purpose of this fetal cohort was to investigate whether any cardiovascular structural differences observed were present prior to birth. Figure 2.1 summarises the recruitment and assessments for the two cohorts. As the neonatal cohort was established first, I will describe this before going on to give details about the fetal cohort.

Figure 2.1: Overview of fetal and neonatal cohorts, recruitment and assessments



2.1 Neonatal cohort

The study population included in this thesis incorporates 47 infants (15 preterm offspring born to a normotensive pregnancy, 27 preterm born to a hypertensive pregnancy, 4 term born to a preeclamptic pregnancy and 1 term born to a normotensive pregnancy) who were recruited between 2012 to 2014 by a previous DPhil candidate (Dr Esther Davis) in addition to a further 208 infants (255 in total) who I subsequently recruited.

2.1.1 Patient selection

Potential participants were identified by their clinical care team from infants delivered at or admitted to the Oxford University Hospitals NHS Foundation Trust. Mothers of the infants formed a further study cohort.

CASES

These constituted infants born to mothers with preeclampsia or pregnancy induced hypertension and/or born preterm. They were divided into four subgroups: Infants born to a

a) preterm, normotensive pregnancy (PT-NT);

b) preterm, preeclamptic pregnancy (PT-PET);

c) term, pregnancy induced hypertension pregnancy (T-PIH);

d) term, preeclamptic pregnancy (T-PET).

CONTROLS

These were defined as term infants born to mothers that had been normotensive throughout pregnancy (T-NT).

A stratified recruitment strategy was employed to recruit similar numbers of mother and infants dyads from hypertensive and normotensive pregnancies across a range of gestations.

2.1.2 Diagnostic definitions

A hypertensive pregnancy was defined according to ISSHP guidelines[182] as a diastolic blood pressure > 90mmHg on two separate occasions within a 24 hour period more than three hours apart presenting after 20 weeks of pregnancy. A diagnosis of preeclampsia was also noted if there was new onset hypertension plus evidence of gestational proteinuria (300mg per 24 hours or more in a 24 hour urine collection, or at least 2+ protein at least twice on consecutive dipstick testing, or protein/creatinine ratio of ≥30mg protein/mmol creatinine).

A preterm birth was defined as birth before 37 weeks gestation with gestational age calculated based on first trimester ultrasound.

2.1.3 Inclusion criteria

Singletons and multiplets were included.

Infants

The subject must have satisfied all the following criteria to be considered eligible for the study:

- Infants delivered at, or admitted to the Oxford University Hospitals NHS Foundation Trust ;
- Available for assessment within the neonatal period and at 3 months of age;
- Parent is willing and able to give informed consent for participation in the study;
- Physical condition is suitable to allow non-invasive cardiovascular testing;
- Mother meets criteria for inclusion in the study and is willing to participate in the study.

Mothers

Mothers must have satisfied all the following criteria to be considered eligible for the study:

- Given birth to an infant admitted to the Oxford University Hospitals NHS Trust ;
- Aged ≥ 16 years old;
- Is able and willing to give informed consent for participation in the study, and is able and willing to give informed consent for her infant's participation in the study.

2.1.4 Exclusion criteria

Infants

The subject was not recruited into the study if ANY of the following applied:

- Parent is unwilling to give consent;
- Unavailable for assessment within the neonatal period and at 3 months of age;
- Physical condition unsuitable to allow for non-invasive testing of cardiovascular system;
- Evidence of congenital cardiovascular disease (with the exception of PDA and ASD) or known genetic or chromosomal disorder or any severe malformation;
- Cardiorespiratory instability at time of proposed measures;
- Active infection at time of proposed study measures;
- Mother of infant is excluded from the study.

Mothers

Mothers were not asked to enter the study if ANY of the following applied:

- Unable or unwilling to give informed consent;
- Aged less than 16 years;
- Physical condition post-delivery such that it would preclude participation in the study.

2.1.3 Subject recruitment

A stratified recruitment approach to ensure balanced representation of preterm and term birth as well as hypertensive and normotensive pregnancies was employed. On designated days of the week, consecutive women who had delivered preterm and/or who had experienced a hypertensive pregnancy were approached to take part in the study. A subgroup of women who had experienced a term, normotensive pregnancy delivering on the same day were also approached to serve as a control group. Identification of eligible infants and mothers was carried out by their clinical care team on the neonatal unit or inpatient wards. Following identification, the mothers were provided with an invitation letter under the direction of the medical team. All invitation letters were followed up by a member of the research team and if they were interested, the research team then provided full information detailing the potential involvement of both infant and mother. Following this, if they were happy to proceed, separate written informed consent was taken from the mother for herself and on behalf of their infant for participation in the study.

A copy of the signed informed consent forms was given to the appropriate parent/guardian and also filed in the notes. The original signed form was retained at the study site.

2.2 Fetal cohort

2.2.1 Patient selection

The fetal cohort comprised of offspring from pregnant mothers receiving antenatal care in the Oxford University NHS Hospitals Foundation Trust who were taking part in one of two longitudinal studies.

The first study was EPOCH from which the neonatal cohort was derived (Section 2.1), whereby a subset of 55 mothers were identified, recruited and scanned prior to birth. The pregnancies were then followed through to delivery and postnatal measurements were acquired i.e. the infant was part of the fetal and neonatal cohort. The allocation of the offspring into the case or control group was determined postnatally depending on whether the infant had been born preterm and/or been exposed to a maternal hypertension (Section 2.1.1. and 2.1.2). These mothers were also approached as to whether they would be happy to donate their umbilical cords to the Oxford Cardiovascular Tissue Bioresource in order to link studies and compare *in vitro* and *in vivo* microvascular measures in the same infant.

Normative fetal cardiac data was supplemented by women taking part in InterBIO-21st, a longitudinal fetal surveillance study, on designated days. These offspring only contributed antenatal data and were only part of the fetal cohort.

2.2.2 Inclusion criteria

Mothers must have satisfied all the following criteria to be considered eligible:

- Receiving antenatal care in the Oxford University Hospitals NHS Trust;
- Aged ≥ 16 years old;
- Is able and willing to give informed consent for participation in the study.

2.2.3 Exclusion criteria

Mothers were not asked to enter the study if ANY of the following applied:

- Unable or unwilling to give informed consent;
- Aged less than 16 years;
- Evidence of fetal congenital cardiovascular disease (with the exception of ASD) or known genetic or chromosomal disorder.

2.2.4 Subject recruitment

Identification of eligible mothers for the EPOCH arm of the fetal cohort was carried out by their clinical care team in outpatient clinics or inpatient wards. Following identification, the mothers were provided with an invitation letter under the direction of the medical team. All invitation letters were followed up by a member of the research team and if they were interested, the research team then provided full information detailing the potential involvement of both offspring and mother. A separate invitation letter was also provided for the Oxford Cardiovascular Tissue Bioresource. Following this, if they were happy to proceed, written informed consent was taken from the mother for herself for participation in the study or studies. Post-delivery, consent for the mother was reaffirmed and consent taken from the mother

on behalf of the infant for participation in the postnatal part of the study (neonatal cohort). All consent forms included permission to access maternal and offspring clinical records and link data between studies.

Identification and consent of eligible mothers for the InterBIO-21st arm of the fetal cohort was carried out by the InterBIO-21st research team. Cardiac measures used in my thesis were in addition to the InterBIO-21st fetal surveillance protocol but were covered by their research ethics and consent forms so further consent was not required.

A copy of all the signed informed consent forms were given to the mother and also filed in the notes. The original signed form was retained at the study site.

3: General Methods

The following chapter describes the methods that were used to investigate the cardiovascular system of the offspring. Firstly, I will present an overview of the study visits. I will then describe the investigations used and how the data was collected and analysed along with their respective intra and inter-observer variability based on 10 randomly selected datasets. Finally, I describe the statistical methods employed in my thesis. Additional methods for investigations or analysis that came about as part of a specific collaboration with other researchers will be described in the corresponding results chapter.

3.1 Study visit outline

3.1.1 Fetal Cohort

Antenatal fetal echocardiograms for those mothers taking part in EPOCH were performed after recruitment and repeated every 4-6 weeks until delivery. Fetal

echocardiography was also performed in a group of women taking part in the InterBIO-21st study. This was a longitudinal study in which women attended every 4 weeks during pregnancy for an obstetric ultrasound scan. On designated days, all mothers attending an assessment had fetal echocardiography performed as part of their scan. Therefore, some fetuses underwent multiple echocardiograms whereas others only had measurements at a single time point. All scans were carried out in the Nuffield Department of Obstetrics and Gynaecology in the John Radcliffe Hospital.

Umbilical cords were collected at birth and human umbilical vein endothelial cells (HUVECs) were isolated and stored according to standard operating procedures (See Section 7.3.2.1 and Appendix).

The data collected was anonymised and coded with study/bioresource specific IDs (e.g. InterBIO 07-00001) to ensure confidentiality and blinded analysis.

3.1.2 Neonatal Cohort

The infants attended two identical assessments (Figure 3.1) at two different time points:

VISIT 1: First week of life (Birth assessment);

VISIT 2: Three months of age (Follow up assessment).

In a proportion of participants, their clinical status, timing of discharge from hospital or patient preference would not allow measurements within this time period. Therefore, the upper limit for the birth assessment was 4 weeks (28 days) for birth assessment and 24 weeks (168 days) for the follow up assessment.

Birth assessments were carried out either in the postnatal wards or neonatal units in the Oxford University Hospital NHS Foundation Trust (John Radcliffe Hospital, Oxford or Horton General Hospital, Banbury) or in the Cardiovascular Clinical Research Facility (CCRF) in the John Radcliffe Hospital. Follow up visits were conducted in CCRF or in the Day Assessment Unit in the Horton Hospital. All were performed in a temperature-controlled room, with the infant at rest, either in their mother's arms, in a crib or a pushchair.

Details of the study procedure and what to expect were described to the mothers allowing them time to ask any further questions. Informed consent was then gained or re-affirmed.

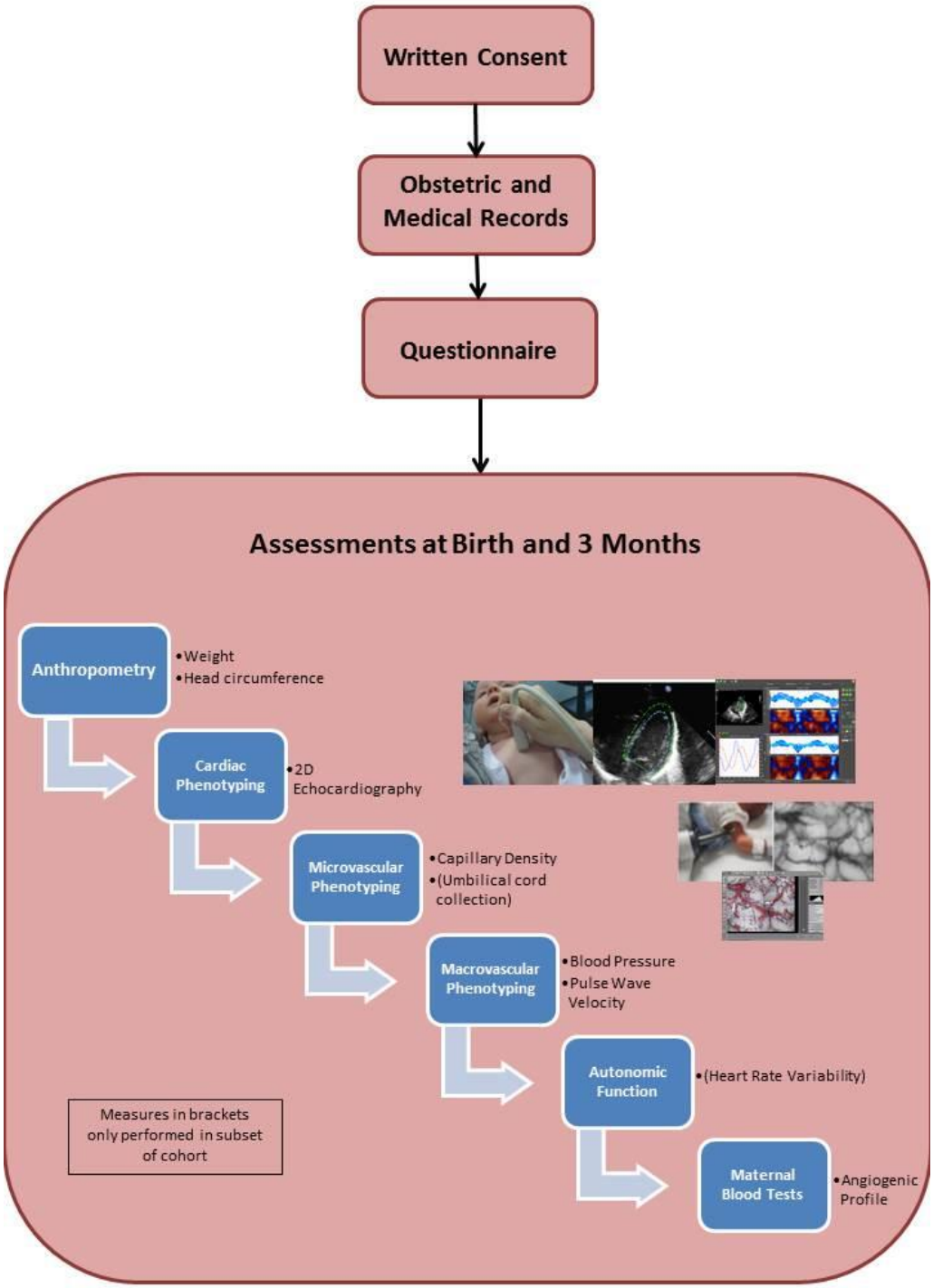
Following consent, offspring underwent anthropometric measurements, echocardiography, microvascular structural imaging and blood pressure and pulse wave velocity measurements. A subgroup of infants also underwent a 5 minute ECG recording in order to calculate heart rate variability measures. Mothers of the infants also underwent venepuncture and completed detailed lifestyle and medical history questionnaires.

3.2 Ethical standard

The clinical studies were approved by the South Central Berkshire Research Ethics Committee ref. 11/SC/0006 (EPOCH) and the South Central Oxford C Research Ethics Committee ref. 08/H0606/139 (InterBIO-21st). The *in vitro* studies were covered by

ethical approval from the Oxford Cardiovascular Tissue Bioresource (ref. 09/H0606/68, 07/H0606/148 and 11/SC/0230).

Figure 3.1: Overview of investigations and methods of data collection utilised for the neonatal cohort.



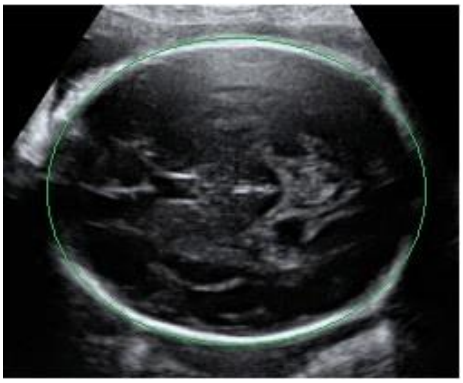
3.3 Anthropometry

3.3.1 Fetal cohort

During fetal life head circumference, abdominal circumference and fetal length were measured according to previously published protocols[224] and the average of three measurements used.

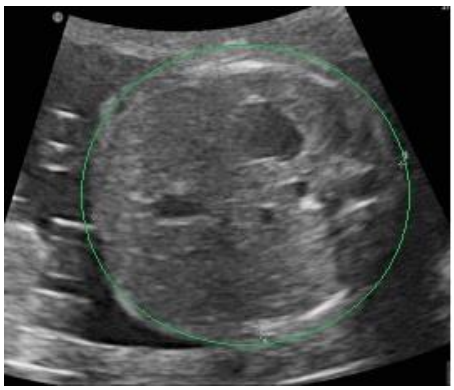
Briefly, a cross-sectional view of the fetal head at the level of the thalami was taken as close as possible to the horizontal to measure head circumference (Figure 3.2).

Figure 3.2: Representative image of measurement of fetal head circumference in a fetus of 32 weeks.



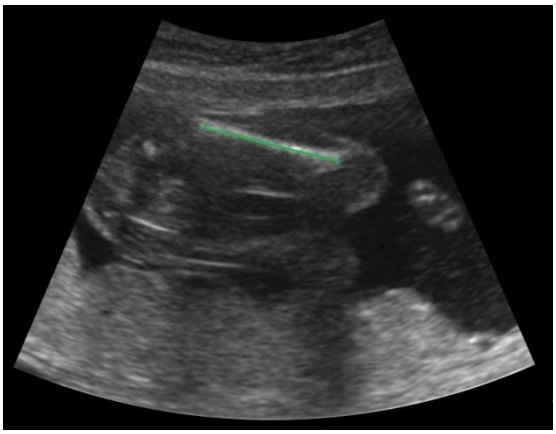
Abdominal circumference was taken in a cross-sectional view of the fetal abdomen as close as possible to circular, with the umbilical vein in the anterior third of the abdomen (at the level of the portal sinus), with the stomach bubble visible (Figure 3.3).

Figure 3.3: Representative image of measurement of fetal abdominal circumference in a fetus of 22 weeks.



Finally, the femur length was measured in the longitudinal view using the fetal thigh closest to the probe as close as possible to the horizontal plane (Figure 3.4).

Figure 3.4: Representative image of measurement of fetal femur length in a fetus of 20 weeks.



Estimated fetal weight (EFW) was calculated using the Hadlock formula[225]:

$$EFW = In(1.326 – (0.00326 \times AC \times FL) + (0.0107 \times HC) + (0.0438 \times AC) + (0.158 \times FL))$$

EFW indicates estimated fetal weight; abdominal circumference AC; FL femur length; HC head circumference.

3.3.2 Neonatal cohort

Weight was measured using digital scales (Charder Model MS4200) to the nearest 0.01kg with the infant fully naked. Head circumference was measured from the supraorbital ridges around to the occiput with a tape measure to nearest mm.

Birth length was not measured as crown-heel length measurements are known to be stressful for neonates and have a low accuracy [226, 227] with methodological variability affecting both clinical decisions and research findings.[227] In addition, many of the preterm infants were in incubators, making measurements impractical. Therefore, body surface area (BSA) was calculated using the Boyd formula[228]:

$$BSA\ (m^2) = 4.688 \times Weight\ (kg)^{(0.8168 - 0.0154 \times \log Weight\ (kg))}$$

This method, based on weight alone, has been validated in the paediatric population with deviations of less than 10% from formulas using both height and weight.[229]

Z-scores for weight were calculated using the International Standard size at birth reference charts from the INTERGROWTH-21st Project[230, 231] using their online application (https://intergrowth21.tghn.org/global-perinatal-package/intergrowth-21st-comparison-application/).

3.4 Cardiac Phenotyping

3.4.1 Echocardiography

Fetal echocardiography was performed using a Philips HD9 ultrasound system (Philips Healthcare, Guildford, Surrey, UK) with a C6-3 curved-array transducer. Postnatally, infants were scanned on a Phillips CX50 ultrasound system (Philips Healthcare, Guildford, Surrey, UK) and at three month follow up on a Philips iE33 system (Philips Healthcare, Guildford, Surrey, UK) with an S12-4 transducer.

3.4.1.1 Image Acqusition

Fetal Cohort

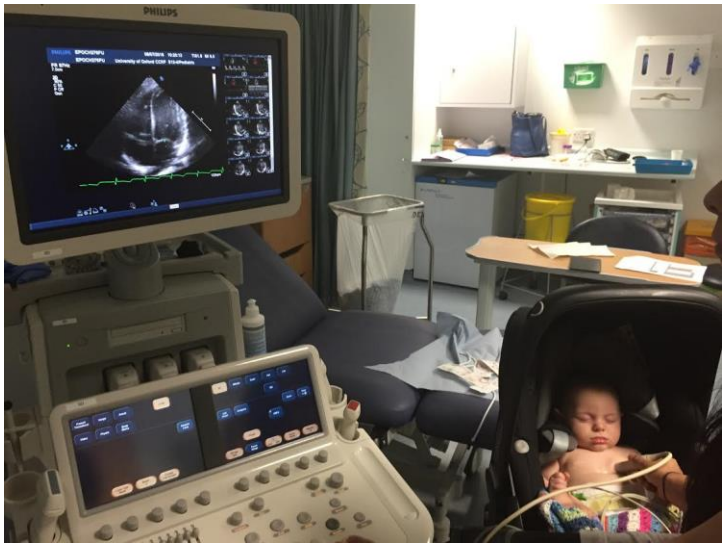
Multiple apical or basal four chamber cine loops were acquired with the septum or free wall aligned parallel to the Doppler beam (<10degrees without further angle correction). In order to obtain the highest frame rate, the scan area was kept as small as possible with the heart taking up >1/3rd of the screen. Gains and depth were optimised to aid post-processing analysis. The cine loops were taken during a period of fetal quiescence in the absence of maternal or fetal breathing movements. They were then retrospectively gated offline using TomTec Image Arena 4.6 (TomTec Imaging Systems GMBH, Unterschleissheim, Bavaria, Germany) where end diastole was defined at the point of mitral valve closure.

Neonatal Cohort

A detailed 2D transthoracic echocardiography protocol was followed that included acquisition of a four chamber view optimised for the left ventricle (LV). For the

duration of the echocardiogram, the infants were placed in a semi-recumbent position at a 45 degree angle. To enhance image resolution for post processing analysis, the frame rate was increased by minimizing the sector width, the gains and depth were optimised and multiple images of the same view were acquired to enable offline selection of the highest quality loop (Figure 3.5).

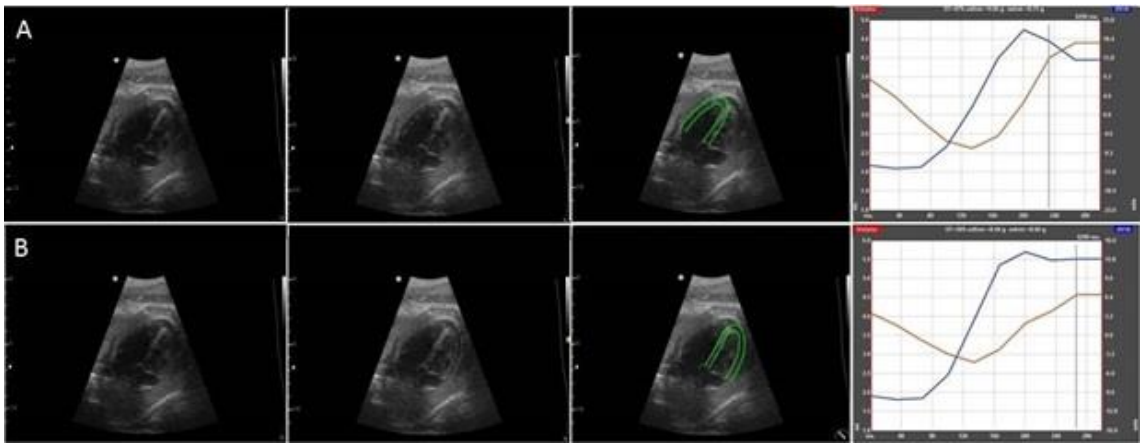
Figure 3.5: An example of a three month old infant undergoing echocardiography



3.4.1.2 Cardiac structure

Ventricular end diastolic volume (EDV) and mass were obtained by manual contouring of the endo and epicardium using TomTec Image Arena 4.6 from the apical four chamber view for fetal and infant echocardiography. The end diastolic frame was manually selected using the point of mitral valve closure as the marker and contours manually set at the inner endocardial and outer epicardial edge. To maximise reproducibility, the entirety of the septum was contoured for both LV and right ventricular (RV) measurements (Figure 3.6).

Figure 3.6: Images taken from a fetus at 39+2 weeks gestation demonstrating the technique used to estimate (A) left and (B) right ventricular mass by contouring the endo and epicardial border in the four chamber view using TomTec Image Arena 4.6.



In addition, LV septal (intraventricular septum, IVS) and posterior wall thicknesses (PWd) and left ventricular internal diameter in diastole (LVIDd) were measured in the neonatal cohort from the parasternal long axis view in 2D using Philips Xcelera 3.3 (Philips Healthcare, Guildford, Surrey, UK). The measurements were made at the base of the LV perpendicular to the mitral annulus through the leaflet tips.

This enabled a second method of calculating postnatal left ventricular mass using the current American Society of Cardiology's recommended formula[232] in in order to corroborate any findings.

$$\text{LV mass} = 0.8 \times (1.04 \times (\text{LVIDd}+\text{PWd}+\text{IVS})^3 - \text{LVIDd}^3) + 0.6$$

LVIDd indicates left ventricular internal diameter in diastole; PWd posterior wall thickness in diastole; IVS intraventricular septum in diastole.

Body size correction for measures during fetal life and in analysis from fetal through early postnatal life was based on adjustment for head circumference, as this provided a directly quantified indicator of growth known to be accurate in the antenatal and

postnatal period.[233] In addition, for neonatal measures, ventricular mass and volumes was adjusted for body size based on estimated body surface area, using the Boyd formula[228] (Section 3.3.2), and these values are reported as mass, or volume, index.

3.4.1.3 Cardiac function

In the neonatal cohort, LV systolic parameters including ejection fraction and stroke volume were captured from automated tracking of the contours of the endocardium using TomTec Image Arena 4.6 (Section 3.4.1.1) and the following equations:

$$\text{Stroke volume (ml)} = \text{EDV} - \text{ESV}$$

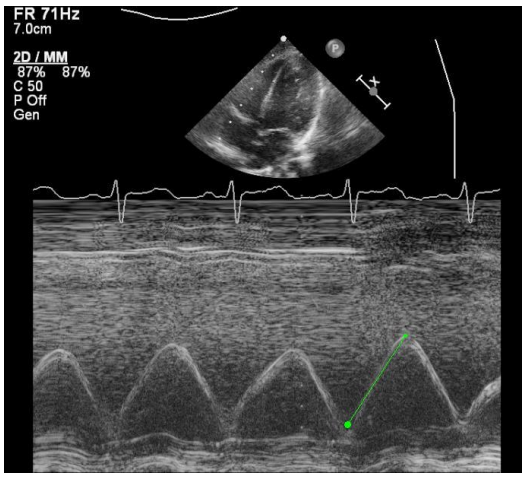
$$\text{Ejection fraction (\%)} = ((\text{EDV} - \text{ESV})/\text{EDV}) \times 100$$

EDV indicates end diastolic volume; ESV end systolic volume.

Manual adjustments of the contours were made, as required, throughout the cardiac cycle to ensure appropriate tracking of all segments and excluded if this was not possible due to image quality.

RV systolic function was quantified by taking an M-mode slice through the tricuspid annulus using the cursor in real time to measure the tricuspid annular plane systolic excursion (TAPSE), analysed offline using Philips Xcelera 3.3.

Figure 3.7: Example of TAPSE measurement from a term infant at follow up assessment



Routine diastolic functional parameters were also assessed. Pulsed wave doppler was measured from the mitral valve tips to assess early and late diastolic inflow and the ratio of these flows were characterised as E/A ratio using Philips Xcelera 3.3. Further Doppler interrogation of the lateral mitral valve annulus using Tissue Doppler Imaging was measured in early diastole (E') and was utilised in the ratio of early diastolic flow to early diastolic tissue velocity (E/E') to assess myocardial relaxation in relation to the filling velocities.

Ten datasets were selected at random to assess the inter and intra-observer variability in measurement of LV and RV in both fetal and neonatal cohorts using TomTec Image arena 4.6 (Table 3.1). Intraclass correlation coefficients (ICCs), the standard method used for reporting variability in echocardiography measures, for diastolic parameters and TAPSE are also reported in Table 3.1.

Table 3.1: Intra and interobserver intraclass correlation coefficients for echocardiography assessment of cardiac structure and function using the same set of cardiac cycles

	Intraclass Correlation Coefficients (ICC)	
Measurement	**Intra-observer**	**Inter-observer**
TomTec Image Arena		
Fetal		
LV mass	0.97 (0.87-0.99)	0.81 (0.41-0.95)
RV mass	0.99 (0.95-1.00)	0.78 (0.35-0.94)
Neonatal		
LV mass	1.00 (0.93-1.00)	0.97 (0.83-0.99)
RV mass	0.96 (0.84-0.99)	0.81 (0.41-0.95)
XCelera		
E/A	0.99 (0.95-1.00)	0.98 (0.94-1.00)
Lateral wall E’	1.00 (1.00-1.00)	0.99 (0.97-1.00)
TAPSE	0.97 (0.87-0.99)	0.90 (0.64-0.97)

ICCs for single measures and associated 95% confidence intervals are displayed.
LV left ventricle; RV right ventricle;
E early; A atrial; TAPSE Tricuspid annular plane systolic excursion.

3.4.1.4 Cardiac shape analysis

Cardiac atlases derived from the four chamber view in 2D echo were developed in collaboration with Dr Pablo Lamata at the Department of Biomedical Engineering, King's College London. The aim was to explore if there were any subtle differences in cardiac shape in those exposed to pregnancy complications, which were not identified using traditional volumes and dimensions and would aid the characterisation of offspring cardiovascular phenotype and remodelling. Methods for cardiac shape analysis are described in (Section 5.3.2.3).

3.5 Microvascular Phenotyping

3.5.1 *In vivo* microvascular imaging

It is possible to measure dermal capillary density non-invasively and it has been shown to be representative of systemic capillary density.[234] Capillary rarefaction is a major determinant of increased vascular resistance and is associated with the development of hypertension (Chapter 1).

Imaging of the axillary small vessel network was performed postnatally with Side Stream Dark Field (SSDF) imaging (Microscan, Microvision Medical, Amsterdam, The Netherlands), as previously reported for neonates[235] (Figure 3.8). This device emits LED light at a frequency of 530nM, which is absorbed by haemoglobin in the microvasculature to produce a dark blood image against a white/grey background for later off-line analysis. Measurements were performed on the same upper inner arm as in previous studies which identified this location as the optimal site for images[235] (Figure 3.8). Three one-minute video clips of adjacent areas, showing a region of

1mm^2, were recorded while the image was monitored to ensure a stable position and steady skin pressure.

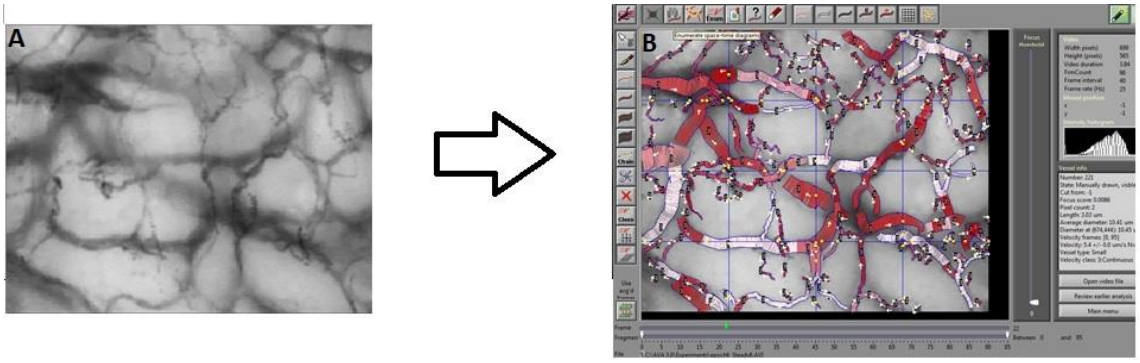
Analysis was performed off-line using dedicated quantitative software developed for the Microscan (AVA 3.0, MicroVision Medical, Amsterdam, The Netherlands).[236] Sections of video clip lasting approximately five seconds long during which there was minimal motion artefact and good image quality were selected for analysis. Capillaries were manually delineated and the dedicated software calculated total vessel density (TVD) and De Backer (DB) score with the average for the three clips used for analysis[237] (Figure 3.9). The De Backer score is another measure of capillary density which is based on the principle that the density of vessels is proportional to the number of vessels crossing arbitrary lines. The screen is divided into nine equal areas by three horizontal and three vertical lines. The DB is calculated using the following formula[237]:

De Backer Score = No of vessels crossing lines/Total length of lines

Inter and intraobserver variability are shown in Table 3.2

Figure 3.8: Example of an infant undergoing microvascular imaging at birth

Figure 3.9: An example of Microscan image on the upper inner arm of a 3 month old infant (A) before and (B) after analysis.



Ten datasets at random were selected in order to calculate intra and inter-observer variability using Coefficient of Variance (CoV), the parameter standardly used to report variability in microvascular measures. [238]

Table 3.2: Intra and interobserver coefficients of variation for *in vivo* microvascular structure.

Measurement	**Intraobserver CoV (%)**	**Interobserver CoV (%)**
Total Vessel Density (mm/mm^2)	4.35	6.54
De Backer Score	4.46	6.10

CoV indicates coefficient of variance

3.5.2 *In vitro* vasculogenic capacity

In order to link microvascular *in vivo* and *in vitro* measures, I worked in collaboration with Dr Grace Yu, a postdoctoral research fellow in Professor Leeson's research group.

I collected umbilical cords at birth from a subgroup of mothers within the study. These were then processed by Dr Yu and human umbilical vein endothelial cells (HUVECs) were isolated for investigation. Methods for the *in vitro* work are described in Section 7.3.2.1 and the Appendix.

3.6 Macrovascular Phenotyping

3.6.1 Peripheral blood pressure

Offspring of mothers with preeclampsia and those born preterm have elevated blood pressure in later life (Chapter 1) although there have been few neonatal studies. At birth and three months, blood pressure measurements were recorded with the baby supine on the right calf which has been shown to be comparable to arm measurements in neonates,[239] with an automated non-invasive digital monitor Carescape V100 Dinamap technology® (GE Healthcare, Chicago, Illinois, USA) using appropriately sized neonatal CRITIKON blood pressure cuffs (GE Healthcare, Chicago, Illinois, USA). Three readings were taken and were averaged for analysis. This monitor has been validated against invasive blood pressures in a neonatal population with mean differences of 3.35mmHg for systolic blood pressure and -0.92mmHg for diastolic blood pressure.[240]

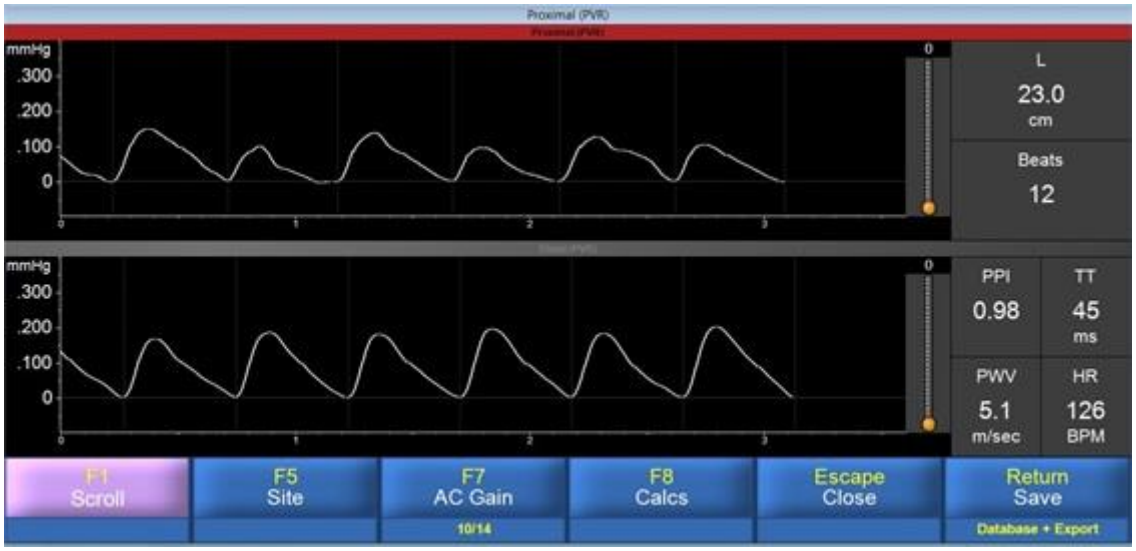
3.6.2 Pulse Wave Velocity

Pulse Wave Velocity (PWV) is seen as the gold standard measurement of arterial stiffness in adults and is described as the forward propagated arterial pressure wave.[241] It is a predictor of cardiovascular events[136, 137] with increased arterial stiffness

also being associated with traditional cardiovascular risk factors (Chapter 1). There have been reports of increased maternal arterial stiffness following a hypertensive pregnancy, although these have been inconsistent.[242, 243] In offspring, increases in pulse wave velocity has been associated with pregnancy complications such as being born small for gestational age [244] and maternal alcohol consumption during pregnancy [245] and also with other cardiovascular risk factors such as diabetes[246] and obesity.[247, 248]

In this study, brachial-femoral PWV was measured postnatally by applying brachial and femoral oscillometric cuffs a known distance apart with the infant supine and at rest and connecting them to a Vicorder (Skidmore Medical, Gloucestershire, UK) device. The cuffs were automatically, partially inflated to diastolic blood pressure and the corresponding oscillometric signal from each cuff was digitally analysed for the pulse time delay between the cuffs and the PWV calculated by using the distance between the two cuffs (Figure 3.10). The feasibility of using this non-invasive technique to assess infant arterial stiffness in population studies has been previously demonstrated.[249]

Figure 3.10: Example of Vicorder reading from an infant at birth



3.7 Autonomic Function

3.7.1 Heart rate variability

Heart rate variability (HRV) analysis is an important indirect and non-invasive measure of cardiac autonomic function. It attempts to derive information from the study of the QRS to QRS (RR or normal to normal NN interval) interval sequence of the electrocardiogram (ECG) in order to study the balance between the sympathetic and parasympathetic arms of the autonomic nervous system (ANS) and gives an indication of the sinoatrial node's ability to adapt to extrinsic signals.

There are two broad categories of HRV parameters: time domain and frequency domain (power spectrum) measures. Time domain analyses are calculated using simple equations directly from the RR intervals. They are less influenced by the quality of the recording but provide little information on HRV at lower frequencies. Frequency domain measures use more complex algorithms to describe a time-averaged estimate

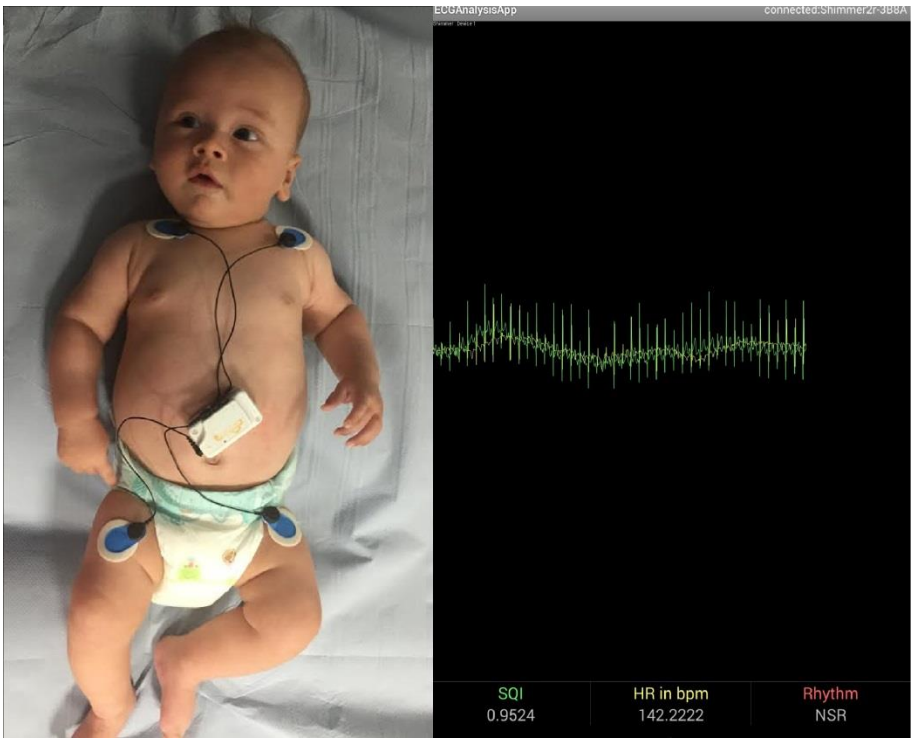
of power across a range of frequencies (high frequency (HF), low frequency (LF) and very low frequency(VLF)) and are thought to provide information on the intrinsic oscillators that control the underlying rhythm of heart rate, namely respiration, baroreceptor reflex and thermoregulation respectively.[250]

Decreased HRV has been demonstrated in various pathological states in the neonate such as respiratory distress syndrome,[251-256] birth asphyxia,[253, 257] intraventricular haemorrhage,[253, 258] small-for gestational age[259] and sudden infant death syndrome [260-264] as well as correlating with clinical illness scales.[265] A few studies have shown that infants born preterm also have attenuated HRV.[266, 267]

3.7.1.1 Data acquisition

At the birth assessment all babies had a short 5-10 minute ECG taken between feeds, lying down without use of a pacifier. The Shimmer® device (Shimmer, Glasnevin, Dublin, Ireland) was used to acquire the data which was connected via Bluetooth to an Android mobile smartphone.[268] The ECG collected consisted of two-lead signals with a 256Hz sampling frequency and 12bit quantization levels. Data was collected through an Android app,[269] processed and stored on the phone before being transferred to a server (Figure 3.11). Details of whether the baby was asleep or awake were noted and the recording was stopped if there was excessive restlessness or crying.

Figure 3.11: Example of ECG recording on an infant at 3 months of age on the Shimmer® device connected to Android mobile smartphone.[268]



3.7.1.2 Data processing and analysis

This was performed in collaboration with Dr Julien Oster in the Institute of Biomedical Engineering. Methods are described in (Section 8.3.2.1).

3.8 Maternal Biomarkers

Mothers had venepuncture performed at the postnatal assessments whilst fasted for at least 4 hours using a sterile technique at the same time as the birth assessment. 20 mls of blood was collected into blood collection tubes (EDTA, fluoride, serum, heparin and citrate) and the samples were centrifuged at 3000xg for 15 minutes at 4^0C, separated into cryovials and stored at -80^0C.

3.8.1 Enzyme-linked immunosorbent assays

We quantified plasma circulating $VEGF_{165}$, sFlt-1, PlGF, and sENG concentrations in maternal blood with commercial enzyme-linked immunosorbent assays (ELISAs) (Quantikine, R&D Systems Europe, Abingdon, UK).

Standard solutions of known concentrations were created by mixing analyte with deionized water to produce a stock solution. This was left for 15 minutes before further agitation prior to making dilutions. From this stock solution, increasing amounts of calibrator diluent was then mixed in to create a series of known and reducing concentrations to be used as standards. Eppendorf tubes were mixed thoroughly before each subsequent dilution.

Maternal blood samples were then thawed on ice and gently agitated to ensure homogeneity. A set volume of the thawed sample was pipetted into each well of a 96 well plate. Assay diluent was used as the control. All samples, standards, and controls were plated in duplicate and incubated at room temperature on an automatic plate shaker for 2 hours.

Next, each well was aspirated and washed using 400µL of an automatic plate washer Hydroflex (Tecan, Männedorf, Switzerland). After washing, a standard volume of conjugate solution depending on the assay was added to each well and the plate was incubated at room temperature for a further 2 hours. The wash cycle was repeated as before followed by the addition of a further 200µL of substrate solution but this time the plate was incubated at room temperature for 30 minutes in darkness. 50µL of stop solution was then added to each well and the plates shaken gently on an automatic

plate shaker at low speed for 5 minutes to ensure thorough mixing of the substrate and stop solution.

Optical density of each well was measured at 450nm using a FLUOstar Omega microplate reader (BMG Labtech, KBioScience, Cary, North Carolina, USA) within 30 minutes. Data was analyzed using Omega Data Analysis software. Duplicate readings for each standard, control, and sample were averaged, and the average zero standard optical density was subtracted. Standard curves were created by generating a four-parameter logistic curve-fit. A coefficient of variance between duplicates <15% was considered acceptable.

3.9 Medical history

Medical and pregnancy history was collected both from medical records of the mother and offspring and a questionnaire at each postnatal assessment.

3.9.1 Maternity records

Maternal demographics at booking were extracted from clinical records including weight, height, BMI, blood pressure and smoking status. Previous obstetric history was also noted. Clinical data from the whole duration of the pregnancy was recorded including haemodynamic changes, development of maternal hypertension, administration of antenatal steroids and delivery details including mode of birth, infant APGAR scores, birthweight and gestation. Diagnostic details for maternal hypertension were confirmed as corresponding with international guidelines. [270]

3.9.2 Infant records

Infants who required additional observation or treatment or who were admitted to the neonatal unit had additional medical records relating to their immediate postnatal course. These records were reviewed and information collected including length and nature of oxygen support, cardiac abnormalities such as persistent patent ductus arteriosus and postnatal infections.

3.9.3 Questionnaires

Validated questionnaires were given to the mothers at both birth and follow up assessments. At birth, data was collected on demographic details, medical, family and pregnancy history relevant to preeclampsia and cardiovascular disease and lifestyle information such as diet, smoking and alcohol intake for both the mother and father of the infant (see Appendix). At three month follow up, data on lifestyle in the postnatal period was collected in addition to information on infant growth, development and feeding (see Appendix).

3.10 Statistical analysis

Statistical analysis was performed using SPSS Version 22 (IBM, Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad Software Inc, La Jolla, CA, USA). Normality of data for continuous variables was assessed visually and also by using the Shapiro-Wilk test. Comparison between groups for continuous variables was carried out using a two-tailed, independent samples t-test for normally distributed variables using Bonferroni

post hoc corrections where appropriate and Mann Whitney U test for non-normally distributed data. Comparison of categorical variables was performed using a Chi-Square test. Bivariate regression models were performed using a forced entry method and unstandardized B Coefficients and 95% confidence intervals are reported. Any variables from bivariate regression analysis with a *p*-value <0.10 were then included in a multivariable regression analysis. *P*-values less than 0.05 were considered statistically significant. Whiskers on Tukey boxplots demonstrate 75^{th} percentile plus 1.5*interquartile range and 25^{th} percentile minus 1.5*interquartile range.

Additional power calculations and statistical techniques are described in the relevant chapter.

4: Establishing Fetal Nomograms of Cardiac Structure

In this thesis I will be exploring the effect of pregnancy complications on offspring cardiovascular phenotype including ventricular mass pre and post birth. I will be using 2D echocardiography and TomTec Image Arena 4.6 in order to quantify measures of cardiac structure. However at present, no nomograms of fetal cardiac mass using 2D echo exist. Therefore, this chapter sets out to develop fetal nomograms of cardiac structure to which datasets from offspring born to pregnancy complications can be later compared. I have then compared my results to other published nomograms using more novel technologies.

4.1 Abstract

Background- Changes in cardiac dimensions have been demonstrated in offspring born to pregnancy complications. Quantification of cardiac dimensions, in addition to its use in the research field, is also important for screening and monitoring of cardiac and extra-cardiac fetal conditions. 2D ultrasound image quality and automated quantification methods have improved in recent years and I therefore assessed their feasibility and reproducibility for fetal ventricular measures from 15 to 42 weeks gestation, developed nomograms and compared measures to reports from other modalities.

Methods- Fetal hearts were scanned using 2D echocardiography (Philips HD-9 and C6-3 curved-array transducer) and cineloops of apical or basal four chamber views were acquired. Left and right ventricular (LV and RV) mass and end-diastolic volumes (EDV) were estimated by manual contouring of the endo and epicardium using TomTec Image Arena 4.6 in end-diastole. Intraclass Correlation Coefficients (ICC) were used to estimate intra- and inter-observer agreement. Nomograms were created from smoothed centiles of mass and volume measurements and constructed using fractional polynomials after log-transformation. Results were compared to those from previous studies using other modalities identified from literature review.

Results- 294 scans from 146 fetuses were included with data ranging from 15+0 to 41+6 weeks gestation. Only 7% of scans were unanalysable and intraobserver variability was good (ICC for LV and RV mass 0.97 (0.87-0.99) and 0.99 (0.95-1.0) respectively). Mass and volume increased exponentially throughout pregnancy

demonstrating good agreement with 3D estimates of mass up to 28 weeks gestation, after which the 2D measurements were in better agreement with previous results from neonatal cardiac magnetic resonance imaging. There was good agreement with 4D volume estimates for the left ventricle.

Conclusions- Current state-of-the-art 2D echocardiography platforms appear to provide accurate, feasible and reproducible fetal ventricular mass and volume measures across a wide range of gestations. In certain circumstances such as extremes of gestation, 2D ultrasound may be the modality of choice.

4.2 Introduction

Two dimensional (2D) echocardiography has for a long time been considered the modality of choice in the clinical setting.[271] In addition to developing nomograms for research purposes, assessment of the fetal heart forms an important part of screening for fetal malformations. Congenital heart disease has a reported incidence of 6 per 1000 live births for moderate to severe forms which rises to 75 per 1000 if more mild defects are included.[272] Structural and functional defects can often have an impact on fetal cardiac dimensions including ventricular mass. Furthermore, extra-cardiac factors, such as diaphragmatic hernias, and maternal conditions, for example diabetes, may also have an effect on fetal cardiac structure. Therefore, estimation of ventricular mass for screening and monitoring purposes can aid in assessing the severity and clinical course of the condition in order to guide management.

In recent years, newer modalities such as 3D and 4D sonography have emerged as potentially more accurate, optional adjuncts.[271] However, the technologies for delivery of 3 and 4D sonography have also led to parallel improvements in 2D technology which has resulted in better image quality and development of off-line quantification packages that offer a variety of automated measures. As no 2D echocardiography references ranges exist for fetal left and right ventricular mass and volumes, the purpose of this chapter was to use the acquired data to produce nomograms from 15 to 42 weeks gestation. I also used the results to evaluate state-of-art 2D echocardiography and quantification approaches for fetal echocardiography to assess their feasibility and reproducibility. Finally, I compared the values obtained against other published results using different modalities.

4.3 Methods

4.3.1 Study population

This chapter includes data from 146 eligible fetuses that underwent cardiac imaging. Inclusion and exclusion criteria are detailed in Section 2.2. Clinical records were also used to ensure that, for the purposes of development of fetal normal ranges, datasets from fetuses who went on to be delivered following any pregnancy complications including intrauterine growth restriction, preterm birth (before 37 weeks gestation) and/or exposure to chronic or new-onset maternal hypertension[182] (Section 2.1.2) were excluded from analysis.

Details of this cohort and how they were recruited are set out in Chapter 2.

4.3.2 Study visit

All mothers attended the research department and had the following assessments and analysis, which are described in detail in Chapter 3:

- Fetal anthropometry (Section 3.3.1)
    - Head circumference
    - Abdominal circumference
    - Femur length
- Fetal echocardiography (Section 3.4.1)
    - Cardiac image acquisition using cineloop
    - Cardiac structure
        - LV mass and volumes
        - RV mass and volumes
- Medical notes data extraction (Section 3.9)

4.3.3 Statistics

Statistical analysis is described in detail in Section 3.10.

4.3.3.1 Additional statistics - Growth trajectories

For the creation of the nomograms, I collaborated with Dr Eric Ohuma, a post-doctoral statistician working on the InterBIO-21st Project who has expertise in modelling fetal and neonatal growth parameters. STATA, version 11.2, software (StataCorp LP, College Station, Texas, USA) was used in order to create smoothed centiles of left ventricular

(LV) and right ventricular (RV) mass, left and right end diastolic volume (EDV), ratio of LV to RV mass and ventricular mass as a function of estimated fetal weight (EFW) (i.e. ventricular mass/EFW) using fractional polynomials according to gestational age. Where appropriate, a multi-level, linear regression analysis was applied to account for repeated measures[273] but there were insignificant differences when compared to analyses that did not account for the hierarchy of the data. LV and RV mass, LV and RV EDV, ratio of LV to RV mass and ventricular mass as a function of EFW exhibited a non-normal distribution; therefore, the data were log-transformed (natural log) to stabilise variance and transform the data to normality. Goodness-of-fit assessment incorporated a visual inspection of the quantile-quantile (q-q) plot of the residuals and a plot of fitted z-scores across gestational ages.

I also performed a literature review of published normative values for fetal LV and RV mass and EDV to identify papers that reported on PubMed between 2000 and 2015 using different modalities in order to compare our results to those previously reported.

4.4 Results

4.4.1 Study population characteristics

317 fetal echocardiograms from 146 eligible fetuses without pregnancy complications who were born at term were analysed. 23 scans were unanalysable due to image quality, fetal position or movement (7%), which left data from 294 scans to be used to build the nomograms. 91 (63%) of the fetuses had one analysable echocardiogram, 44 (30%) had two, 18 (12%) had three, 10 (7%) had four, 3 (2%) had five and one (0.6%)

fetus had six. The range of gestational age at scan was 15+0 to 41+5 weeks. The cohort characteristics of the fetuses contributing to the nomograms are presented in Table 4.1. Intraclass Correlation Coefficients (ICC) with 95% confidence intervals for intra and inter-observer variability for measures of cardiac dimensions using TomTec Image Arena yielded 0.97 (0.87-0.99) and 0.81 (0.41-0.95) for the left ventricular mass and 0.99 (0.95-1.0) and 0.78 (0.35-0.94) for the right, respectively.

Table 4.1: Fetal cohort characteristics

	n=146
Maternal Demographics & Anthropometrics	
Age at delivery, years	31.3±4.5
BMI at booking, kg/m^2	23.8±3.8
Smokers, n (%)	16 (11)
Offspring Demographics & Anthropometrics	
Gestational age, weeks	39.9±1.3
Males, n (%)	80 (55)
Birth order$^{\Psi}$	1±1
Caesarean section, n (%)	23 (16)
Birthweight, grams	3423±445
Birthweight z-score	0.29±0.9

Values as Mean±Standard Deviation unless stated otherwise
$^{\Psi}$ Median±Interquartile range

4.4.2 Cardiac mass and volume

Normal ranges for left and right ventricular mass and end-diastolic volume are displayed in Tables 4.2a and 4.2b with 3rd, 50th and 97th centiles displayed. The nomograms for these measures are presented in Figure 4.1 and show that both left and right ventricular mass and EDV increase exponentially from 15 weeks through gestation.

Table 4.2a: Normal ranges for fetal ventricular mass

	LV Mass (g)			RV Mass (g)		
Week	P_3	P_{50}	P_{97}	P_3	P_{50}	P_{97}
16	0.07	0.15	0.34	0.06	0.11	0.23
18	0.13	0.29	0.65	0.12	0.23	0.46
20	0.22	0.49	1.07	0.21	0.41	0.82
22	0.35	0.75	1.62	0.33	0.66	1.31
24	0.51	1.08	2.27	0.49	0.97	1.93
26	0.70	1.46	3.02	0.68	1.35	2.68
28	0.93	1.89	3.84	0.90	1.79	3.56
30	1.19	2.37	4.71	1.16	2.29	4.55
32	1.48	2.89	5.63	1.43	2.84	5.63
34	1.80	3.44	6.56	1.73	3.43	6.80
36	2.14	4.01	7.51	2.04	4.06	8.05
38	2.51	4.61	8.45	2.38	4.71	9.35
40	2.90	5.22	9.38	2.72	5.39	10.70
42	3.31	5.84	10.30	3.07	6.10	12.10

LV mass:

$P_3 = \exp(4.020472 + (-9.473844 \times (GA/10)^{-1}) + (-1.88 \times (0.526187 + (-0.0053485x(GA)))))$

$P_{50} = \exp(4.020472 + (-9.473844 \times (GA/10)^{-1}))$

$P_{97} = \exp(4.020472 + (-9.473844 \times (GA/10)^{-1}) + (1.88 \times (0.526187 + (-0.0053485x(GA)))))$

RV mass:

$P_3 = \exp(4.253648 + (-10.2729 \times (GA/10)^{-1}) + (-1.88 \times (0.3644328)))$

$P_{50} = \exp(4.253648 + (-10.2729 \times (GA/10)^{-1}))$

$P_{97} = \exp(4.253648 + (-10.2729 \times (GA/10)^{-1}) + (1.88 \times (0.3644328)))$

Table 4.2b: Normal ranges for fetal ventricular volume

	LV EDV (ml)			RV EDV (ml)		
Week	P_3	P_{50}	P_{97}	P_3	P_{50}	P_{97}
16	0.04	0.08	0.16	0.03	0.07	0.20
18	0.09	0.17	0.33	0.06	0.16	0.40
20	0.15	0.30	0.59	0.12	0.29	0.71
22	0.24	0.47	0.94	0.21	0.49	1.12
24	0.35	0.70	1.38	0.34	0.75	1.66
26	0.49	0.97	1.92	0.50	1.08	2.31
28	0.65	1.29	2.54	0.70	1.46	3.08
30	0.83	1.65	3.25	0.93	1.91	3.95
32	1.03	2.04	4.02	1.19	2.42	4.92
34	1.25	2.46	4.86	1.48	2.97	5.97
36	1.47	2.91	5.74	1.79	3.57	7.10
38	1.71	3.38	6.67	2.13	4.21	8.30
40	1.96	3.87	7.64	2.49	4.88	9.55
42	2.21	4.37	8.63	2.87	5.58	10.85

LV EDV:

$P_3 = \exp(3.917406 + (-10.25886 \times (GA/10)^{-1}) + (-1.88 \times (0.3618528)))$

$P_{50} = \exp(3.917406 + (-10.25886 \times (GA/10)^{-1}))$

$P_{97} = \exp(3.917406 + (-10.25886 \times (GA/10)^{-1}) + (1.88*(0.3618528)))$

RV EDV:

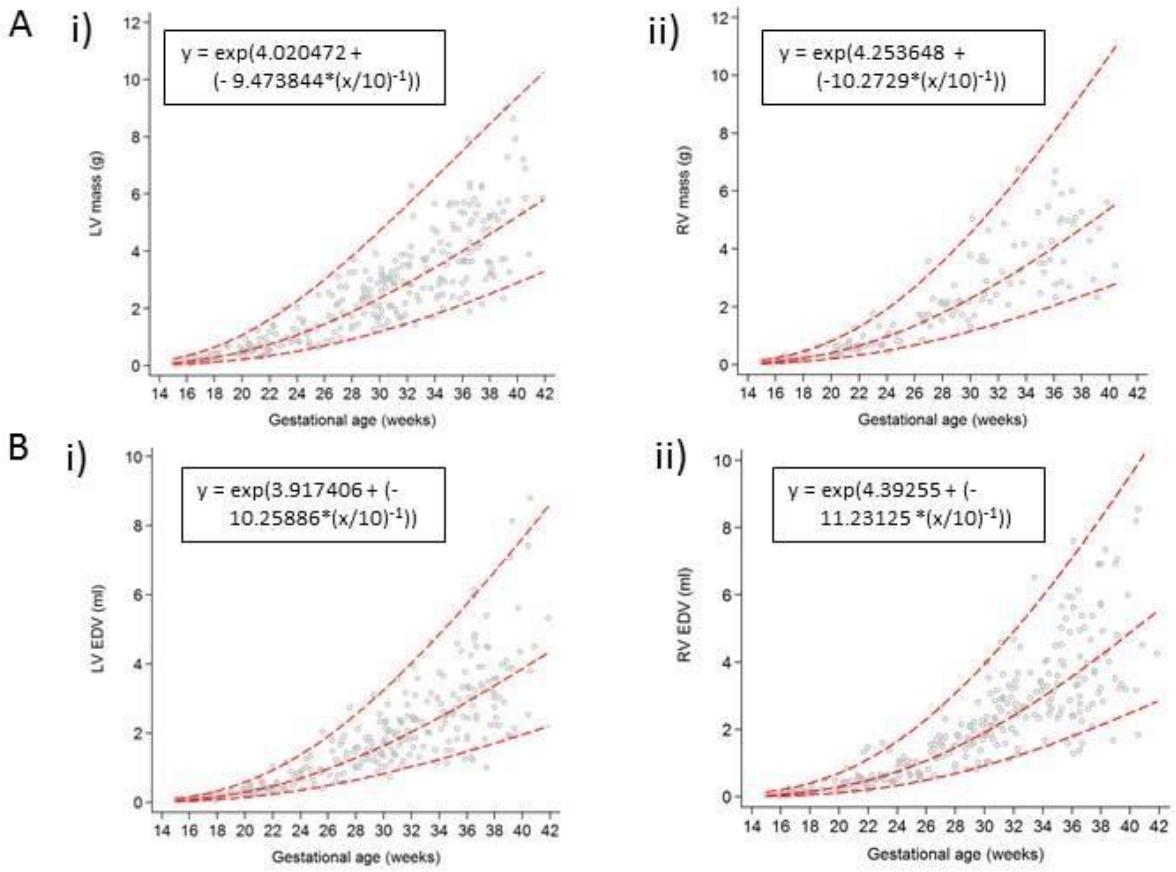
$P_3 = \exp(4.39255 + (-11.23125 \times (GA/10)^{-1}) + (-1.88 \times (0.3213458 + 0.5765573 \times (GA/10)^{-2})))$

$P_{50} = \exp(4.39255 + (-11.23125 \times (GA/10)^{-1}))$

$P_{97} = \exp(4.39255 + (-11.23125 \times (GA/10)^{-1}) + (1.88 \times (0.3213458 + 0.5765573 \times (GA/10)^{-2})))$

LV indicates left ventricular; RV right ventricular; EDV end-diastolic volume; P percentile; GA gestational age in weeks at scan.

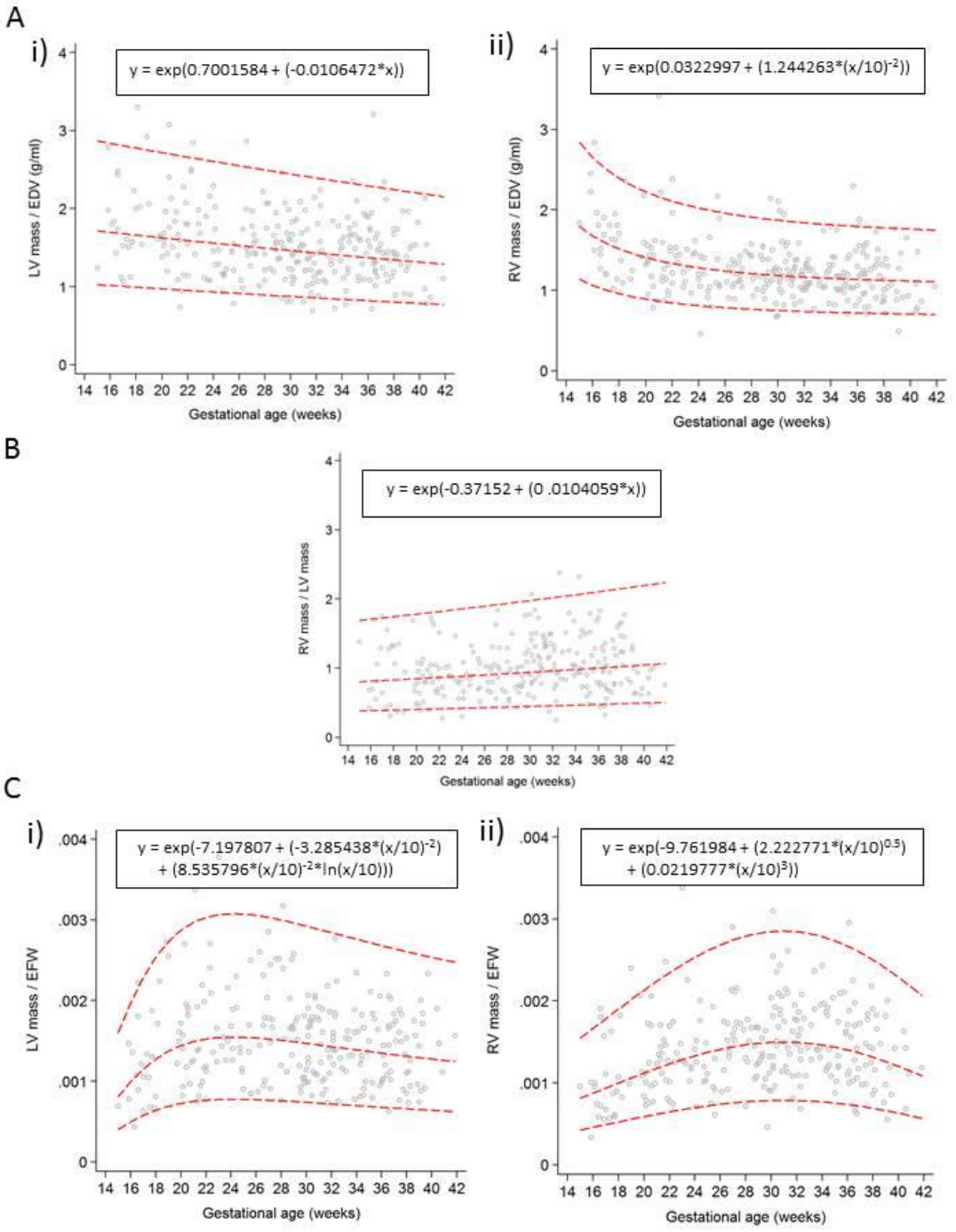
Figure 4.1: Trajectories of (A)(i) left and (ii) right ventricular mass and (B)(i) left and (ii) right end-diastolic volume. Dashed lines indicate the 3rd, 50th and 97th percentile. Equations shown are for the 50th percentile. LV indicates left ventricular; RV right ventricular; EDV end-diastolic volume.



4.4.3 Patterns of mass change

To investigate whether mass changes in proportion to cardiac volume in the fetal circulation trajectories for mass to end diastolic volume ratios were calculated for both ventricles. Left and right mass/EDV ratios decreased slightly as pregnancy progressed from 1.7g/ml and 1.7g/ml at 16 weeks to 1.3g/ml and 1.1g/ml at 40 weeks for left and right ventricles respectively (Figure 4.2A). I also studied ventricular dominance *in utero*, and plotted RV to LV mass ratio, which increased through gestation from 0.81 at 16 weeks until term when masses in the two ventricles were equal (ratio of 1.0) (Figure 4.2B). Development of the fetal myocardium seemed to stayed in line with overall body growth, with the ratio of left ventricular mass to estimated fetal weight being $1x10^{-3}$ at both 16 and 40 weeks and $0.8x10^{-3}$ and $1.2x10^{-3}$ for the right respectively (Figure 4.2C).

Figure 4.2: (A)(i) left and (ii) right mass/end-diastolic volume ratio and (B) right/left ventricular mass ratio from 15 to 42 weeks gestation. (C) Trajectories of (i) left and (ii) right ventricular mass to estimated fetal weight ratios from 15 to 42 weeks. Dashed lines indicate the 3rd, 50th and 97th percentile. Equations shown are for the 50th percentile. LV indicates left ventricular; RV right ventricular; EDV end-diastolic volume; EFW estimated fetal weight.



4.4.4 Comparison to previous studies

Previous ultrasound studies which have published normal values and/or equations for human fetal ventricular mass are displayed in Table 4.3. Figure 4.3A shows trajectories reported in these papers for ventricular mass using novel technologies (3D echo and real-time 3D echo)[274, 275] overlaid on my 2D echo data. This shows good to excellent agreement between 2D and 3D echo for both the left and right ventricle mass up until 28 weeks gestation. Beyond this point the estimates from 3D appear unrealistic.

Comparison could not be made with Messing et al. for 4D ultrasound using spatio-temporal image correlation (STIC) with Virtual Organ Computer-aided AnaLysis (VOCAL)[276] as equations were not provided for mass.

Ventricular mass estimates are derived from subtracting intraventricular from total volume and multiplying the remainder by estimated fetal myocardial density (1.050 g/cm^3).[277] I therefore compared volume estimates from my 2D data to those available using 4D echo from two other studies (Figure 4.3B).[276, 278] These figures show excellent agreement between 2D and 4D estimates for the left ventricle. Unfortunately, the published equation for the RV EDV in the Messing et al. study was incorrect and could not be plotted.[276] The results from Hamill et al. indicate an over-estimation of right ventricular volume by 2D methods compared to 4D.[278]

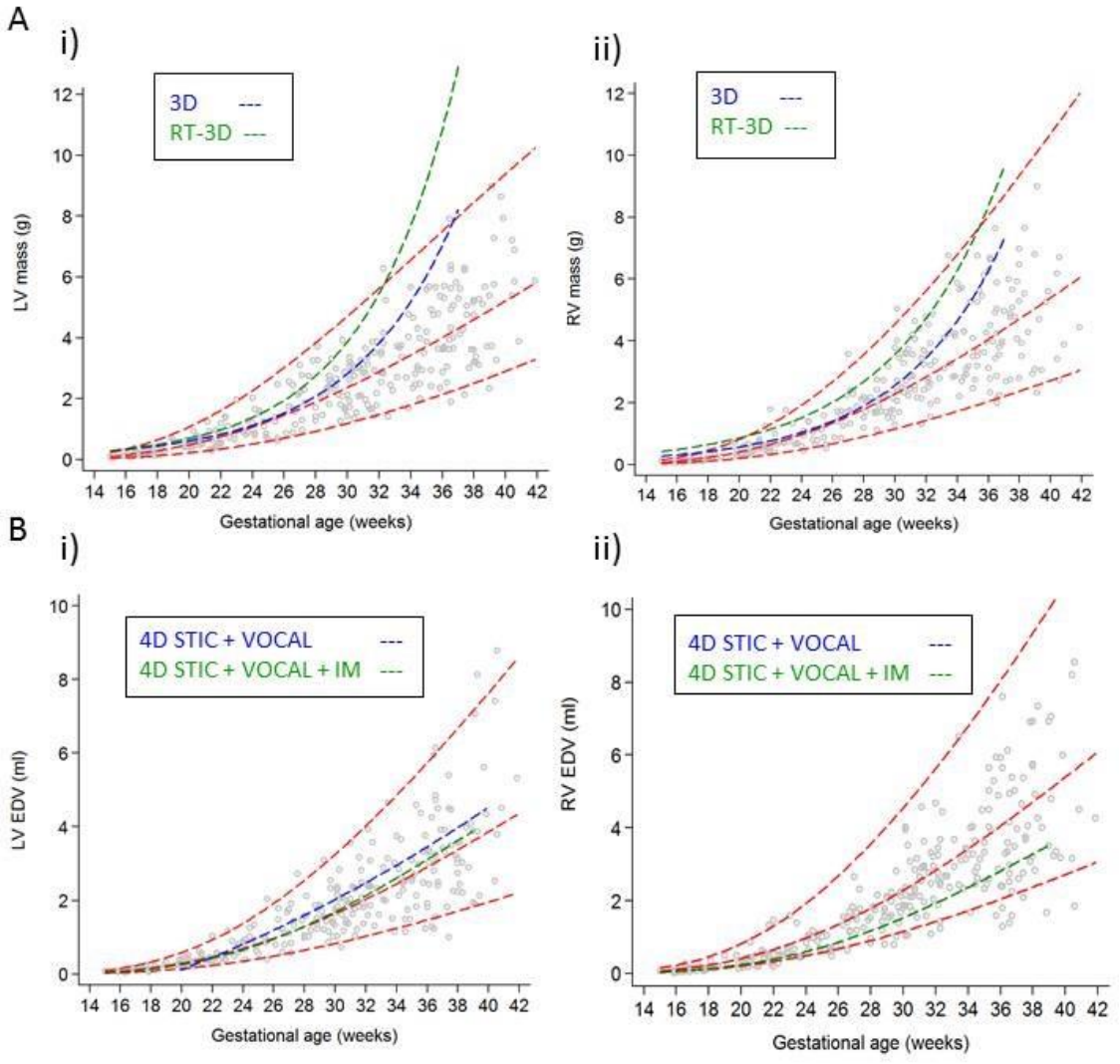
Table 4.3: Comparison of the present study to previously published studies

Authors	Year	Modality	No. of datasets	Range of gestation (weeks)	Structural ventricular parameters measured	Unanalysable datasets, n (%)
Current study		2D	317	15.0 – 41.7	LV and RV mass LV and RV EDV	23 (7%)
St John Sutton et al.[288]*	1983	M-mode	78	20.0-38.0	LV mass LV and RV diastolic & systolic diameters Septal & free wall thicknesses	2 (2.6%)
Bhat et al.[274]	2004	3D	90	15.5 - 37.0	LV and RV mass	15 (17%)
Messing et al.[279]*	2011	3D STIC + VOCAL	121	21.0 - 38.0	LV and RV mass	15 (12%)
Zheng et al.[275]	2013	Real-time 3D	59	16.7 - 34.6	LV and RV mass LV and RV EDV LV and RV ESV	7 (12%)

LV indicates left ventricular; RV right ventricular; 3D three dimensional;
STIC spatiotemporal image correlation; VOCAL Virtual Organ Computer-aided Analysis;
EDV end-diastolic volume; ESV end-systolic volume; 2D two dimensional.

Top line reports results from current study
*No equation/table of normal values provided by study

Figure 4.3: Trajectories of (A)(i) left and (ii) right ventricular mass in the present study from 15 to 42 weeks gestation compared to previous studies using 3D[274] (blue) and real-time 3D (RT-3D)[275] (green) echo. Trajectories of (B)(i) left and (ii) right ventricular end-diastolic volume compared to previous studies using 4D ultrasound using spatio-temporal image correlation (STIC) with virtual organ computer-aided analysis (VOCAL)[278] (blue) and 4D STIC with VOCAL and inversion mode (IM)[276] (green). Dashed red lines indicate the 3rd, 50th and 97th percentile from the present study. 50th Percentile from previous studies shown.



4.5 Discussion

The work in this chapter has developed, for the first time, fetal nomograms of mass and volume in both the left and the right ventricle in a large cohort using 2D echo. Over the past decade, there has been a huge improvement in 2D technology, with better image quality and automated algorithms for measurements. This study demonstrates 2D ultrasound is feasible in the majority of fetuses, with an image quality that allows reproducible measures of mass across a wide range of gestations. As 2D echocardiography is relatively simple and fast, using widely available equipment, these nomograms are likely to be of value across a range of healthcare settings. My results demonstrate that 2D measures of mass and volume are consistent with other studies and that mass increases exponentially as gestation progresses[274, 275, 279, 280] with right and left ventricular masses becomes roughly equal as term approaches.[281] This study has also shown that total heart weight increases linearly with total body weight[281] consistent with the finding that left and right ventricular mass to estimated fetal weight ratios stay relatively stable throughout gestation.[280]

4.5.1 Previous studies

Nomograms of fetal heart volumetry have been published in the past using 2D, 3D and 4D methods.[274, 276, 278, 282-287] They all demonstrate an increase in volume over gestation but with wide variation between studies, probably as a result of the use of different methods for estimation. There has been very little published in the literature regarding estimates of ventricular mass. Previous post-mortem and imaging studies using M-

mode have demonstrated that total left ventricular weight increases with gestational age and body weight.[281, 288] Another study evaluated the use of 2D echo using area-length calculations methods and found that LV mass was significantly greater in the third than in the second trimester, although no equations were produced in order to calculate normal ranges.[280]

The other published studies on estimation of fetal ventricular mass with echocardiography have used more novel technologies such as 3D and 4D echo.[274, 275, 279] Zheng et al.[275] used real-time 3D and generated values consistently greater than those from a study using 3D echo by Bhat et al.,[274] which were closer to values from my 2D method. Indeed, there was good to excellent agreement between my 2D measures and those reported for 3D echo for both the left and right ventricle up until 28 weeks gestation. After this time the 3D estimates appear to generate values that are strikingly unrealistic, becoming increasingly higher than that generated by my 2D study, so that by 40 weeks the values are substantially greater than would be expected based on what is known about cardiac size after birth based on neonatal echocardiography from my cohort (Chapter 5). This may be because estimated trajectories from Zheng et al. are based on data from fetuses only up to 35 weeks gestation, at which point the estimate for left ventricular mass was 9.15g compared to 6.07g for 3D echo and my 2D estimate of 3.72g.[275] Interestingly, my 2D estimates from this time point are most consistent with a previous study looking at left ventricular mass in preterm infants using cardiovascular resonance imaging.[289] This is considered a gold standard imaging modality for quantification of ventricular mass in adults[290] and based on imaging of infants at a mean corrected gestational age of 34+6 weeks, they

found that mean LV mass normalized to weight at scan was around 1.39 g/kg. Bhat et al. also only included fetuses up to 37 weeks gestation by which time estimated LV mass was lower at 8.24g but still substantially larger than the 4.31g estimated by 2D echo.[274]

Ventricular mass is usually calculated by subtracting intraventricular from total volume and multiplying the remainder by estimated fetal myocardial density (1.050 g/cm^3).[277] Therefore I extracted estimates of EDV from these studies in order to investigate correlation between my 2D method for volumes and 4D values. Comparing my results to those derived from 4D ultrasound I demonstrated an excellent agreement between 2D and 4D estimates for left ventricular end diastolic volume.[276, 278] The results seem to suggest an over-estimation of right ventricular volume by my 2D method compared to 4D.[278] Intriguingly, my fetal estimates for RV EDV at 27 weeks and 37 weeks were in good agreement with previously published measurements in preterm and term infants of similar gestations (1.26ml vs 1.8ml at 27 weeks and 3.88ml vs 3.7ml at 37 weeks for fetal and neonatal values respectively)[291] so it may be that 4D methods underestimate ventricular volume. It has been reported, though, that in some cases of pathology, the use of 2D echocardiography for volume measurements can underestimate the severity of the diagnosis.[276] However, this does not preclude using 2D ultrasound as a screening tool where newer technologies are available.

4.5.2 Benefits of 2D ultrasound

The benefits of using 2D ultrasound over more novel technologies are that it is faster, cheaper and more widely accessible, although the Tomtec automated software may not be widely available especially in low income settings. 2D ultrasound is still seen as the primary imaging modality for fetal cardiac imaging with other techniques seen as an optional adjunct.[271] In addition, newer techniques such as 3D ultrasound or 4D ultrasound using STIC require a significant learning period both for acquisition and analysis.[274, 276, 278] They also involve either manually defining the contours serially at each plane as in the case with 4D with STIC[276] or tracing along endo and epicardial surfaces in 3D[274] both of which are significantly more time consuming than extraction of data from 2D echocardiograms. There are limitations to newer methods, such as inability to perform measurements at extremes of gestation[276, 287] and reliance on the fetus being in an optimum position with a significant period of quiescence[276, 278, 287] as well as acoustic shadowing and dropout.[278, 287] This results in a high proportion of scans being unsuitable for analysis; for example in a previous study using 3D ultrasound to estimate mass, a sixth were found to be of insufficient quality for analysis[274] and in another investigating cardiac volumes, acquisition using STIC was only possible in 71% of examinations.[287] However, this is not an isolated problem with newer technologies; a previous study using M-mode to describe ventricular geometry and function reported a rejection rate of 21% which is much higher than the 7% in this study.[292] Finally, even though my acquisition was not gated, measurements could be timed for end diastole by offline gating using mitral valve closure unlike 3D ultrasound where the four chamber view can often only be analysed in mid-diastole.[274]

4.5.3 Limitations

2D methods have been criticized in the past as they have been shown to have a high level of interobserver variability compared to 3D and 4D methods especially for the fetal right ventricle.[274, 276] However, I found that using newer quantification packages for analysis, both inter and particularly intraobserver variability was low. In addition, there was some variability in what was considered normal mass and volume from my 2D assessment throughout gestation and it has been reported that in some cases of pathology, the use of 2D for volume measurements can underestimate the severity of the diagnosis.[276] However, this does not preclude using this modality as a screening tool where newer technologies are available, especially if serial measurements are taken by the same operator.

Another potential limitation of my study was that I did not provide validation against an inanimate or animal model for my measures of mass. This may have been useful as I was using a 2D single-plane method which may have resulted in inaccuracies due to geometrical assumptions. Single-plane methods using 2D ultrasound have previously been used in studies where estimation of volumes and mass have been technically challenging. One study compared single-plane to biplane measures of left atrial volumes in adults which are notoriously hard to visualise.[293] In this large study of 527 patients, estimates from the two methods were strongly correlated, but agreement for categorical classification was suboptimal due to over-estimation with the single-plane method. This method was also used in a study measuring right ventricular mass and volumes in neonates at birth and at one month old, again due to the difficult anatomical location and complex anatomy of the right ventricle.[294] Again, estimates of

volume using this method correlated well with angiography, but this time there was a trend of under-estimation.[295] It is important to note that fetal echocardiography is known to be technically challenging and biplane views are not always feasible. Therefore using only the four chamber view, which is usually easily obtained and can be adequately visualised in 100% of fetuses by 13 weeks gestation,[296, 297] makes this method for determination of mass widely applicable.

4.6 Conclusions

This study has created, for the first time, fetal nomograms of ventricular mass and volume using 2D echo from 15 to 42 weeks gestation. My results suggest that current state-of-the-art 2D echocardiography ultrasound platforms and off-line analysis software provides accurate, feasible and reproducible measures that allow estimation of fetal ventricular mass in a wide range of gestations. In certain circumstances such as extremes of gestation, 2D ultrasound may be a modality of choice, and at other times provide a screening or monitoring test, after which other novel modalities may be used as an adjunct. The application and further validation of these nomograms within future clinical and research studies will be of value to fully evaluate their potential utility.

5: PRETERM BIRTH & OFFSPRING CARDIAC PHENOTYPE

In this chapter I will be exploring the effect of preterm birth on offspring cardiovascular phenotype. I will primarily be exploring differences in cardiac structure in preterm infants building on previous work from Professor Leeson's group which described unique changes in young adults born preterm. I will be investigating whether these changes are present at birth or whether they become apparent over the first three months of life. The work will also include studying whether there is a fetal component to any structural changes seen and finally if there are any related changes in cardiac function or ventricular shape.

5.1 Abstract

Background- Hearts of adults who were born preterm have an increased mass and reduced function. Premature exposure of the immature heart to *ex utero* physiological

stresses induces a similar phenotype in experimental models during early postnatal life. Therefore I studied whether babies born preterm exhibit a similar disproportionate cardiac hypertrophy.

Methods- Cardiac ultrasound imaging was performed on 392 infants (128 born preterm and 264 born at term) at different time points between 15 weeks gestation to three months of postnatal age to establish trajectories of *in utero* and postnatal cardiac structural and functional development. Left and right ventricular mass, size and function were quantified and geometric ventricular computational models created to identify shape changes. Differences were related to changes in body size as well as maternal, pregnancy and postnatal history.

Results- At birth, preterm offspring had a lower cardiac mass and volume, relative to body size, compared to those born at term, with a more globular heart. However, by three months, ventricular shape was similar between groups and both left and right ventricular mass indexed to body surface area were significantly higher than expected for post-menstrual age, exceeding values in three month old term born offspring (left p=0.001, right p=0.03). Increase in mass relative to body size was two-fold greater in preterm offspring (left 57.8±41.9% vs 27.3±29.4 %, p<0.001; right 39.3%±38.1 vs 16.6±40.8 %, p=0.002), with greater changes associated with lower gestational age at birth (left p<0.001; right p=0.001). Greater mass increase associated with reduced diastolic function (lateral E/E') by three months of age (p=0.01).

Conclusions- Preterm offspring have a disproportionate increase in left and right ventricular mass during the first three months of life consistent with cardiac

phenotypes seen in adults born preterm and experimental models. Postnatal life may provide an important window for interventions relevant to long term cardiovascular health.

5.2 Introduction

As adults, individuals born preterm have reduced left ventricular volumes and function with increased left ventricular mass.[298] Similar changes are observed in the right ventricle, but are significantly more pronounced, with preterm born adults having smaller right ventricular volumes, greater myocardial mass and significant impairment in right ventricular systolic function.[108] These cardiac changes may develop because preterm birth disrupts proper organogenesis through interruption of normal *in utero* development. Birth triggers phenotypic and functional changes in cardiomyocytes, which transition from a fetal hyperplastic to hypertrophic growth pattern.[96, 299] In experimental preterm models, exposure of these immature, hypertrophic-pattern cardiomyocytes to the high-resistance, postnatal, arterial circulation, in combination with the relative hyperoxia of the *ex utero* environment[299] results in disproportionate cardiac hypertrophy and fibrosis.[96, 299] Consistent with this hypothesis, the cardiac changes in both ventricles in adult life are proportional to the degree of prematurity.[108, 109] Therefore, to study whether there is evidence in humans for an early cardiac hypertrophic response to the *ex utero* environment in those born preterm, I performed fetal and neonatal echocardiography at different time points between early pregnancy and three months of postnatal life. The phenotypic switch in cardiomyocytes is thought to take place in the first two weeks of postnatal life.[300] I

therefore chose three months of age as the timing for the follow up assessment as at this point, even the most preterm in my cohort would be a few weeks post term equivalent age. I used this data to compare trajectories for change in cardiac mass, volumes and shape in preterm and term infants taking into account variation in growth and other perinatal factors.

5.3 Methods

5.3.1 Study population

This chapter includes data from infants in whom echocardiography was performed at birth and three months of age. In addition, results from echocardiographic datasets from fetuses which were acquired prior to birth are reported (Figure 5.1), including a subset who also had postnatal measures performed. Inclusion and exclusion criteria are detailed in Section 2.1 and 2.2 and diagnostic definitions in Section 2.1.2. Details of these cohorts and how they were recruited are set out in Chapter 2.

5.3.2 Study visit

5.3.2.1 Fetal Cohort

Mothers attended the research department and underwent the following assessments and analysis (Figure 5.1), which are described in detail in Chapter 3:

- Fetal anthropometry (Section 3.3.1)
    - Head circumference
    - Abdominal circumference

    - Femur length
- Fetal echocardiography (Section 3.4.1)
    - Cardiac image acquisition using cineloop
    - Cardiac structure
        - LV mass and volumes
        - RV mass and volumes
- Medical notes data extraction (Section 3.9)

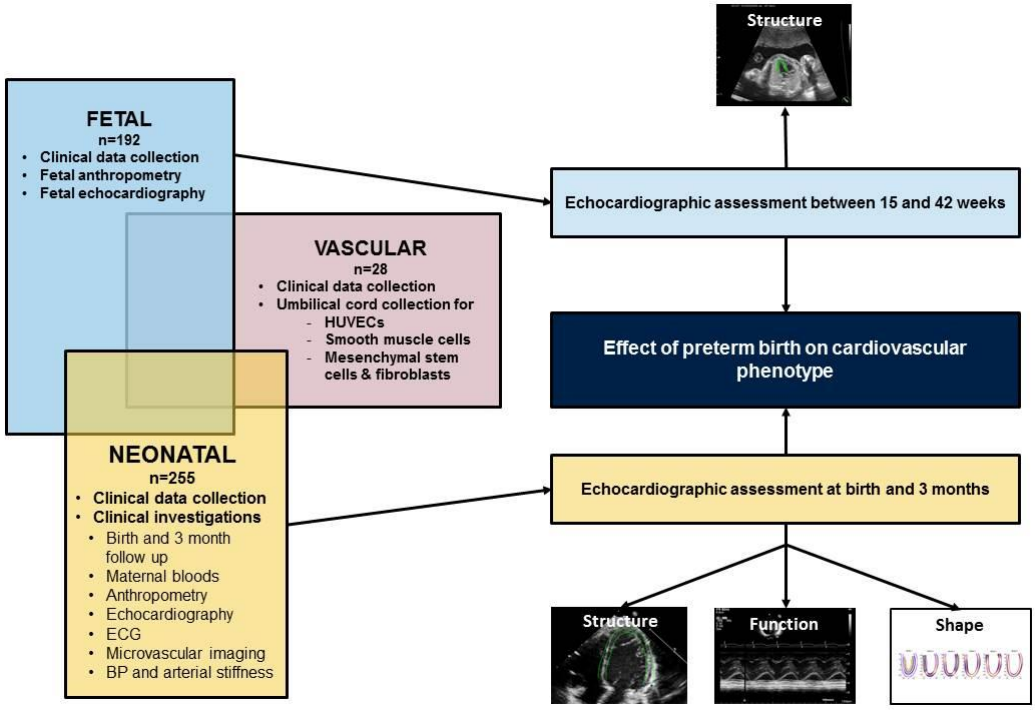
5.3.2.2 Neonatal Cohort

Mother and infants underwent the following assessments and analysis at birth and three months (Figure 5.1), which are described in detail in Methods (Chapter 3):

- Anthropometry (Section 3.3.2)
    - Weight
    - Head circumference
- Echocardiography (Section 3.4.1)
    - Cardiac structure
        - LV mass and volumes
        - RV mass and volumes
    - Cardiac function
        - LV systolic and diastolic function
        - RV systolic function
    - Cardiac shape analysis
        - LV shape

- Blood pressure (Section 3.6.1)
- Medical Records and Questionnaires (Section 3.9 and Appendix)

Figure 5.1: Overview of fetal and neonatal research programme performed between 2011 and 2015 within Oxford with specific data collections used for cardiac analysis identified in the right column.



5.3.2.3 Additional methods - Cardiac shape analysis

Cardiac shape analysis was performed in collaboration with Dr Pablo Lamata, Department of Biochemical Engineering, King's College London. The morphology of the left ventricle in the four chamber view was analysed through the construction of a computational statistical shape model, through adaptation of a technical approach we have previously described for three dimensional medical images. The endocardial and epicardial contours were exported from TomTec Image Arena 4.6 as text files that encode these contours and were fitted to a parametric description of a line. As a

result, each contour was described with 52 coefficients, with each ventricle in the four chamber view therefore being reported using 104 coefficients (two contours, endocardium and epicardium). The left ventricles were aligned to their centre of mass and set in vertical direction by the line that joins the centre of the base and the apex. The mean shape in the population was calculated, and a Principal Component Analysis (PCA) of all cases was performed, finding the main modes of anatomical variation. The analysis was performed using a set of functions developed in Matlab for this purpose (Mathworks, Natick, Massachusetts, U.S.A.).

Although this was using a two dimensional view to produce information on shape, we had previously performed an analysis on a subset of my data where two, three and four chamber views of the same ventricle were available. We found that the vast majority of shape variation was captured by using the four chamber view alone. As two and three chamber views are not always easily acquired in the infant population, in order to minimise data loss due to incomplete datasets, we decided to proceed with shape analysis using only the four chamber view.

5.3.3 Statistics

Statistical analysis is described in detail in Section 3.10.

5.3.3.1 Additional statistics - Growth trajectories

To further explore the timing of mass and volume changes in preterm infants, I studied trajectories of cardiac development using the combined dataset of echocardiographic measures of LV and RV mass at different time points during fetal and neonatal life

again in collaboration with the statistician Dr Eric Ohuma. As volumes pre and postnatally would not be comparable due to circulatory changes at birth, I limited analysis to mass. STATA, version 11.2, software (StataCorp LP, College Station, Texas, USA) was used to create smoothed centiles of right and left ventricular mass, left and right EDV and ratio of mass as a function of head circumference (HC) using fractional polynomials according to gestational age. To account for body size differences between preterm and term infants at different time points, we adjusted the left and right ventricular mass for head circumference (i.e. left ventricular mass / HC) on the basis that this measure would be the most consistently accurately, quantifiable body measure throughout fetal and neonatal life unlike weight or body surface area which are more standard measures used in the literature. This is because weight cannot be directly or accurately measured in fetal life and therefore pre and postnatal values cannot be directly compared. In addition, equations for body surface area have not been validated for fetuses. Where appropriate, we applied a multi-level, linear regression analysis to account for repeated measures[273] but there were insignificant differences when compared to analyses that did not account for the hierarchy of the data. Left and right mass, volume and ratio of mass as a function of HC exhibited a non-normal distribution; therefore, the data were log-transformed (natural log) to stabilise variance and transform the data to normality. Goodness-of-fit assessment incorporated a visual inspection of the quantile-quantile (q-q) plot of the residuals and a plot of fitted z-scores across gestational ages.

5.3.3.2 Additional statistics - Cardiac shape analysis

Analysis of shape variation between preterm and term infants was performed by a non-paired t-test. Shape differences were first tested in each PCA mode under three conditions (1) at birth, (2) at follow up or (3) by their growth. Then, the PCA modes, which accounted for the most variation within the experimental sample, were combined through a linear discriminant analysis (LDA) in an attempt to identify the morphological signature of a premature birth. Cross-validation (leave-1 out) was used to test the generality of differences found in the LDA, and to select the optimal set of PCA modes to be combined to differentiate groups.

5.3.3.3 Power calculation

The sample size n=134 for term offspring and n=121 preterm offspring provided me with 80% power at a significance level of α=0.05 to detect a difference of at least 0.38 standard deviations (SDs) between groups in left ventricular mass index at 3 months.

5.4 Results

5.4.1 Study population characteristics

Maternal and offspring demographic and anthropometric characteristics in the preterm and term groups are presented in Table 5.1. The mothers who had preterm deliveries had a similar body mass index (BMI) at pregnancy booking and prevalence of smoking but were on average a year older and were more likely to have had a hypertensive pregnancy disorder and a caesarean delivery (p<0.001). There was no bias by gender and birth order for term and preterm deliveries. Gestational age in the

preterm group was six weeks younger than the term group (34.0±2.2 vs 39.7±1.3 weeks) and they were significantly lighter at birth, with a lower birthweight z-score. Those with postnatal measures had similar characteristics to the full study group at birth (Table 5.2) and still had significantly lower weight and smaller head circumference at three months. Their blood pressure was also lower at birth but by three months only diastolic blood pressure was lower.

Table 5.1: Maternal, fetal and postnatal characteristics

	Preterm (n=128)	Term (n=264)
Maternal Demographics & Anthropometrics		
Maternal age at delivery, years	32.7±5.7	31.7±5.2*
BMI at booking, kg/m^2	25.4±5.1	24.9±4.5
Smokers, n (%)	11 (9)	22 (8)
Maternal hypertension during pregnancy, n (%)	70 (55)	99 (38)***
Offspring Birth Characteristics		
Gestational age at delivery, weeks	34.0±2.2	39.7±1.3
Males, n (%)	65 (51)	124 (47)
Birth order †	1 (1)	1 (1)
Caesarean section, n (%)	78 (61)	59 (22)***
Birthweight, grams	2074±578	3360±507***
Birthweight z-score	-0.38±1.1	0.20±1.0***
Persistent patent ductus arteriosus, n (%)	2 (2)	0 (0)
Offspring Physiological Measures at Birth	(n=106)	(n=121)
Age at assessment, days	6.6±5.4	4.0±5.5***
Weight, grams	2053±587	3315±563***
Head circumference, cms	30.7±2.4	34.5±1.6*
sBP, mmHg	74.1±14.7	81.6±13.4***
dBP, mmHg	40.9±9.9	45.1±9.4***
Offspring Physiological Measures at 3 months	(n=106)	(n=123)
Age at assessment, days	99.1±15.1	98.0±13.8
Weight, grams	4960±967	6051±894***
Head circumference, cms	39.1±2.1	40.8±1.7***
sBP, mmHg	93.7±12.2	96.8±12.4
dBP, mmHg	50.0±12.4	54.4±12.2**

† Median±Interquartile range

p values that are statistically significant are asterisked. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

BMI indicates Body Mass Index

Table 5.2: Comparison of full and neonatal cohort characteristics

	Full Cohort		Neonatal Cohort	
	Preterm (n=128)	**Term (n=264)**	**Preterm (n=121)**	**Term (n=134)**
Maternal Demographics & Anthropometrics				
Maternal age at delivery, years	32.7±5.7	31.7±5.2	33.0±5.7	32.3±5.3
BMI at booking, kg/m^2	25.4±5.1	24.9±4.5	25.5±5.1	25.7±6.8
Smokers, n (%)	11 (9)	22 (8)	7 (6)	4 (3)
Maternal hypertension during pregnancy, n (%)	70 (55)	99 (38)	70 (58)	81 (60)
Offspring Birth Characteristics				
Gestational age at delivery, weeks	34.0±2.2	39.7±1.3	33.9±2.2	39.4±1.3
Males, n (%)	65 (51)	124 (47)	60 (50)	59 (44)
Birth order, n (%)	1 (1)	1 (1)	1 (1)	1 (1)
Caesarean section, n (%)	78 (61)	59 (22)	77 (64)	36 (27)
Birthweight, grams	2074±578	3360±507	2053±587	3315±563
Birthweight z-score	-0.38±1.1	0.20±1.0	-0.38±1.1	0.16±1.1
Persistent Patent ductus arteriosus, n (%)	2 (2)	0 (0)	2 (2)	0 (0)

337 echocardiographic datasets were acquired during fetal life of which images were suitable for measurement of LV mass in 318 and RV mass in 323. Of 229 infants in whom echocardiography was performed at birth, 211 had LV measures included in analysis (17 unanalysable due to infant movement, 1 excluded due to subsequent diagnosis of Turner's syndrome). 227 attended for scan at three months of age of which 213 had analysable images, with 183 babies having LV mass estimations at both time points. Image acquisition was optimised for LV assessment but of the available scans at birth, 127 had analysable RV views with 81 infants having measures at both

time points. To investigate change in mass over the first three months of life analysis was restricted to the group with measures at both time points. For trajectory changes, all analysable echocardiographic data were included and statistical approaches used as appropriate to allow for missing values in longitudinal datasets.

5.4.2 Postnatal increase in ventricular mass in preterm born infants

Infants born preterm had a smaller LV mass at birth compared to term born infants. This difference persisted even when indexed to body surface area (Table 5.3) and they also had smaller end diastolic volume (EDV) and EDV index at birth. This meant there was no significant difference in mass/EDV ratio compared to the term group, consistent with a small but proportional cardiac structure at birth (Table 5.3). By 3 months of age, preterm infants still had smaller LV EDV but this was no longer different indexed to body surface area. Furthermore, there was no significant difference in absolute LV mass and, as a result, left ventricular mass index (based on either body surface area or EDV) was now significantly greater in the preterm group (Table 5.3). The percentage postnatal mass change in the preterm group was more than double that in the term group (change in LV mass index 57.8±41.9 vs 27.3±29.4 %, p<0.001). Disproportionate myocardial changes were also evident in the linear measures of wall thickness. Preterm offspring had smaller interventricular septal (IVS) thickness at birth but similar thickness at 3 months, compared to term born infants, with a significantly greater posterior wall thickness (PWd) (Table 5.3). These findings were also supported by the subgroup analysis of 136 infants who had measurements of LV mass at birth and three months estimated by the ASE-recommended method.[301] The preterm group

(n=73), again showed an LV mass change double that of their term counterparts (n=63) (170.2±95.1 vs 88.9±60.9%, p<0.001).

Similar results were found for the RV, with the preterm group having a lower mass and EDV when compared to term born infants at birth but significantly increased RV mass indices by three months of age. Interestingly, preterm neonates already had an increased RV mass/EDV ratio at birth (Table 5.3) but there was still a significant two-fold greater change in RV mass over the first three months of life (39.3±38.1 vs 16.6±40.8 %, p=0.002).

The percentage change in both LV and RV mass index during the first three months of postnatal life varied with gestational age at birth (LV mass index change r= -0.49, p<0.001; RV mass index change r=-0.37, p=0.001 and Figure 5.2A). Gestational age was the main predictor of mass change in multivariate models, which took account of maternal pregnancy hypertension, birthweight z-score, 5 minute APGAR score and mode of delivery (Tables 5.4 and 5.5). In addition, although birthweight z-score was significantly, positively correlated with gestational age (r=0.23, p<0.001), it was an independent predictor of left ventricular mass change (Tables 5.4 and 5.5). Interestingly, babies born by caesarean sections had lower AGPAR scores across gestation (p<0.001) and for the preterm group alone (p=0.003). Both parameters were associated with mass change, although neither were significant in multivariable models (Tables 5.4 and 5.5). Similar patterns of mass change were seen in both genders. There were insufficient numbers of infants with persistent patent ductus arteriosus to investigate any additional impact on mass change.

5.4.3 Mass change relative to normal cardiac growth trajectories

I additionally studied whether there was a fetal component to the differential cardiac development in preterm offspring based on the combined dataset of echocardiographic measures of LV and RV mass at different time points from 15 weeks of fetal life to 3 months postnatal life. The growth trajectory for preterm offspring datasets was overlaid onto the fetal and neonatal cardiac growth pattern of term born infants (Figure 5.2B). All preterm measures at birth fell below the 95th centile for expected cardiac mass based on gestational age with mean z-scores within the group modelled from the expected cardiac fetal mass being -0.16±0.53 for the LV and -0.13±0.50 for the RV. By follow up, however, several of the preterm infants were exceeding the 97% centile for expected cardiac mass with an absolute difference in the 50th centile at 49 weeks postmenstrual age (3 months of postnatal age for a baby born at term) between preterm and term LV mass being 1.61g. To allow for change in body size during this period, trajectories were created based on LV and RV mass indexed to head circumference,[233] and these showed similar patterns to the absolute measures of mass (Figure 5.2C).

Table 5.3: Cardiac structure and function at birth and 3 months

	Birth			Follow Up		
	Preterm	**Term**	***p* value**	**Preterm**	**Term**	***p* value**
Left Ventricle						
Volumes	n=93	n=118		n=101	n=111	
EDV (ml)	2.7±1.0	4.2±1.2	**<0.001**	8.3±2.1	9.2±2.0	**0.001**
EDV Index (ml/m^2)	16.8±5.5	18.5±4.3	**0.002**	27.6±5.8	26.8±5.0	0.32
ESV (ml)	1.1±0.4	1.6±0.7	**<0.001**	3.5±1.0	3.7±1.0	0.19
ESV Index (ml/m^2)	7.0±2.4	6.8±2.6	0.40	11.7±3.3	10.9±2.7	0.12
Mass						
IVS diameter (cms)	0.33±0.08	0.39±0.08	**<0.001**	0.44±0.08	0.43±0.08	0.29
PWd (cms)	0.29±0.06	0.30±0.07	0.41	0.38±0.07	0.35±0.06	**0.007**
Mass (g)	3.1±1.0	4.7±1.2	**<0.001**	8.6±1.7	8.9±1.8	0.25
Mass Index (g/m^2)	18.8±3.9	20.7±3.9	**0.001**	29.2±6.5	26.0±4.8	**<0.001**
Mass/EDV	1.2±0.3	1.2±0.2	0.38	1.1±0.3	1.0±0.2	**0.005**
Function						
Systolic Function	n=72	n=95		n=62	n=66	
Ejection fraction (%)	59±8	64±8	**<0.001**	58±7	60±8	0.39
Stroke Volume (ml)	1.7±0.7	2.7±0.7	**<0.001**	4.8±1.2	5.7±1.5	**0.001**
Diastolic Function	n=95	n=122		n=90	n=109	
EA	1.0±0.2	1.0±0.3	0.45	1.1±0.2	1.0±0.2	0.31
Lateral E’	6.1±1.8	6.6±1.7	**0.02**	9.4±2.3	10.2±2.2	**0.01**
Lateral E/E’ ratio	8.5±2.5	7.9±2.5	0.06	10.1±2.7	9.4±2.7	**0.03**
Right Ventricle						
Volumes	n=51	n=76		n=67	n=75	
EDV (ml)	1.9±0.9	3.4±1.5	**<0.001**	4.7±2.0	5.3±1.8	0.09
EDV Index (ml/m^2)	11.1±4.4	14.7±5.7	**<0.001**	15.4±5.7	15.2±4.4	0.59
Mass						
Mass (g)	2.8±1.2	4.1±1.2	**<0.001**	6.5±2.2	6.8±1.8	0.24
Mass Index (g/m^2)	16.3±5.6	17.9±4.3	0.08	21.5±6.2	19.3±4.5	**0.03**
Mass/EDV	1.6±0.6	1.4±0.5	**0.03**	1.5±0.5	1.4±0.6	**0.04**
Function	n=83	n=118		n=92	n=117	
TAPSE	0.7±0.2	0.9±0.2	**<0.001**	1.4±0.3	1.5±0.3	**0.007**

EDV indicates end-diastolic volume; ESV end-systolic volume; LVIDd left ventricular internal diameter in diastole; IVS intraventricular septum; PWd posterior wall in diastole

Figure 5.2: (A) Tukey boxplots showing (i) left and (ii) right ventricular mass index change between birth and three months is significantly greater with increasing prematurity using a one-way ANOVA. (B) Trajectories of (i) left and (ii) right ventricular mass from 15 weeks gestation through to 3 months postnatal life for term (red with grey points) and preterm (blue with blue points) infants demonstrating a relative cardiac hypertrophy of the preterm group in postnatal life. (C) Trajectories of (i) left and (ii) right ventricular mass from 15 weeks gestation through to 3 months postnatal life indexed to head circumference for term (red with grey points) and preterm (blue with blue points) infants demonstrating a similar increase in mass in the preterms *ex utero* despite the correction for size. Dashed lines indicate the 3rd, 50th and 97th centiles. LV indicates left ventricular; RV right ventricular; HC head circumference.

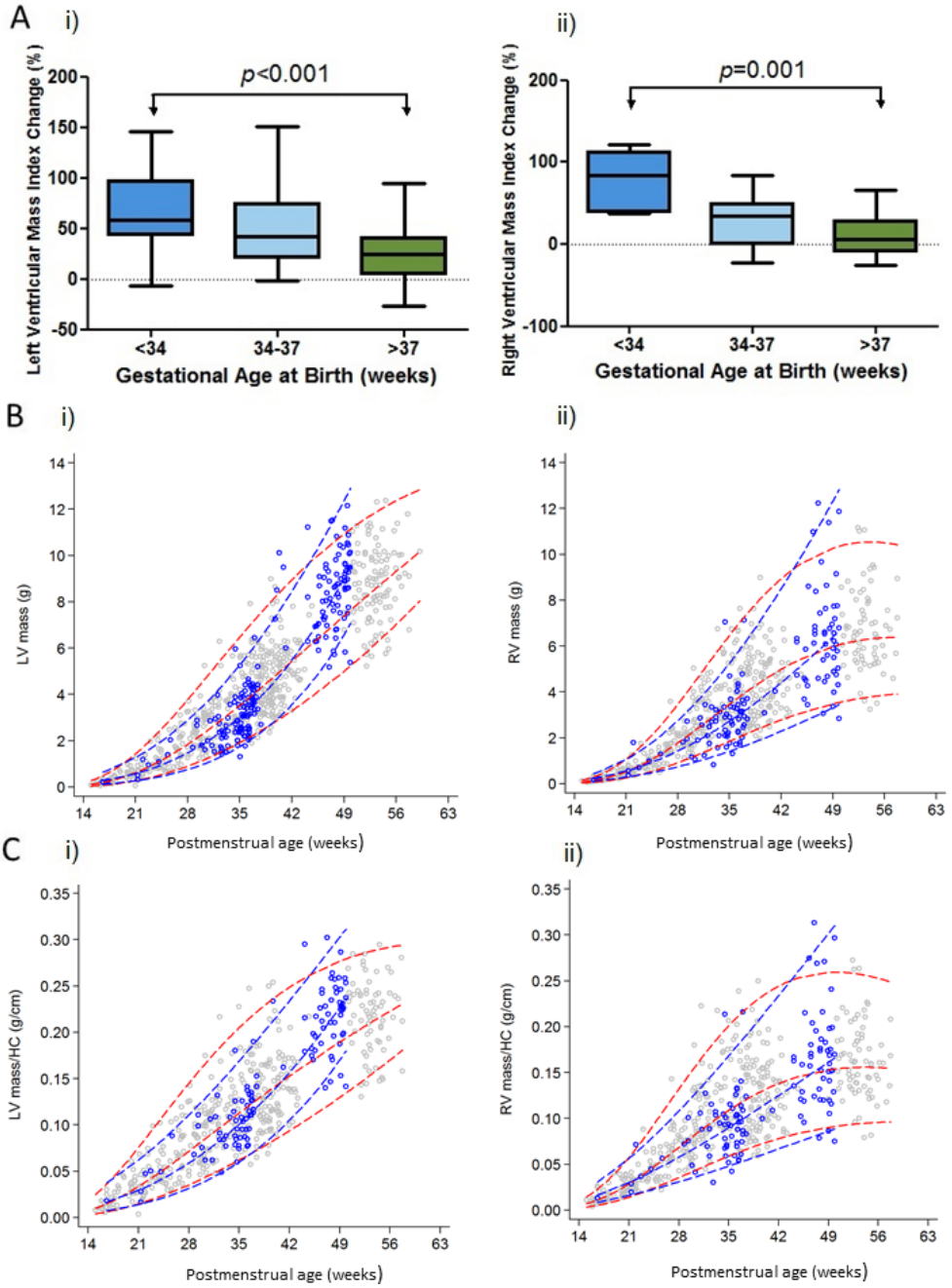


Table 5.4: Bivariate regression coefficients for maternal and perinatal characteristics and change in ventricular mass

	Change in LVMI (%)			Change in RVMI (%)		
	B	**95% CI**	***p*-value**	**B**	**95% CI**	***p*-value**
Maternal Factors						
Age at delivery	0.70	-0.37 - 1.76	0.20	-0.15	-1.79 - 1.49	0.86
BMI at booking	0.03	-1.07 - 1.14	0.95	0.57	-1.09 - 2.22	0.50
Smoking	16.87	-17.60 - 51.34	0.34	-4.89	-39.78 - 30.00	0.78
Maternal hypertension	10.93	-0.42 - 22.27	0.06	18.72	0.85 - 36.59	**0.04**
Perinatal Factors						
Gestational age, weeks	-6.02	-7.62 - -4.43	**<0.001**	-5.23	-8.22 - -2.25	**0.001**
Caesarean section	19.56	8.53 - 30.59	**0.001**	25.67	7.64 - 43.71	**0.006**
Birthweight z-score	-9.53	-14.26 - -4.80	**<0.001**	2.34	-6.49 - 11.16	0.60
Sex	-3.11	-14.41 - 8.19	0.59	8.01	-10.20 - 26.22	0.38
APGAR score (5mins)	-7.49	-14.95 - -0.04	**0.05**	-7.46	-18.53 - 3.61	0.18
Antenatal steroid exposure	32.07	21.17 - 42.97	**<0.001**	33.45	13.98 - 52.92	**0.001**
Patent ductus arteriosus	*	*	*	*	*	*
Days of ventilation	12.24	-6.31 - 30.79	0.19	*	*	*

*Not enough cases to compute coefficients

BMI indicates Body Mass Index; HTN hypertension

Table 5.5: Multivariable regression coefficients for maternal and perinatal characteristics and change in ventricular mass

	B	95% CI	*p*-value
Change in LVMI (%)			
Maternal hypertension	6.46	-3.77 - 16.69	0.21
Gestational age, weeks	-5.14	-6.93 - -3.35	**<0.001**
Caesarean section	-5.90	-16.60 - 4.81	0.28
Birthweight z-score	-4.66	-9.17 - -0.14	**0.04**
APGAR score (5mins)	-0.40	-7.27 - 6.47	0.91
Change in RVMI (%)			
Maternal hypertension	14.01	-3.08 - 31.09	0.11
Gestational age, weeks	-4.41	-7.43 - -1.40	**0.005**
Caesarean section	14.96	-3.36 - 33.28	0.11

LVMI indicates left ventricular mass index; RVMI right ventricular mass index;

5.4.4 Altered left and right ventricular systolic and diastolic function

I then evaluated whether there were changes in function related to preterm birth. In the LV, there was reduced stroke volume and ejection fraction at birth in the preterm group related to both the reduction in EDV index and an increase in end systolic volume (ESV) index. LV stroke volume, but not ejection fraction, remained reduced at three months of age (Table 5.3 and Figure 5.3A). In the right ventricle, tricuspid annular plane systolic excursion (TAPSE), a measure of RV systolic function, was reduced at both birth and three months in the preterm group (Table 5.3 and Figure 5.3B). For diastolic function parameters at birth there was a significant reduction in lateral E' but, although lateral E/E' ratio was greater, the difference was not significant.

By three months this difference had increased so that the lateral E/E' ratio was significantly higher in the preterm group (Table 5.3 and Figure 5.3C). Interestingly, the increase in lateral E/E' was proportional to the increase in LV mass index change between birth and three months in this group (r=0.21 p=0.01).

5.4.5 Shape changes during postnatal life

I then studied whether there were changes in ventricular shape during the postnatal period to suggest a unique preterm abnormality in cardiac development. The major mode that differentiated between groups was, as expected, a general size mode, (mode 1). Analysis confirmed the significant difference in size at birth with convergence to a similar size by three months (Figure 5.4A). To study specific shape changes, this size mode was removed from analysis and a linear discriminant analysis used to identify the optimum number of further modes which accounted for the majority of the rest of the shape variation between groups. Modes from 2 onwards were combined in an increasing fashion. Further modes beyond mode 6 did not increase the area under the curve for differentiation of preterm and term groups and the five modes (2-6) all persisted in the cross-validation test (Figure 5.4B). Collectively, these described variation between a 'globular' and a 'conical' heart (Figure 5.4C). At birth, the preterm heart tended to be more globular with a slightly narrower mitral annulus relative to mid-ventricular width (p<0.001) but this difference between groups had disappeared by three months of age (p=0.24).

Figure 5.3: (A) Image demonstrating the technique used to measure ejection fraction by contouring the endo and epicardial border in the four chamber view using TomTec Image Arena 4.6 Ejection fraction is significantly higher in the term (green) compared to the preterm (blue) infant at birth, but there is no significant difference by 3 months of age. (B) Examples of M-mode measurements of tricuspid annular plane systolic excursion (TAPSE) in term (left) and preterm (right) infants at 3 months of age. TAPSE is significantly reduced in the preterm group both at birth and at 3 months old. (C) Examples of doppler interrogation of the lateral mitral valve annular using Tissue Doppler Imaging in early diastole (E') in term (left) and preterm (right) infants and their corresponding Pulsed wave Doppler from the mitral valve tips to assess early and late diastolic inflow (E/A ratio) at 3 months of age. This demonstrates a decreased lateral E' in the preterm infant which contributes to a significantly increased lateral E/E' ratio at 3 months in the preterm group. Error bars represent the standard error of the mean. *p<0.05; **p<0.01; ***p<0.001

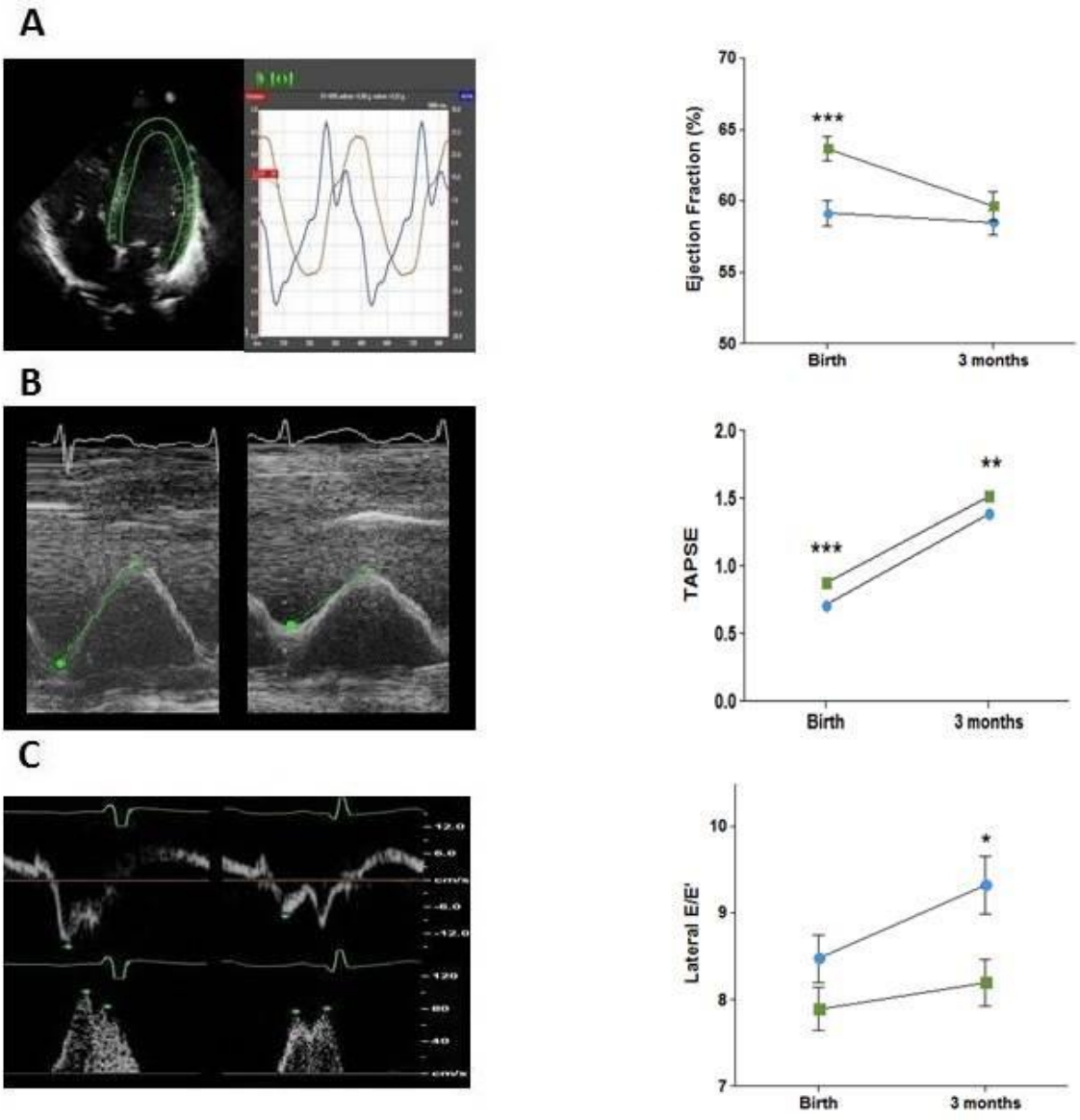
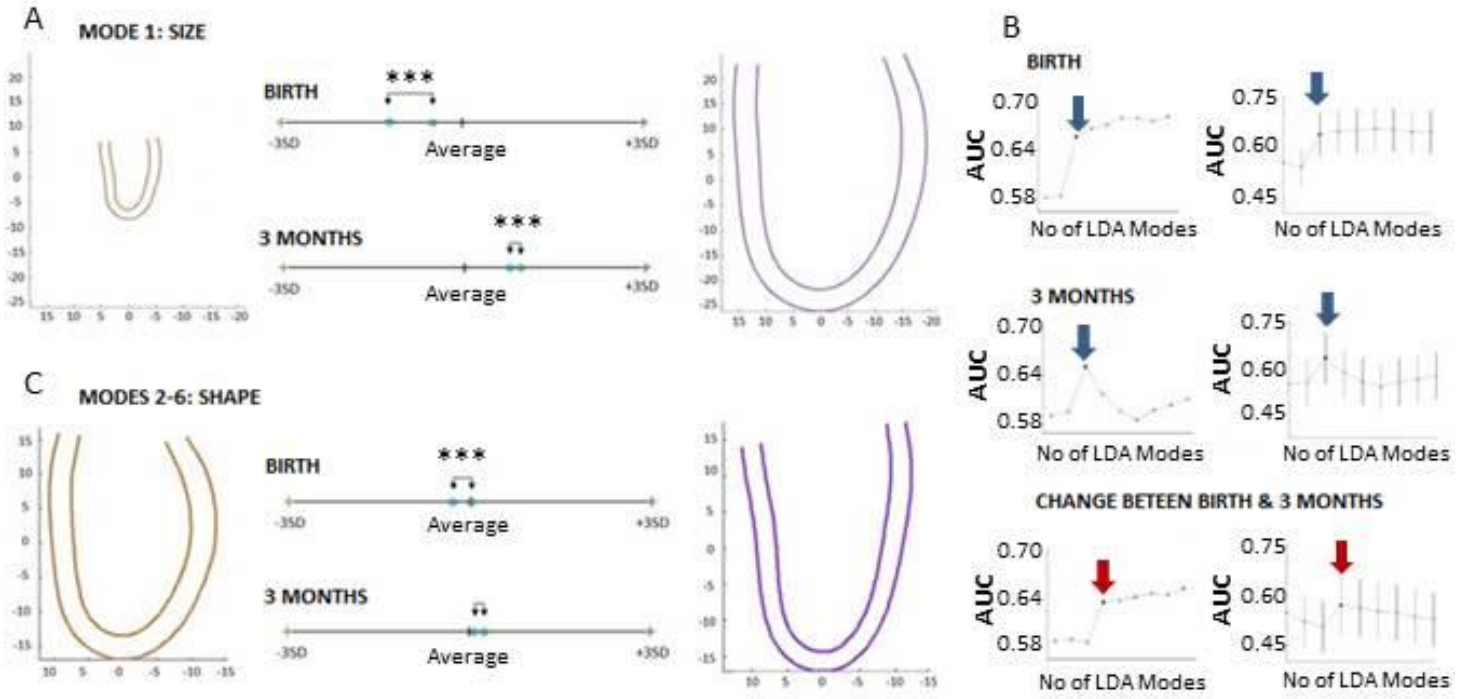


Figure 5.4: (A) Significant shape differences between term (green) and preterm (blue) infants at birth are mainly accounted for by size with convergence by three months. (B) Area Under the Curve using Linear Discriminant Analysis from mode 2 onwards including a cross-validation test. (C) Shape variations using modes 2-6 in a linear discriminant analysis. Brown and purple contours demonstrate -3 and +3 standard deviations away from the mean. Boxplots indicate where groups lie on this scale with dots, whiskers and circles indicating the median, range and outliers respectively; term in green and preterm in blue. AUC indicates Area Under the Curve; PCA Principal Component Analysis; LDA Linear Discriminant Analysis. ***p<0.001



5.5 Discussion

This study shows infants born preterm have a greater increase in both left and right ventricular mass over the first three months of life, disproportionate to increases in body and total cardiac size. The average mass change in our cohort was double that of their term-born counterparts, with the degree of change proportional to their degree of prematurity. A reduction in left ventricular diastolic function also emerged during these three months with a persistent reduction in right ventricular systolic function. Our study included a high proportion of late preterm infants which account for 75% of preterm births, suggesting the findings are of relevance to a large cohort of individuals.

5.5.1 Previous studies

The increase in ventricular mass at three months of age is strikingly similar to our previous reports of increased left and right ventricular mass in adults born preterm.[108, 109] Increased intraventricular septal thickness compared to expected reference values has been reported in children born preterm[65] and my current findings extend observations back to the neonatal period. Based on prospective measures I demonstrate that patterns of cardiac hypertrophy, similar to those observed in adult life, are not evident at birth but emerge during the early postnatal period. Some studies report 'term equivalent' comparisons rather than post-delivery age but as I was able to develop term data, chose to model preterm trajectories instead which confirmed the postnatal pattern of increased mass. In fact, the 0.3g difference in left ventricular mass between groups at three months of age was substantially smaller than the 1.6g difference between the two modelled 50th centiles at 49 postmenstrual

weeks, suggesting that if I had used prematurity-corrected ages, greater differences may have been seen between groups. However, longitudinal follow up is required to confirm this.

The importance of the postnatal period for cardiac hypertrophy in preterm offspring is also seen in experimental models. A preterm-born sheep model had a five- to seven-fold increase in cardiomyocyte hypertrophy during postnatal life, with increased interstitial myocardial fibrosis and altered cardiac maturation, demonstrated by an increase in nuclear ploidy and increased number of bi- and tri-nucleated cells.[96] Similarly, a rat model of preterm birth conditions demonstrated increased left ventricular hypertrophy during the postnatal period and, furthermore, that these changes progressed to heart failure in later life when challenged with low dose angiotensin II infusion and exposure to hypertension.[97]

5.5.2 Cardiac structural differences in preterm infants

Cardiac growth *in utero* is relatively proportional to fetal growth[274] and an M-mode echocardiography study of healthy newborns found body size at birth correlated with cardiac dimensions.[302-304] The absolute measures of cardiac size at birth in my cohort were similar to those previously reported in babies of similar size, born at similar and different gestations.[303, 304] I also found that variation in cardiac mass in my preterm cohort was proportional to ventricular size and that these measures fell within normal ranges for postmenstrual age on my fetal nomograms. Therefore, if there were significant fetal influences on preterm cardiac development, they appear to be subtle. Although mass was increased at three months, at birth the preterm infants in my

cohort tended to have slightly lower left ventricular mass index compared to term infants. One explanation may be the use of the Boyd formula, which is thought to overestimate body surface area[305] and would tend to lead to overcorrection of indexed measures in smaller infants, although use of this formula, based on weight alone, removed methodological inaccuracies in measurement of length in a baby. Alternatively, the difference may reflect the different developmental stage of the preterm infant compared to the term infant, in particular, the variation in dominance of right and left circulations that may mean the left heart is relatively less developed. *In utero*, the fetus is exposed to a right ventricular dominant circulation and fetuses up to 2.7kg in estimated body weight have a right ventricular wall thickness greater than the left ventricle.[306] This association changes as fetal bodyweight increases and there is a trend towards a reduction in right ventricular mass compared to left ventricular mass toward term[274] with further falls in right ventricular mass index postnatally in term neonates.[294, 307] At birth my preterm group had an average weight of 2.1kg and therefore would be at an earlier stage, with a more prominent right compared to left dominance. This fits with the observation of similar right ventricular mass index at birth in my cohort compared to a significant reduction in left ventricular mass index.

This right ventricular dominance at birth might also be expected to impact on cardiac shape. Left ventricular shape was analysed in my cohort using a novel adaptation of a computational atlas approach we have previously used for three-dimensional images.[109] Application of this technique to ultrasound images proved highly effective as it was able to identify that those born preterm had a more globular shape left ventricle at birth but that these differences were lost by three months of age. Globular

shaped left ventricles are seen in those with significant growth restriction[178] but this was not evident in my preterm cohort. The more likely explanation is that the difference reflects their cardiac developmental stage and relative right ventricular dominance at birth leading to altered left ventricular shape, which resolves following the switch to the systemic dominant circulation in postnatal life.

Preterm birth is associated with a degree of postnatal catch up growth in body size[308] and change in cardiac volumes over the first few weeks of life in very preterm infants, as also seen in my cohort, has been shown to relate to size at birth.[309] Therefore, for my neonatal analysis I indexed for individual growth trajectories across my cohort of early and late preterm babies. Neither indexing for body surface area in my neonatal comparisons nor indexing for head circumference, a good marker of skeletal growth,[233] in my fetal to neonatal comparisons attenuated the large change in both LV and RV mass seen in the preterm group related to gestational age.

5.5.3 Influence of other perinatal factors

Other perinatal factors linked with preterm birth might have influenced the disproportionate increase in cardiac mass. The use of antenatal steroids was not included in the multivariable model due to the significant co-linearity between the two variables. However, I cannot be sure that steroid use does not have an effect on LV and RV mass. Multivariable analyses highlighted a small independent influence of birthweight z-score on the left ventricle, consistent with a previous finding of mass increase proportional to birth-size in very preterm infants.[309] Maternal hypertension was associated with right ventricular mass, which requires further investigation, but

this effect was not significant once gestational age and mode of delivery were put in the model. The associations between birth by caesarean section, a low APGAR score and a greater increase in mass are intriguing. Although, again, these variables were not significant in multivariable analyses, it might be hypothesized that these babies might have been exposed to *in utero* hypoxia which has been shown to cause cardiac remodelling in animal models.[310] Blood pressures in the cohort were appropriate for gestational age[311] and with diastolic blood pressure still lower at three months in the preterm group as expected[312] and therefore could not explain the hypertrophy. The mass change was also independent of a range of other key potential perinatal factors and was similar in both males and females.

5.5.4 Functional differences in preterm infants

A finding of potential relevance to the cardiac hypertrophy was that both right ventricular global function, as measured by TAPSE, and left ventricular volumetric measures of function, were reduced at birth in my preterm group. My values match those previously reported for the first few days of life for preterm and term infants,[303, 313] with the majority of the variance in right ventricular function being explained by gestational age alone in a previous study.[313] This may reflect a relative immaturity of the myocardium in preterm infants rather than birthweight, as groups of term newborn infants of differing birthweights have been demonstrated to have similar left ventricular function.[304] Content of contractile elements is known to be reduced in the myocardium in preterm models and myofibril shortening is less efficient due to differences in calcium homeostasis.[314-316] Previous studies have shown improvements

by 28 days[317] and, by three months, left ventricular ejection fraction was similar to term born infants in my cohort. This normalisation of left ventricular systolic function may partly be explained by the disproportionate cardiac hypertrophy observed in preterm offspring during postnatal life, which could reflect a compensatory response of the impaired myocardium to maintain cardiac output. However, this would appear to be at the expense of a reduction in left ventricular diastolic function due to the associated structural changes[314, 318] and is not mirrored by improvements in RV function, which remains reduced up to three months of age.

5.5.5 Future work

Longitudinal follow up of my cohort will be important to understand whether the changes in cardiac structure and function in these individuals persist into later childhood and adolescence. If so, then the changes observed may be of direct relevance to the increased cardiac mass present in adults born preterm and highlight a critical postnatal window that could be targeted to prevent later disease. Our group have recently shown extreme nutritional variations during this period, including exclusive human milk consumption and intravenous lipids, can predict adult cardiac outcomes.[160,158] However, although these interventions modified cardiac chamber size and function, our group have not identified one that influences the cardiac hypertrophy in adults born preterm. In experimental models, pharmacological interventions during the postnatal period have been shown to normalise some features of the cardiac phenotype.[97] As those born preterm display a specific limitation in exercise capacity[319, 320] as well as significantly higher blood pressures in later life,

early postnatal interventions to improve myocardial development and health may be warranted.

5.5.6 Limitations

In the neonatal cohort alone, due to stratified recruitment, the incidence of maternal hypertension was similar between groups which meant my term "control" group does not represent normative data from uncomplicated pregnancies. However, it does limit the extent to which hypertension is acting as a confounding factor in my study. Also, due to the addition of datasets from uncomplicated pregnancies in the fetal cohort there was a greater proportion of hypertensive pregnancies in the preterm group when modelling cardiac growth trajectories this therefore needs to be taken into account during interpretation.

Other limitations of the study include the fact that I used an automated computer software package to derive left and right mass and volume measurements based on a single plane 4 chamber view which would result in significant geometrical assumptions. Neonatal and especially fetal echocardiography is technically challenging and biplane views are not always feasible. Therefore using only the four chamber view, which is usually easily obtained and can be adequately visualised in 100% of fetuses by 13 weeks gestation,[296, 297] allowed me to collect a large number of datasets using the same method from early fetal life through to 3 month postnatal age. In addition, in my previous chapter, I have shown that fetal estimates of mass and volume are in good agreement with more 3D and 4D methods up to 28 weeks gestation and may even be more accurate than these more novel methodologies

(Chapter 4). I also managed to replicate our findings using linear measurements of wall thickness and a subgroup analysis using the ASE-recommended method for mass determination.

I also used this same algorithm, which is designed for the left ventricle, to compute mass and volume estimations for the right ventricle. There are currently no clear recommendations regarding the optimal method for calculating mass measurements in the right ventricle due to its complex shape. Therefore, absolute values of RV mass and volume in our study need to be interpreted with caution and validated with future studies using for example magnetic resonance imaging. However, I used the same technique to produce estimates throughout my study with good reproducibility (Table 3.1) so the difference in growth patterns observed is most likely a true finding. A similar single-plane method has also been used in a small, previous study measuring right ventricular mass and volumes in neonates at birth and at one month old due to its complex shape and anatomy, with estimates very similar to those from my cohort.[294]

Another limitation was that longitudinal echocardiographic measurements were not possible in my entire cohort, with some loss to follow up. This is unfortunately a problem with recruiting from a tertiary centre, with many babies being transferred to local hospitals and living too far away to return for reassessment. However, a high percentage of the scans that I did acquire were analysable (94% and 93% for fetal and neonatal cohorts respectively) and the number of babies with longitudinal neonatal follow up was high. Finally, only 55 infants had both antenatal and postnatal

measurements performed when ideally each fetus should have been tracked across gestation and into postnatal life. However, data comparing the full and neonatal cohorts suggest minimal differences between them and all mothers were selected from the same population setting.

5.6 Conclusions

Preterm infants undergo a disproportionate increase in left and right ventricular mass in the postnatal period, over and above what would be expected *in utero,* which is associated with diastolic dysfunction and a persistent reduction in right ventricular function. These changes reflect those observed in experimental models of preterm delivery and those that have been seen in adults who were born preterm. The postnatal period appears to be a critical period of cardiac hypertrophy in preterm infants and may offer a window for interventions to prevent long term cardiovascular consequences of preterm birth.

6: MATERNAL HYPERTENSION & OFFSPRING CARDIAC PHENOTYPE

In the previous chapter, I identified maternal hypertension as a predictor of increased ventricular mass change over the first three months of life in preterm born offspring. In this chapter, I am now exploring the effect of maternal hypertension in the absence of prematurity in order to investigate its effect on cardiac development. For the purposes of this chapter, I therefore excluded any infants born before 37 weeks gestation from my cohort from analysis.

6.1 Abstract

Background- Exposure to *in utero* maternal hypertension imparts a greater risk of cardiovascular disease in the offspring. Increases in cardiac ventricular mass are associated with the development of hypertension and have been demonstrated in

offspring born to hypertensive pregnancies in adolescence and in infants in the context of preterm birth. I sought to identify whether offspring born at term to hypertensive pregnancies demonstrated an increase in left or right ventricular mass over the first three months of postnatal life.

Methods– 134 infants born at term (54 normotensive pregnancy, 80 hypertensive pregnancy) were recruited to undergo cardiac ultrasound at birth and three months of age. Left ventricular (LV) and right ventricular (RV) structure and function were quantified.

Results- There were no differences in left and right ventricular mass indexed to body surface area at birth (left 20.9±3.7vs20.6±4.0g/m^2, p=0.64, right 17.5±3.7vs18.1±4.7g/m^2, p=0.57). By three months, however, LV and RV mass index were significantly increased in the hypertensive group (left 24.9±4.6vs26.8±4.9g/m^2, p=0.04; right 17.1± 4.2vs21.1±3.9g/m^2, p<0.001). There was no difference in ventricular function or in blood pressure between groups at either time points. Increased LV mass index at three months was significantly associated with microvascular density loss over the first three months of life (total vessel density change (%) B -0.06, p=0.04) and RV mass index was correlated with markers of the severity of the hypertensive disorder including maximum maternal systolic blood pressure (mmHg) (B=0.09, p<0.001), mode of delivery (B=4.33 p<0.001), diastolic blood pressure of the infants at three months (mmHg) (B=0.09, p=0.04) and maternal smoking (B=4.4, p=0.10).

Conclusions- I have demonstrated, for the first time, a disproportionate increase in mass over the first three months of life in infants born to a hypertensive pregnancy without differences in blood pressure. The relationship of these changes to long term cardiac phenotype and clinical cardiovascular disease remains to be seen.

6.2 Introduction

There is a growing body of evidence that infants exposed *in utero* to maternal hypertensive disorders of pregnancy have increased blood pressure later in life.[215, 216] A recently published study has shown that exposure to hypertensive disorders of pregnancy is associated with greater relative wall thickness and reduced left ventricular end-diastolic volume in adolescent offspring.[223] Intriguingly, I have also demonstrated in Chapter 5 that exposure to hypertension *in utero* is a predictor of increased ventricular mass change over the first three months of life in preterm-born individuals. In this study, I aimed to explore the effect of maternal hypertension on postnatal cardiac development in term-born individuals without any other pregnancy complications. I also explored whether any particular clinical features of the maternal hypertensive disorder associated with changes in ventricular mass and if changes in cardiac mass were linked to other measures of cardiovascular development.

6.3 Methods

6.3.1 Study population

This chapter includes data from term infants in whom echocardiography was performed at birth and three months of age. Inclusion and exclusion criteria and

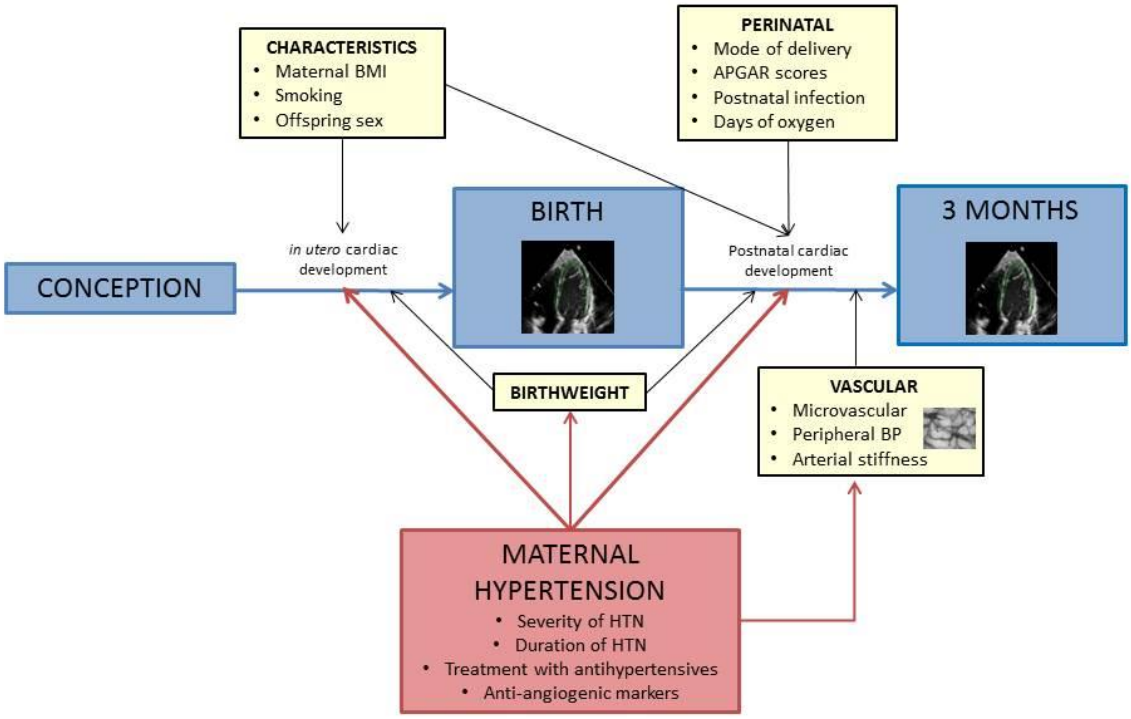
diagnostic definitions are detailed in Section 2.1. Details of these cohorts and how they were recruited are set out Chapter 2.

6.3.2 Study visit

An overview of the study design is shown in Figure 6.1. Mother and infants underwent the following assessments and analysis at birth and three months, which are described in detail in Methods (Chapter 3):

- Anthropometry (Section 3.3.2)
    - Weight
    - Head circumference
- Echocardiography (Section 3.4.1)
    - Cardiac structure
        - LV mass and volumes
        - RV mass and volumes
    - Cardiac function
        - LV systolic and diastolic function
        - RV systolic function
- Blood pressure (Section 3.6.1)
- Medical Records and Questionnaires (Section 3.9 and Appendix)

Figure 6.1: An overview of the study design. Postnatal echocardiography was performed in a cohort of term infants at birth and three months of age in order to investigate the effects of maternal hypertension on cardiac structure and function and their association with other maternal, offspring and perinatal characteristics.



6.3.3 Statistics

Statistical analysis is described in detail in Section 3.10.

6.3.3.1 Power calculation

The sample size n=54 for offspring exposed to a normotensive pregnancy and n=80 offspring exposed to a hypertensive pregnancy provided me with 80% power at a significance level of α=0.05 to detect a difference of at least 0.5 standard deviations (SDs) between groups in left ventricular mass index at 3 months.

6.4 Results

6.4.1 Study population characteristics

Maternal and offspring demographic and anthropometric characteristics in the normotensive and hypertensive groups are presented in Table 6.1. Mothers who experienced a hypertensive pregnancy had a higher BMI and a higher systolic and diastolic blood pressure at booking. Babies born to hypertensive pregnancies were born earlier and had smaller head circumferences at birth. However, on subgroup analysis, this was only true for those babies born to preeclamptic pregnancies and not those exposed to pregnancy induced hypertension (Table 6.2). Infants from a preeclamptic pregnancy were also found to be lighter and had a lower birthweight z-score than those born to pregnancy induced hypertension or normotensive pregnancies. Of note, there were no differences in blood pressure between groups either at birth or three months of age (Table 6.1 and Table 6.2).

Of the 122 infants in whom echocardiography was performed at birth, 117 had LV measures included in analysis (4 unanalysable due to infant movement, 1 excluded due to subsequent diagnosis of Turner's syndrome). 120 attended for scan at three months of age of which 110 had analysable images. Image acquisition was optimised for LV assessment but of the available scans at birth, 76 had analysable RV views with 75 infants having measures at follow up.

6.4.2 Disproportionate increase in mass in offspring born to hypertensive pregnancies

Infants born to a hypertensive pregnancy had a similar LV and RV mass indexed to body surface area at birth (Figure 6.2, Table 6.3). There was also no difference in the ratio of mass/end diastolic volume between groups in the LV, although RV mass/EDV was increased at birth due to a significantly smaller ventricular volume in the hypertensive group (Figure 6.2B, Table 6.3). By three months, however, LV mass when indexed to both body size and ventricular volume was significantly increased in the hypertensive group (Figure 6.2A, Table 6.3). In the RV, mass indexed to body surface area was also now increased and the RV mass/EDV stayed significantly greater and ventricular volume remained reduced (Figure 6.2B, Table 6.3). There was no difference in left or right ventricular function between groups (Table 6.3).

Table 6.1: Cohort characteristics

	Normotensive (n=54)	**Hypertensive (n=80)**
Maternal Demographics & Anthropometrics		
Maternal age at delivery, years	32.7±4.0	32.0±6.0
Body Mass Index at booking, kg/m^2	23.1±3.5	26.7±5.1***
Booking sBP, mmHg	107.9±9.0	120.0±12.0***
Booking dBP, mmHg	64.8±8.0	73.5±10.3***
Smokers, n (%)	2(4)	2(3)
Offspring Demographics & Anthropometrics		
Birth		
Gestational age at delivery, weeks	39.8±1.3	39.3±1.3*
Males, n (%)	29(54)	30(38)
Birth order †	1(1)	1(1)
Caesarean section, n (%)	14(26)	22(28)
Age at assessment, days	5.3±7.6	3.2±3.1
Birthweight, grams	3433±529	3250±568
Birthweight z-score	0.28±1.0	0.10±1.2
Head circumference, cms	35.0±1.5	34.2±1.7**
sBP, mmHg	82.6±13.8	81.1±13.1
dBP, mmHg	44.7±9.3	45.3±9.6
3 Months		
Age at assessment, days	99.6±14.8	96.7±13.1
Weight, grams	6180±816	5974±937
Head circumference, cms	41.1±1.6	40.7±1.7
sBP, mmHg	96.3±11.7	97.6±12.6
dBP, mmHg	52.7±12.0	55.9±12.4

† Median±Interquartile range. *p<0.05; **p<0.01; ***p<0.001

Table 6.2: Subgroup cohort characteristics

	Normotensive (n=34)	PIH (n=23)	PET (n=18)
Maternal Demographics & Anthropometrics			
Maternal age at delivery, years	32.6±4.6	32.7±5.1	32.5±6.7
Body Mass Index at booking, kg/m^2	23.0±3.1	27.6±6.1[c]	25.8±4.9[a]
Booking sBP, mmHg	107±9	123±11[c]	117±13[b]
Booking dBP, mmHg	65±8	76±8[c]	72±13
Smokers, n (%)	2(6)	0(0)	1(6)
Offspring Demographics & Anthropometrics			
Birth			
Gestational age at delivery, weeks	39.9±1.3	39.7±1.1	38.9±1.4[a]
Males, n (%)	20(59)	6(26)	8(44)
Birth order [†]	1(0)	1(1)	1(0)
Caesarean section, n (%)	7(21)	6(26)	8(44)
Age at assessment, days	6±9	4±5	3±2
Birthweight, grams	3448±558	3541±492	3042±502**[a]
Birthweight z-score	0.26±1.0	0.65±1.0	-0.29±1.0*
Head circumference, cms	35.0±1.5	35.2±1.5	33.6±1.5**[b]
sBP, mmHg	82±14	83±14	79±11
dBP, mmHg	45±9	46±10	44±10
3 Months			
Age at assessment, days	101±15	95±11	97±15
Weight, grams	6235±813	6207±830	5913±867
Head circumference, cms	41.1±1.7	41.1±1.6	40.7±1.9
sBP, mmHg	96±11	97±12	99±14
dBP, mmHg	52±12	57±11	57±13

Comparison of PIH and PET group *p<0.05; **p<0.01; ***p<0.001
Comparison to NT group [a]p<0.05; [b]p<0.01; [c]p<0.001

Figure 6.2: (A) Tukey boxplots demonstrating i) left ventricular mass index and ii) left ventricular mass/end diastolic volume at birth and iii) left ventricular mass index and iv) left ventricular mass/end diastolic volume at three months in term offspring born to normotensive and hypertensive pregnancies. (B) Tukey boxplots demonstrating i) right ventricular mass index and ii) right ventricular mass/end diastolic volume at birth and iii) right ventricular mass index and iv) right ventricular mass/end diastolic volume at three months in term offspring born to normotensive and hypertensive pregnancies. NT indicates normotensive pregnancy; HTN hypertensive pregnancy; LVMI left ventricular mass index; LV left ventricle; RVMI right ventricular mass index; RV right ventricle; EDV end diastolic volume.

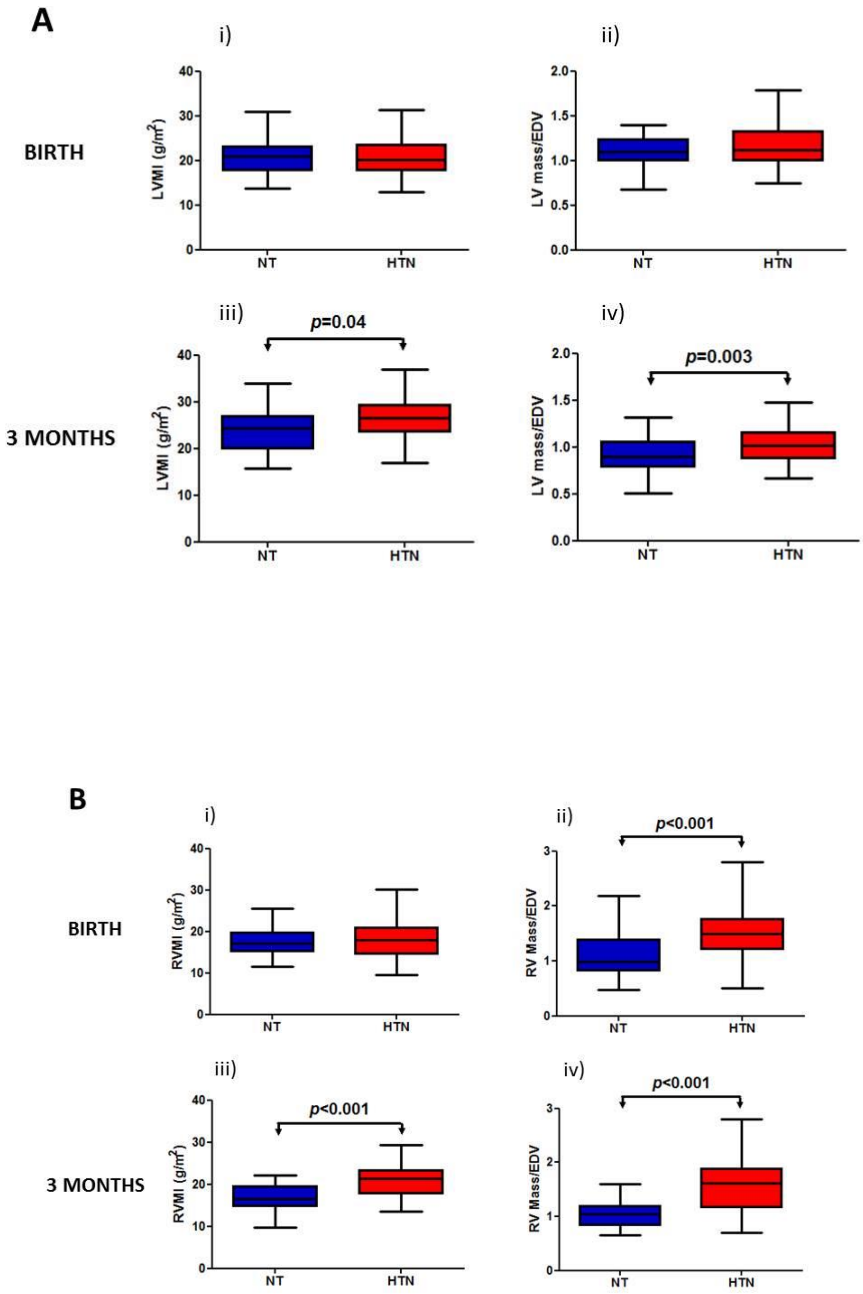


Table 6.3: Cardiac structure and function

	Birth			Follow Up		
	NT	**HTN**	***p*-value**	**NT**	**HTN**	***p*-value**
Left Ventricle	n=48	n=69		n=48	n=62	
Volumes						
EDV (ml)	4.6±1.2	4.0±1.2	**0.02**	9.6±2.0	9.0±2.0	0.11
EDV Index (ml/m^2)	19.4±4.5	17.9±4.1	0.06	27.5±4.6	26.4±5.2	0.25
Mass						
Mass (g)	4.9±1.2	4.6±1.2	0.18	8.7±1.6	9.1±1.9	0.17
Mass Index (g/m^2)	20.9±3.7	20.6±4.0	0.64	24.9±4.6	26.8±4.9	**0.04**
Mass/EDV	1.1±0.2	1.2±0.3	0.11	0.9±0.2	1.0±0.2	**0.003**
Function						
Systolic Function						
Ejection fraction (%)	62.2±9.4	64.8±7.6	0.13	59.1±7.2	60.0±8.8	0.65
Stroke Volume (ml)	2.8±0.7	2.6±0.7	0.27	5.8±1.3	6.9±8.0	0.49
Diastolic Function						
EA	1.0±0.3	1.1±0.3	0.83	1.0±0.2	1.0±0.2	0.40
Lateral E’	6.8±1.5	6.5±1.8	0.49	10.2±2.0	10.3±2.2	0.77
Lateral E/E’ ratio	8.0±2.3	7.8±2.7	0.69	8.3±3.0	8.0±2.3	0.84
Right Ventricle	n=32	n=44		n=34	n=41	
Volumes						
EDV (ml)	3.9±1.5	2.9±1.2	**0.001**	5.8±1.4	5.1±1.9	0.06
EDV Index (ml/m^2)	16.8±5.3	12.7±4.7	**0.001**	16.4±3.2	14.4±4.8	**0.04**
Mass						
Mass (g)	4.1±1.0	4.1±1.3	0.86	6.1±1.6	7.4±1.7	**0.001**
Mass Index (g/m^2)	17.5±3.7	18.1±4.7	0.57	17.1±4.2	21.1±3.9	**<0.001**
Mass/EDV	1.1±0.5	1.5±0.4	**<0.001**	1.1±0.3	1.6±0.7	**<0.001**
Function						
TAPSE	0.89±0.2	0.86±0.2	0.36	1.5±0.3	1.5±0.3	0.65

6.4.3 Predictors of mass increase

Left ventricular mass index at three months was significantly associated with loss in microvascular density over the first three months of life (TVD change %, B -0.06, *p*=0.04). However there were no other maternal, offspring, perinatal or pregnancy factors that were found to predict left ventricular mass (Table 6.4). Increased right ventricular mass indexed to body size in the offspring at follow up was, however, associated with a number of factors related to the severity of hypertensive disease including higher booking blood pressure, higher maximum blood pressure, longer duration of hypertension, treatment with antihypertensives and higher levels of sENG in the maternal circulation at birth (Table 6.4). Delivery by caesarean section and a higher diastolic blood pressure in the offspring at three months was also correlated with increased RV mass, with a borderline association for maternal smoking (Table 6.4). In a multivariable model, maximum maternal systolic blood pressure during the pregnancy, mode of delivery and smoking were all independent predictors of right ventricular mass index and accounting for 55% of the variance (Table 6.5).

Table 6.4: Bivariate regression coefficients for maternal, offspring and pregnancy characteristics and ventricular mass at 3 months

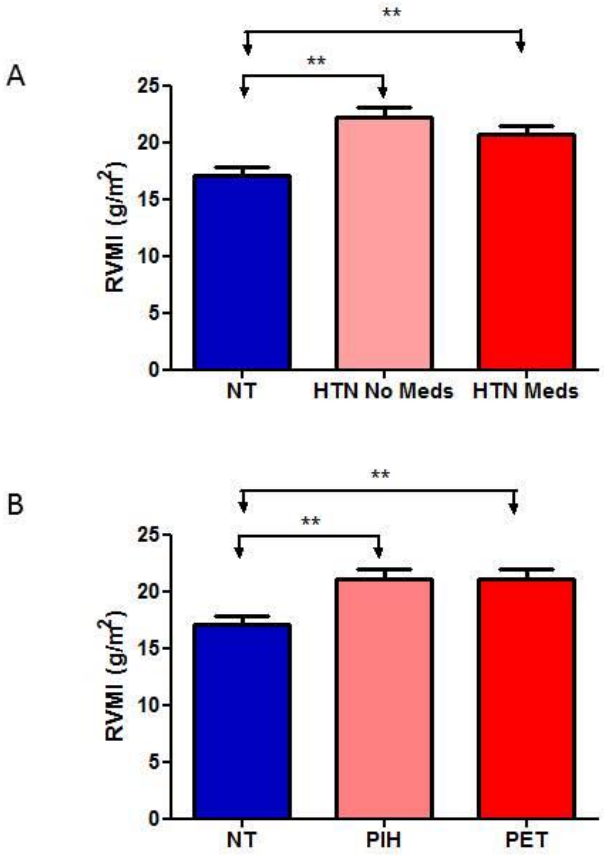
	LVMI 3months			RVMI 3 months		
	B	**95% CI**	***p*-value**	**B**	**95% CI**	***p*-value**
Maternal factors						
Maternal BMI, kg/m^2	0.06	-0.13-0.26	0.53	0.17	-0.04-0.38	0.11
Smoking	5.32	-0.23-10.87	0.06	4.4	-0.85-9.6	0.10
Severity of HTN						
Booking sBP, mmHg	0.004	-0.08-0.08	0.92	0.12	-0.04-0.20	**0.005**
Booking dBP, mmHg	0.06	-0.03-0.15	0.19	0.15	0.05-0.24	**0.003**
Maximum sBP, mmHg	0.04	-0.001-0.09	0.06	0.09	0.04-0.13	**<0.001**
Maximum dBP, mmHg	0.05	-0.02-0.11	0.17	0.12	0.05-0.20	**0.001**
Duration of HTN, weeks	0.27	-0.12-0.66	0.18	0.64	0.10-1.17	**0.02**
Treatment with anti-hypertensives	1.57	-0.29-3.43	0.10	2.23	0.15-4.30	**0.04**
sFlt-1 levels, pg/mL	-9.84E-005	-0.001-0.001	0.83	0.001	0.00-0.002	0.12
sENG levels, ng/mL	0.07	-0.11-0.24	0.43	0.30	0.17-0.43	**<0.001**
PlGF levels, pg/mL	0.003	-0.08-0.09	0.94	0.05	-0.02-0.11	0.15
VEGF levels, pg/mL	0.008	-0.02-0.04	0.63	-0.003	-0.03-0.03	0.83
Perinatal factors						
Caesarean section	1.36	0.63-3.35	0.18	4.33	2.25-6.42	**<0.001**
Sex	-0.56	-2.41-1.29	0.55	1.38	-0.69-3.44	0.19
Birthweight z-score	-0.37	-1.17-0.43	0.37	-0.09	-1.08-0.90	0.85
APGARS at 5 mins	-1.96	-5.08-1.16	0.22	-1.17	-4.14-1.80	0.43
Postnatal infections	-1.26	-6.17-3.64	0.61	2.58	-3.85-9.01	0.43
Need for oxygen	1.52	-1.40-4.44	0.31	-0.81	-7.26-5.65	0.80
TVD change, %	-0.06	-0.12- -0.003	**0.04**	-0.04	-0.10-0.02	0.22
Vascular measures						
sBP at birth, mmHg	0.002	-0.07-0.07	0.96	-0.01	-0.09-0.07	0.76
dBP at birth, mmHg	0.02	-0.08-0.11	0.72	-0.01	-0.12-0.10	0.86
sBP at 3 months, mmHg	0.009	-0.07-0.09	0.82	0.07	-0.02-0.16	0.13
dBP at 3 months, mmHg	-0.03	-0.11-0.05	0.44	0.09	0.007-0.18	**0.04**
PWV at birth, m/s	0.06	-0.39-0.51	0.79	-0.18	-0.64-0.29	0.45
PWV at 3 months, m/s	-0.21	-0.76-0.33	0.44	0.24	-0.31-0.79	0.39

Table 6.5: Multivariable regression coefficients for maternal, offspring and pregnancy characteristics and right ventricular mass index at 3 months

	RVMI 3 months		
	B	**95% CI**	***p*-value**
Smoking	10.62	4.36 - 16.88	**0.002**
Booking sBP, mmHg	-0.05	-0.14 - 0.04	0.25
Maximum sBP, mmHg	0.09	0.01 - 0.18	**0.03**
Duration of HTN, weeks	0.33	-0.17 - 0.83	0.19
Treatment with anti-hypertensives	-0.39	-3.28 - 2.51	0.79
sENG levels, ng/mL	0.08	-0.08 - 0.24	0.31
Caesarean section	2.59	-4.72 - -0.46	**0.02**

I then performed a subgroup analysis on the hypertensive group to investigate if there were any features of the hypertensive disorder that had an influence on right ventricular mass. There was no difference in offspring RV mass index at 3 months between hypertensive subgroups that had or had not received antihypertensive medication during pregnancy, although both were significantly higher than the normotensive group (Figure 6.3A). Intriguingly, there was also no difference between subgroups exposed to pregnancy induced hypertension and preeclampsia, although, again, both had an increased RV mass index compared to the control group (Figure 6.3B).

Figure 6.3: (A) Bar charts to show increased right ventricular mass index at three months in offspring born to untreated hypertensive and treated hypertensive pregnancies compared to those from normotensive pregnancies. (B) Bar charts to show increased right ventricular mass index at three months in offspring born to a pregnancy complicated by pregnancy induced hypertension and preeclamptic pregnancies compared to those from normotensive pregnancies. Bar charts are presented as Mean±Standard Error of the Mean. RVMI indicates right ventricular mass index; NT normotensive pregnancy; HTN no meds untreated hypertensive; HTN Meds hypertensive treated pregnancy; PIH pregnancy induced hypertension; PET preeclamptic. ** p<0.01.



6.4.4 Decreased right ventricular end diastolic volume in offspring born to hypertensive pregnancies

I went on to investigate the reduction in right ventricular end diastolic volume in offspring born to hypertensive pregnancies which I had demonstrated in Table 6.3. There was a significant association between a smaller right ventricular index at birth and severity of hypertensive disorder when the groups were subdivided based on need for antihypertensive treatment and classification of the hypertensive disorder (Figure 6.4A). However, these differences had attenuated by three months of age (Figure 6.4B).

Next, I performed bivariate regression analyses to study possible predictors of right ventricular volume in my cohort at birth. A smaller right EDV indexed to body surface area was significantly associated with markers of maternal hypertensive severity such as a higher maximum maternal blood pressure, treatment with antihypertensives, higher maternal sENG levels at birth and a lower birthweight z-score (Table 6.6). Intriguingly a smaller EDV index was also associated with capillary rarefaction over the first three months of life, which is also seen in offspring exposed to *in utero* hypertension (Chapter 7). In a multivariable model, only maternal maximum systolic blood pressure was an independent predictor of a smaller right ventricular volume (Table 6.7).

Figure 6.4: (A) Bar charts to show a significant association between a reduction in right ventricular end diastolic volumes at birth and (i) severity or (ii) classification of the hypertensive disorder in the mother. (B) Bar charts to show that differences in right ventricular end diastolic volume index have attenuated by three months in offspring born to a hypertensive pregnancy when divided into (i) severity or (ii) classification of the hypertensive disorder. Bar charts are presented as Mean±Standard Error of the Mean. *P* values are presented as one-way ANOVA test between groups. RVEDVI indicates right ventricular end diastolic volume index; NT normotensive; HTN no meds untreated hypertensive; HTN Meds hypertensive treated pregnancy. PIH pregnancy induced hypertension; PET preeclamptic.

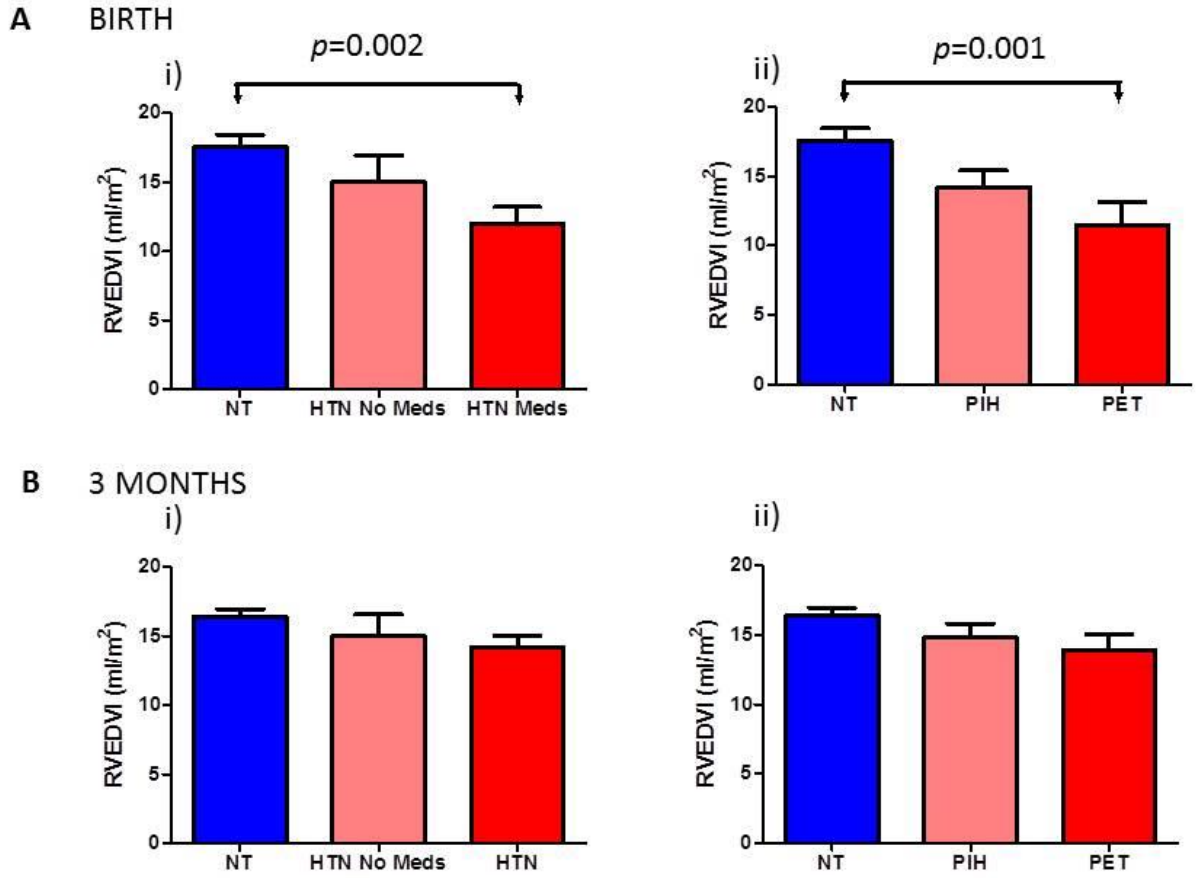


Table 6.6: Bivariate regression coefficients for maternal, offspring and pregnancy characteristics and right ventricular volume at birth

	RVEDVI Birth		
	B	**95% CI**	***p*-value**
Maternal factors			
Maternal BMI, g/m^2	-0.07	-0.35-0.22	0.64
Smoking	-3.62	-10.07-2.82	0.26
Severity of HTN			
Booking sBP, mmHg	-0.07	-0.20-0.05	0.25
Booking dBP, mmHg	-0.04	-0.19-0.10	0.53
Maximum sBP, mmHg	-0.09	-0.15- -0.02	**0.009**
Maximum dBP, mmHg	-0.20	-0.30- -0.09	**<0.001**
Duration of HTN, weeks	-0.60	-1.38-0.19	0.13
Treatment with anti-hypertensives	-4.94	-7.74- -2.15	**0.001**
sFlt-1 levels, pg/mL	0.00	-0.002-0.001	0.83
sENG levels, ng/mL	-0.30	-0.55- -0.05	**0.02**
PlGF levels, pg/mL	0.11	-0.24-0.46	0.52
VEGF levels, pg/mL	-0.009	-0.05-0.04	0.68
Perinatal factors			
Caesarean section	-0.97	-4.47-2.53	0.58
Sex	-0.71	-3.74-2.33	0.64
Birthweight z-score	1.92	0.58-3.27	**0.006**
APGARS at 5 mins	0.24	-3.82-4.30	0.91
Postnatal infections	4.51	-3.30-12.32	0.25
Need for oxygen	0.08	-11.00-11.17	0.99
TVD change, %	0.11	0.02-0.19	**0.02**
Vascular measures			
sBP at birth, mmHg	0.004	-0.11-0.12	0.94
dBP at birth, mmHg	-0.04	-0.21-0.12	0.60
sBP at 3 months, mmHg	-0.02	-0.13-0.10	0.80
dBP at 3 months, mmHg	-0.02	-0.15-0.11	0.76
PWV at birth, m/s	-0.30	-0.97-0.36	0.36
PWV at 3 months, m/s	-0.42	-1.23-0.38	0.29

Table 6.7: Multivariable regression coefficients for maternal, offspring and pregnancy characteristics and right ventricular end diastolic volume index at birth

	RVEDVI Birth		
	B	**95% CI**	***p*-value**
Maximum sBP, mmHg	-0.15	-0.28 - -0.02	**0.03**
Treatment with anti-hypertensives	-1.60	-7.54 - 4.34	0.59
sENG levels, ng/mL	-0.006	-0.30 - 0.28	0.97
Birthweight z-score	0.26	-1.46 - 1.97	0.76
TVD change, %	0.02	-0.10 – 0.13	0.79

6.5 Discussion

In this study I have demonstrated, for the first time, a disproportionate increase in left and right ventricular mass in infants exposed to maternal hypertensive disorders of pregnancy in the early postnatal period prior to the appearance of any blood pressure changes. There were no significant differences in mass indexed to body size between groups at birth, which suggests a postnatal phenomenon. In the left ventricle, the increase in mass was associated with capillary rarefaction over the same time period, whereas in the right, there were correlations with the severity of hypertension as well as mode of delivery, maternal smoking and higher diastolic blood pressure in the offspring at 3 months suggesting different pathological mechanisms. Exposure to maternal hypertension was also associated with a smaller right ventricular volume index at birth and three months with a graded relationship between severity and classification of the hypertensive disorder and right ventricular volume seen at birth.

6.5.1 Previous studies

Increases in cardiac mass are seen in early primary hypertension[321] and it is also associated with the development of high blood pressure.[322, 323] Cardiac hypertrophy has also been observed in normotensive at-risk groups, including children, with a family history of hypertension.[324-327] Preeclampsia also increases the risk of later hypertension in the offspring[215, 216, 218, 328] and animal models have demonstrated adverse changes to left ventricular structure and function after exposure to gestational hypertension.[329] This is the first study to demonstrate a difference in cardiac mass in infants born to hypertensive pregnancies and although there were no differences in blood pressures between groups at birth or at three months of age, a greater right ventricular mass index at three months was associated with a higher diastolic blood pressure in the infants at follow up.

A previous research group has studied ventricular mass in offspring of hypertensive pregnancies. In one study they found children born to a hypertensive pregnancy had a higher blood pressure at age 12 years and a significant correlation between systolic blood pressure and left ventricular mass, although no significant difference in mass between groups were observed. [330] In another, no differences in left ventricular mass in children born to hypertensive pregnancies compared to normotensive pregnancies at average age 12 and 18 years of age were found although numbers were small[331] and early increased left ventricular mass was sustained throughout adolescence.[332] The failure to find any differences between groups may be because these children were studied in adolescence which could give rise to large variation due to puberty.

However, a recently published study using a large prospective birth cohort study has demonstrated that offspring exposed to hypertensive disorders of pregnancy who underwent echocardiography at a mean age of 17.7 years old have an increased relative wall thickness and lower mean left ventricular end diastolic volumes suggesting concentric remodelling.[223] No differences were demonstrated in cardiac function,[223] consistent with my findings.

Hypertensive pregnancies have also been shown to have similar cardiac effects in the mother. Maternal left ventricular hypertrophy and systolic and diastolic dysfunction have been reported to be more prevalent in pregnancies complicated by hypertension, with a dose response found according to disease onset and severity[333-335] some of which are still present a few years postpartum.[336] Few studies have investigated right ventricular changes, but increased rates of hypertrophy and reduced function are reported.[336] Intriguingly, strong maternal-offspring but not paternal-offspring correlations in left ventricular mass have been reported,[337] suggesting that pregnancy history may be of importance to cardiac phenotype. Preeclampsia is associated with inflammation[202, 203] and this shared pathological state may be key to the cardiac sequelae seen in both the mother and infant. Certainly, prenatal exposure to inflammatory stimulants has been shown to induce offspring cardiac hypertrophy in animal models.[338]

6.5.2 Possible mechanisms

The association of increased left ventricular mass with increased capillary rarefaction over the first three months of life is fascinating. If there is similar reduction in capillary

density in the myocardium as seen peripherally, it could hypothesised that this may lead to a relatively ischaemic environment which may cause fibrosis and an increase in mass. Our group have recently demonstrated a similar association in a maternal cohort 5 to 10 years after preeclampsia.[339] These results are consistent with animal models in which rats with induced myocardial hypertrophy have associated capillary rarefaction.[340] Furthermore, commonly prescribed antihypertensive agents, such as calcium channel antagonists, ACE inhibitors and angiotensin receptor blockers have all been shown to increase capillary density[341] and have been shown to reverse myocardial hypertrophy in animals.[342, 343] Critically, there was no such association between mass and capillary loss in my preterm cohort, suggesting that the mechanism behind the relative hypertrophy caused by prematurity and maternal hypertension may be quite different.

Interestingly, it was right and not left ventricular mass that seemed to be associated with disease onset and levels of hypertension in my offspring cohort. Associations with mode of delivery may have been an additional marker of the severity of the hypertensive disease. The similar increases in right ventricular mass in those exposed to pregnancy induced hypertension and preeclampsia is consistent with previous studies looking at the adolescent left ventricle.[223] There was no significant difference in ventricular mass at three months between hypertensive groups that did or did not require treatment, although use of medication was correlated with increased mass change in the cohort as a whole, suggesting that in that analysis it was purely a marker for the presence of hypertension. It would be interesting to further investigate the effect of antihypertensive treatment on cardiovascular development of the offspring

as in my study, numbers were small and there is a possibility that some antihypertensives may exert a protective effect as seen in animals[342, 343] although this may be offset by the high level of hypertension seen in mothers who require treatment.

The differences in ventricular volume seen in the right ventricle may be due to the increased afterload in a poorly invaded preeclamptic placenta in a right ventricular dominant *in utero* circulation. This may have also had an effect on right ventricular mass although there were no increases in mass seen at birth, with increased right ventricular mass/EDV ratios due to volumetric changes. It therefore could be that increased afterload causes ventricular fibrosis prior to the development of hypertrophy although right ventricular volumes had started to normalise by three months of age in the hypertensive group and there was no difference in right ventricular systolic function at either time point.

A more plausible explanation linking postnatal right ventricular mass changes to a hypertensive pregnancy could be the effects on the pulmonary vasculature. The late fetal and perinatal period is a critical window for the development of the pulmonary circulation making it vulnerable to insults.[344] Studies have shown the long lasting detrimental effects of perinatal insults on the pulmonary vasculature in animal and human models,[345, 346] potentially mediated by epigenetics.[347] One previous study has demonstrated pulmonary vascular dysfunction in adolescent offspring of preeclamptic pregnancies living at high altitudes with 30% higher pulmonary artery pressures.[221] These adolescents also had increased markers for oxidative stress and their siblings

born to normotensive pregnancies had normal vascular function, suggesting that these differences were caused by exposure to preeclampsia. Maternal smoking may act as an additional insult by contributing to fetoplacental insufficiency and explain its association with right ventricular mass change.

6.5.3 Limitations

A potential limitation is again my use of automated software to derive measures from single 4-chamber views as discussed in Section 5.5.6. Another limitation is that as the images were optimised for the left ventricle, I was not able to acquire right ventricular measurements in the whole cohort which may have introduced some bias. However, the findings of significantly increased mass were consistent in both the left and the right ventricle. In addition, although predictors for increased mass were different between the left and the right ventricle, markers of hypertensive disease severity were consistently significantly associated with RV mass index, suggesting that my findings are likely to be robust.

I also did not have access to data on markers of placental resistance in the fetal circulation during pregnancy for our infants. This would have been useful in order to investigate whether the cardiovascular system of the offspring has been exposed to increased afterload which could have contributed to the increase in ventricular mass. What is interesting, however, is that changes in mass only became apparent after birth, suggesting a postnatal phenomenon. Nonetheless, in future studies, it would be interesting to relate cardiovascular data on the offspring prior to birth to subsequent *ex utero* changes. Other future work would include follow up of this cohort to establish

whether these changes track into later life and their relationship with peripheral blood pressure.

6.6 Conclusions

I have demonstrated, for the first time, a disproportionate increase in ventricular mass over the first three months of life in infants born to a hypertensive pregnancy without differences in blood pressure. Left ventricular mass indexed to body size at three months was associated with microvascular density loss and right ventricular mass index was correlated with the severity of the hypertensive disease in addition to mode of delivery, diastolic blood pressure in the offspring at follow up and maternal smoking. The relationship of these changes to long term cardiac phenotype and clinical cardiovascular disease remains to be seen.

7: Maternal Biomarkers, Pregnancy Complications & Offspring Microvascular Phenotype

In this chapter I will be investigating the effect of maternal hypertension on offspring microvascular phenotype and its interaction with preterm birth. I will then explore the relationship between *in vivo* measures of capillary density and *in vitro* measures of angiogenic capacity at birth through a collaboration with Dr Grace Yu, postdoctoral fellow from Professor Leeson's group. Finally, I will relate my findings to circulating maternal biomarkers.

7.1 Abstract

Background- Offspring born to hypertensive pregnancies have increased blood pressure in later life. I tested the hypothesis that this is because an impact of hypertensive pregnancy on fetal vasculogenic capacity leads to abnormal postnatal microvascular remodelling.

Methods and *Results*- 255 infants born following either complicated (hypertension and/or preterm) or normal pregnancy were recruited for quantification of postnatal microvascular structure at birth and three months of age. Vasculogenic cell potential was assessed in umbilical vein endothelial cells from 55 offspring based on *in vitro* microtubule formation (matrigel and co-culture) and proliferation. Maternal angiogenic profile (sFlt-1, sENG, VEGF, PlGF) was measured from post-partum plasma samples. At birth, offspring born after both hypertensive and normotensive pregnancies had similar microvessel density but during the first three postnatal months there was an almost two-fold greater reduction in total vessel density (TVD) after hypertensive pregnancy (%change -17.7%±16.4 vs -9.9%±18.7, p=0.002). This *in vivo* postnatal change was predicted by the vasculogenic capacity of the endothelial cells of the infant at birth (Total tubule length, r=0.49, p=0.02, branching r=0.57, p=0.004). Reduced *in vivo* and *in vitro* vascular development was related to increased sFlt-1 levels in the maternal circulation at birth in both hypertensive and normotensive pregnancies.

Conclusions - Offspring born to hypertensive pregnancies have reduced vasculogenic capacity at birth that predicts microvessel density loss over the first three postnatal

months. Degree of microvessel reduction is proportional to levels of anti-angiogenic factors in the maternal circulation at birth.

7.2 Introduction

A hypertensive pregnancy identifies both a mother and offspring with an increased risk of later hypertension, cardiovascular diseases and stroke.[213, 215, 216, 218] As this is a common pregnancy complication,[196] a mechanistic understanding of why the offspring carry this risk is of interest to a large proportion of the population.

Hypertensive pregnancies are characterised by placental dysfunction, which leads to an adverse cardiovascular maternal circulating milieu, including deranged angiogenic factors, inflammation and oxidative stress.[348] Pathophysiologically, this leads to reduced microvascular density, increased peripheral resistance and hypertension in the mother.[349-352] The fetus experiences the same circulating stressors[353, 354 355] and, plausibly, these could have similar disruptive impacts on the rapidly developing offspring vasculature, with long term relevance to later risk of cardiovascular diseases. Consistent with this pathogenic pathway, offspring of experimental hypertensive pregnancy models have altered vascular structure[356] and childhood retinal vascular size can be predicted by levels of circulating maternal angiogenic factors during pregnancy.[357] However, at birth, microvascular density was, unexpectedly, found to be increased in babies after more severe, preterm, hypertensive pregnancies.[349, 358, 359]

A critical phase of microvascular development occurs during the first three months of postnatal life when the disorderly fetal microvascular plexus remodels into the mature

ex utero horizontal papillary loop structure.[360] Therefore I hypothesised that, although fetal vascular development may be protected in some offspring *in utero*, the impact of an adverse circulating milieu during pregnancy on fetal vascular cell potential may alter the ability of the neonate to develop their postnatal microvasculature.[355, 356, 361]

7.3 Methods

7.3.1 Study population

This chapter includes data from infants in whom microvascular assessments were performed at birth and three months of age. In addition, a subset of these infants had *in vitro* assessments performed on endothelial cells isolated from their umbilical cords and information on maternal angiogenic factor levels (Figure 7.1). Inclusion and exclusion criteria and diagnostic definitions can be found in Section 2.1. Details of these cohorts and how they were recruited are set out in Chapter 2.

7.3.2 Study visit

Mother and infants underwent the following assessments and analysis at birth and three months (Figure 7.1), which are described in detail in Methods (Chapter 3):

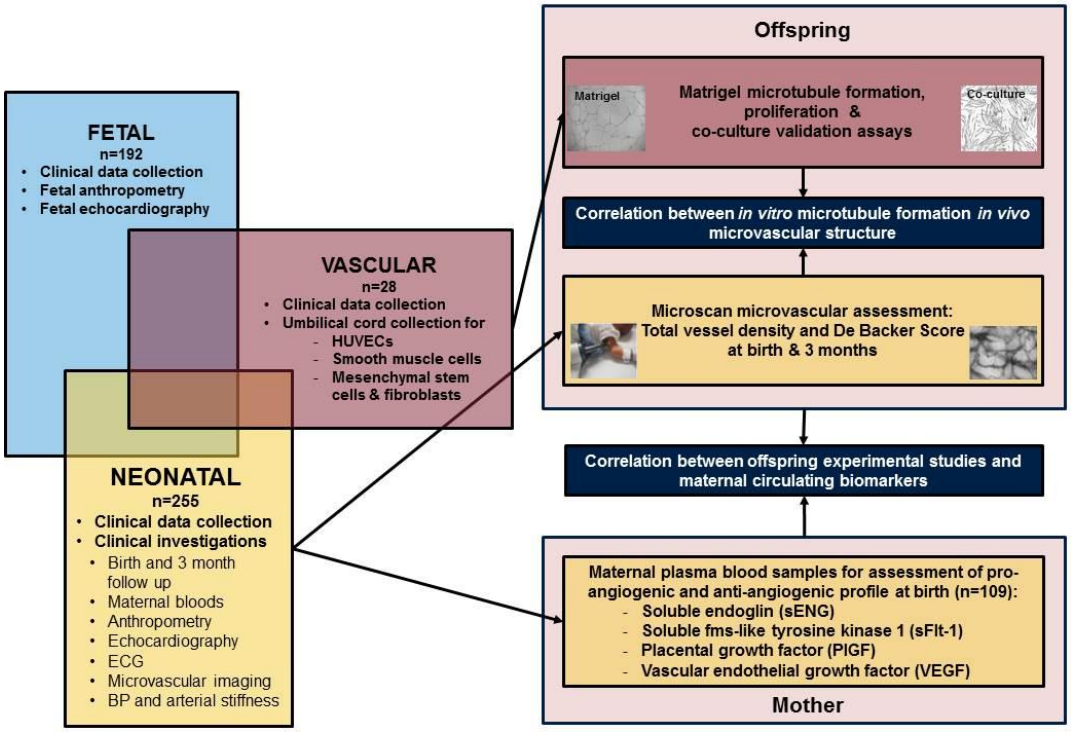
- Anthropometry (Section 3.3.2)
    - Weight
    - Head circumference
- *In vivo* microvascular imaging (Section 3.5.1)
    - Total vessel density

    - De Backer score

- *In vitro* vasculogenic capacity – birth only (Sections 3.5.2, 7.3.2.1 and Appendix)
- Blood pressure (Section 3.6.1)
- Maternal biochemistry (Section 3.8)
- Medical records and questionnaires (Section 3.9)

In addition, in a subgroup of mothers, maternal biochemistry taken at 34 weeks gestation was also available through a collaboration with the OxWATCH study (South Central Portsmouth ref. 12/SC/0492). This was in order to confirm that measures taken at the birth assessment reflected levels during late pregnancy.

Figure 7.1: Overview of study design using clinical and experimental assessments in offspring born to hypertensive pregnancies. HUVEC indicates human umbilical endothelial cell.



7.3.2.1 Additional methods - *in vitro* vasculogenic capacity

Umbilical cords were collected immediately after delivery and placed in Hanks Balanced Salt Solution (HBSS; with phenol red; PAA with 1% Penicillin/Streptomycin) to be stored at 4˚C until processing for cell isolation.

Human umbilical vein endothelial cells (HUVECs) were isolated and stored according to standard operating procedures by a dedicated team managed by Dr Grace Yu, postdoctoral research fellow (see Appendix). Angiogenic capacity was then assessed by two complementary approaches: microtubule formation assays, including matrigel and co-culture with human bone marrow stromal mesenchymal stem cells (BMMSC); and CyQUANT® NF Cell Proliferation assay (see Appendix).

7.3.3 Statistics

Statistical analysis is described in detail in Section 3.10.

7.3.3.1 Power calculation

The sample size n=104 for offspring exposed to a normotensive pregnancy and n=151 offspring exposed to a hypertensive pregnancy provided me with 80% power at a significance level of α=0.05 to detect a difference of at least 0.35 standard deviations (SDs) between groups in total vessel density percentage change between birth and three months.

7.4 Results

7.4.1 Study population characteristics

Maternal and offspring demographic and anthropometric characteristics in the normotensive and hypertensive groups are presented in Table 7.1 and 7.2 with characteristics of predefined subgroups in Table 7.3. Hypertensive and normotensive pregnancy groups were well matched for maternal age, incidence of maternal smoking, sex ratio, birth order, gestational age and age at birth and follow up assessments. BMI and blood pressure (booking, highest and discharge) were higher in mothers from the hypertensive group (p<0.001) and the infants had a lower birthweight z-score (p=0.002) and were more likely to have iatrogenic delivery (p=0.02). Pulse Wave Velocity measures were similar between groups (data not shown).

Microvascular measures were available for 227 infants at birth (four unsuitable due to clinical condition, one abandoned due to non-compliance of infant, 18 equipment unavailable/equipment failure, four unanalysable, one excluded due to Turner's syndrome), 216 infants at three months (29 lost to follow up, one abandoned due to non-compliance of infant, six equipment unavailable/failure, two unanalysable, one excluded due to Turner's syndrome) and for 197 infants at both time points.

Table 7.1: Characteristics of maternal cohort

	Neonatal		***in vivo*/*in vitro* Comparison**	
	Normotensive (n=104)	**Hypertensive (n=151)**	**Normotensive (n=18)**	**Hypertensive (n=10)**
Maternal Demographics & Anthropometrics				
Maternal age, years	32.6±4.6	32.7±6.0	32.8±3.3	34.2±3.8
BMI at booking, kg/m^2	23.7±4.0	26.9±6.9***	23.1±2.7	29.8±8.3**
Smokers, n (%)	6 (6)	5 (3)	0 (0)	0 (0)
Booking sBP, mmHg	107.6±9.8	118.4±20.6***	108.6±10.8	127.9±26.7*
Booking dBP, mmHg	65.2±8.5	72.9±14.9***	64.8±8.9	78.0±13.9**
Highest sBP, mmHg	123.2±10.5	162.2±16.8***	123.4±11.6	166.5±20.2***
Highest dBP, mmHg	76.4±8.5	100.8±10.5***	75.6±6.8	103.2±14.9***
Discharge sBP, mmHg	113.7±11.4	129.8±12.3***	114.2±12.3	128.1±11.3**
Discharge dBP, mmHg	67.3±8.2	79.8±9.9***	67.9±8.5	76.5±4.9**
Maternal Biomarkers				
sFlt-1, pg/mL	971.4±1162.3	1297.1±1341.6	-	-
sENG, ng/mL	7.3±5.6	11.3±6.7***	-	-
VEGF, pg/mL	61.2±102.4	54.5±80.3	-	-
PlGF, pg/mL	12.1±7.7	17.2±27.0	-	-

sBP indicates systolic blood pressure; dBP diastolic blood pressure.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Table 7.2: Characteristics of offspring cohort

	Neonatal		*in vivo*/*in vitro* Comparison	
	Normotensive (n=104)	**Hypertensive (n=151)**	**Normotensive (n=18)**	**Hypertensive (n=10)**
Offspring Demographics & Anthropometrics				
Birth				
Gestational age, weeks	36.8±3.5	36.8±3.2	39.3±2.2	36.6±2.5**
Males, n (%)	52 (50)	67 (44)	10 (56)	5 (50)
Birth order †	1±1	1±1	1±1	1±1
Caesarean section, n (%)	37 (36)	76 (50)*	13 (72)	4 (40)
Age at birth assessment, days	5.74±0.7	4.85±0.4	2.3±2.8	5.2±5.6
Birthweight, grams	2796±854	2670±851	3321±684	2686±839*
Birthweight z-score	0.14±1.00	-0.26±1.19**	0.35±1.06	-0.14±1.47
Head circumference, cms	32.8±2.8	32.7±2.7	34.4±1.69	33.2±2.39
sBP, mmHg	78.6±14.6	78.3±14.5	78.9±17.1	84.3±13.9
dBP, mmHg	42.8±9.8	43.7±10.0	41.8±8.2	50.2±7.8*
Follow up				
Age at follow up, days	99.9±15.4	97.4±13.6	101.9±9.6	108.2±11.1
Weight, grams	5640±1051	5468±1092	6202±748	6058±823
Head circumference, cms	40.1±2.0	40.0±2.1	40.8±1.6	41.3±1.5
sBP, mmHg	95.6±11.7	95.2±12.9	99.9±12.2	92.9±11.6
dBP, mmHg	53.0±12.7	51.9±12.4	54.2±14.1	48.3±9.9

† Median±Interquartile range.

sBP indicates systolic blood pressure; dBP diastolic blood pressure.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Table 7.3: Characteristics of cohort subgroups

	Preterm-born NT (n=50)	Preterm-born PET (n=71)	Term-born PIH (n=43)	Term-born PET (n=37)	Term-born NT (n=54)
Maternal Demographics & Anthropometrics					
Age at delivery, years	32.4±5.3	33.7±6.1	32.6±5.3	31.0±6.3	32.7±3.9
BMI at booking, kg/m^2	24.3±4.3	26.4±5.4	28.6±9.6	25.9±3.9	23.2±3.5
Smokers, n (%)	4 (8.2)	3 (4.2)	1 (2.3)	1 (2.8)	2 (3.6)
Booking sBP, mmHg	107.3±10.8	116.7±27.1	122.0±11.0	117.4±12.9	107.8±9.0
Booking dBP, mmHg	65.6±9.0	72.3±18.8	74.0±9.4	72.8±11.5	64.9±8.0
Highest sBP, mmHg	124.2±10.6	166.9±19.6	156.2±11.9	160.2±12.9	122.4±10.4
Highest dBP, mmHg	77.2±9.1	103.3±12.9	98.6±6.2	98.5±8.5	75.7±7.9
Discharge sBP, mmHg	113.1±12.6	130.4±15.3	128.8±9.2	129.8±8.9	114.2±10.4
Discharge dBP, mmHg	66.4±8.2	81.1±10.3	79.6±10.1	77.8±8.7	68.0±8.1
Offspring Demographics & Anthropometrics					
Birth					
Gestational age, weeks	33.6±2.09	34.2±2.32	39.7±1.05	38.8±1.32	39.7±1.37
Males, n (%)	22 (45)	37 (52)	12 (28)	18 (50)	30 (55)
Birth order [†]	1±(1)	1±(1)	1±(1)	1±(1)	1±(1)
Caesarean section, n (%)	22 (45)	55 (76)	11 (26)	10 (28)	15 (27)
Age at birth assessment, days	6.5±6.1	7.2±6.0	3.1±3.6	2.7±1.3	5.1±7.5
Birthweight, grams	2104±552	2018±606	3443±554	3050±457	3413±544
Birthweight z-score	-0.02±1.02	-0.65±1.07	0.43±1.19	-0.29±1.03	0.28±0.98
Head circumference, cms	30.5±2.0	30.9±2.6	34.9±1.6	33.5±1.4	34.9±1.5
sBP, mmHg	73.4±14.3	74.3±14.9	82.4±13.7	80.5±13.2	82.6±13.7
dBP, mmHg	40.5±10.2	41.2±9.8	45.2±9.9	46.3±9.6	44.6±9.3
Follow up					
Age at follow up, days	100.4±16.4	98.2±14.0	95.3±13.9	98.4±12.3	99.5±14.7
Weight, grams	5039±947	4913±971	6064±958	5907±913	6139±860
Head circumference, cms	39.0±1.9	39.3±2.3	40.9±1.6	40.5±1.7	41.0±1.7
sBP, mmHg	94.5±11.7	92.4±12.7	95.6±12.5	100.4±12.3	96.4±11.7
dBP, mmHg	52.9±13.7	47.4±10.9	54.4±12.9	58.3±11.2	53.0±12.1

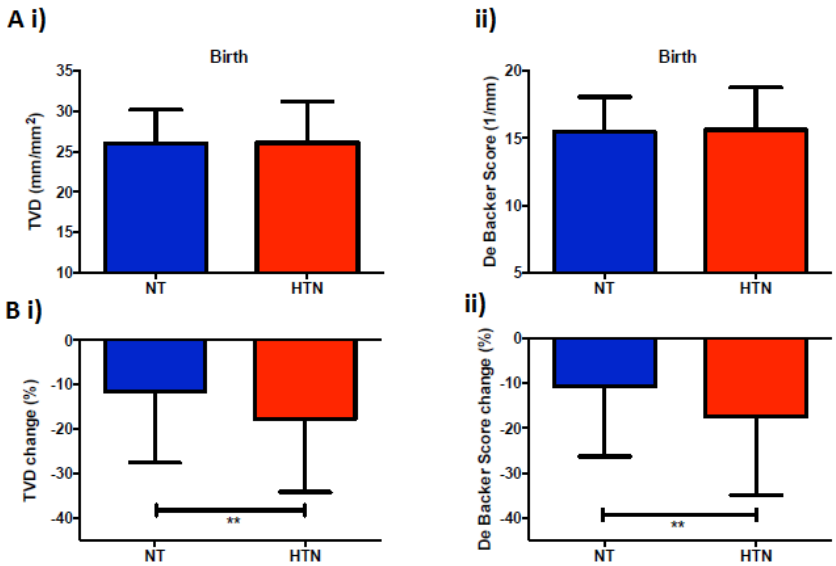
[†] Median±Interquartile range.

NT indicates normotensive; PET preeclampsia; PIH pregnancy induced hypertension; sBP systolic blood pressure; dBP diastolic blood pressure

7.4.2 Reduction in microvascular density in offspring of hypertensive pregnancies

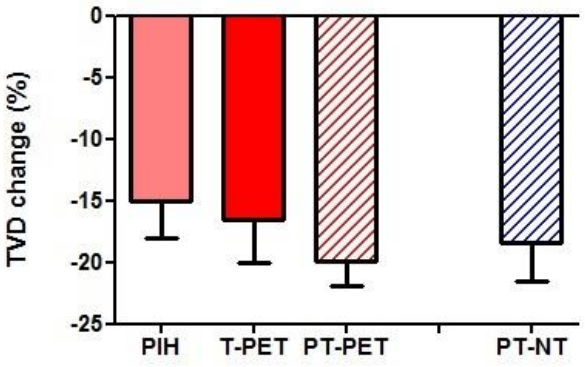
There were no differences in TVD or DB score between the offspring born to normotensive and hypertensive pregnancies at birth (Figure 7.2A). However, there was a significantly greater reduction in TVD as well as DB score between birth and three months of age in those born to hypertensive pregnancy compared to normotensive pregnancy (Figure 7.2B). The reduction in TVD and DB score between birth and three months was almost double in the hypertensive group compared to the normotensive group (TVD change -9.9%±-18.7 versus -17.7%±-16.4, p=0.002; DB change -9.6±-17.2 versus -17.3±-17.7, p=0.002).

Figure 7.2: (A) Total vessel density (i) and De Backer scores (ii) are similar in offspring born to normotensive and hypertensive pregnancies at birth. (B) Reduction in total vessel density (i) and De Backer scores (iv) between birth and three months is greater in the hypertensive group. Bar plots are presented as Mean±Standard Deviation. TVD indicates total vessel density; NT normotensive pregnancy; HTN hypertensive pregnancy. ** p<0.01.



There was a trend towards a graded reduction between birth and three months depending on the degree of hypertensive disorder of pregnancy, with offspring born to a pregnancy-induced hypertensive pregnancy (PIH) showing the smallest change and the preterm preeclamptic group (PT-PET) exhibiting the greatest reduction, although this did not reach statistical significance (PIH versus PT-PET, *p*=0.18; Figure 7.3).

Figure 7.3: Trend towards an increased offspring microvascular reduction over the first three months of life and severity of hypertensive disorder of pregnancy with preterm normotensive pregnancy group shown for comparison. Bar plots are presented as Mean±Standard Error of the Mean. TVD indicates total vessel density; PIH offspring exposed to pregnancy-induced hypertension; T-PET term preeclamptic pregnancy; PT-PET preterm preeclamptic pregnancy; PT-NT preterm normotensive pregnancy.

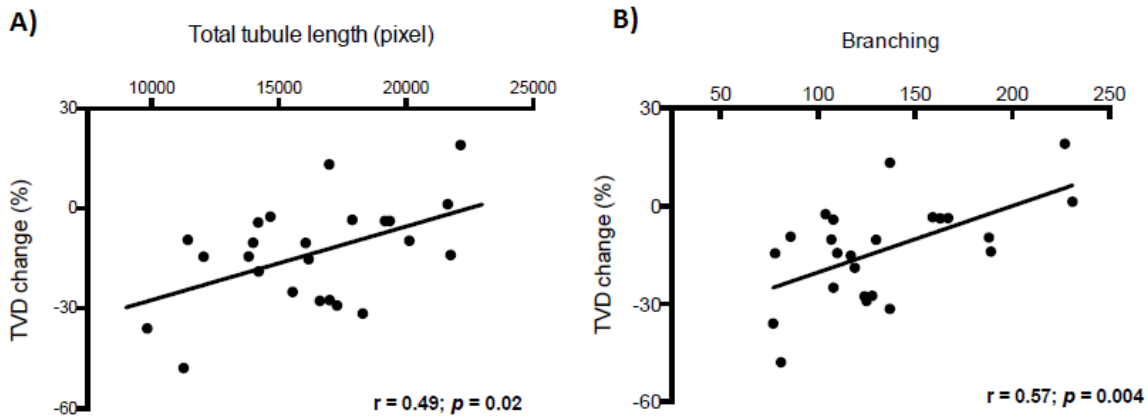


7.4.3 Correlation of *in vivo* and *in vitro* measures

Umbilical-derived cell samples and *in vivo* microvascular measures were available in the same individuals for 28 participants (10 following hypertensive and 18 normotensive pregnancies) and sample characteristics are presented in Table 7.1. *In vitro* tubule length and branching was not associated with *in vivo* measures at birth but there was a graded positive association between *in vitro* total tubule length and the loss of *in vivo* microvascular density over the first three months of life (r=0.49, *p*=0.02,

Figure 7.4A). Similarly, there was a graded positive association between *in vitro* branching and change in *in vivo* TVD over the first three months of life (r=0.57, *p*=0.004, Figure 7.4B).

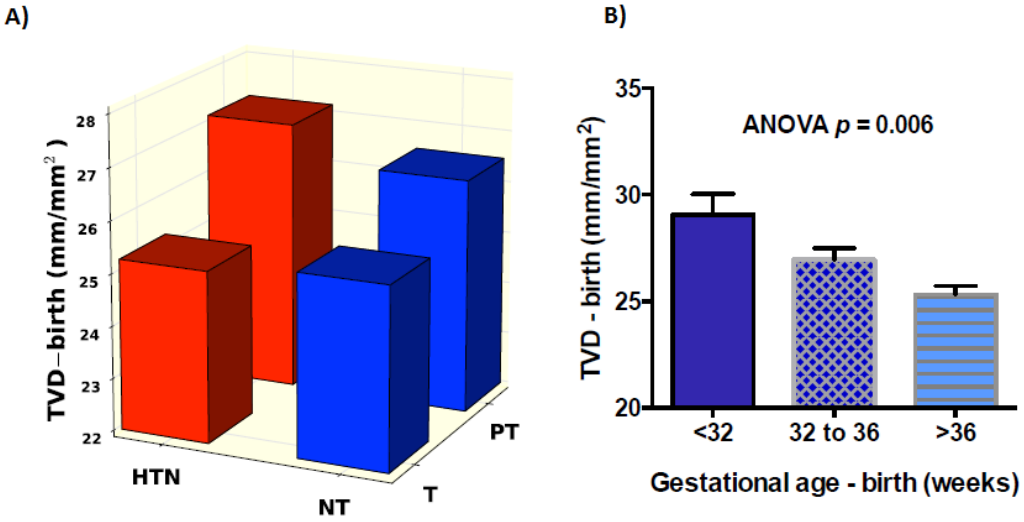
Figure 7.4: *In vitro* tubule network measurements (total tubule length (A) and branching (B)) correlate with change *in vivo* microvasculature over the first three months using matching samples from the same infant (n=28). TVD indicates total vessel density.



7.4.4 Preterm birth and microvascular density

In view of the close association between preterm birth and severe hypertensive pregnancy disorders I specifically studied the impact of preterm birth on vascular measures. In hypertensive pregnancies, babies born preterm (<37 weeks gestation) had a higher TVD at birth than those born at term (*p*=0.02) and this association was also evident in normotensive pregnancies (preterm hypertensive versus preterm normotensive p=0.47, Figure 7.5A). There was a graded relationship between gestational age and TVD at birth, with earlier birth related to greater vessel density loss (r=-0.19, *p*=0.005, Figure 7.5B).

Figure 7.5: Total vessel density is higher in offspring born preterm to both hypertensive and normotensive pregnancies compared to term pregnancies (A) and there is a graded relationship between gestational age and microvessel density at birth (B). Bar plots are presented as Mean±Standard Error of the Mean. *P*-value represents the result of a one-way ANOVA test between groups. TVD indicates total vessel density; NT normotensive pregnancy; HTN hypertensive pregnancy; T term; PT preterm.



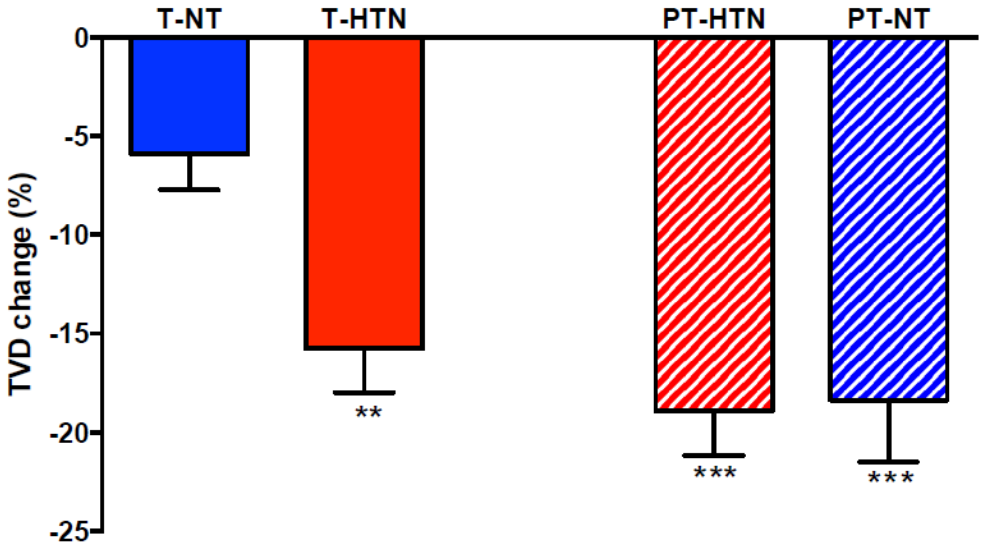
However, the offspring groups exposed to preterm birth and/or maternal hypertension were all associated with significantly greater reductions in microvessel density over the first three months compared to term offspring born to a normotensive pregnancy (Figure 7.6).

7.4.5 Other clinical predictors of microvascular density

To study the role of other potentially relevant factors such as growth restriction, mode of delivery and offspring blood pressure on these associations, I initially performed bivariate regression analyses to identify factors that associated with *in vivo* vascular

measures (Table 7.4). For total vessel density change, there were associations with maternal age at delivery, smoking, exposure to maternal hypertension, gestational age, birthweight z-score, blood pressure at birth and age at follow up. However, there was significant co-linearity between antenatal steroid exposure and preterm birth.

Figure 7.6: Both hypertensive and normotensive preterm offspring show a significant reduction in microvessel density during the first three months of life similar to that seen in term hypertensive pregnancies. Bar plots are presented as Mean±Standard Error of the Mean. *P*-values between each pregnancy complication group and the normotensive group are displayed. TVD indicates total vessel density; T-NT term-normotensive pregnancies; T-HTN term-hypertensive pregnancies; PT-HTN preterm-hypertensive pregnancies; PT-NT preterm-normotensive pregnancies. ** $p<0.01$; *** $p<0.001$.



In order to investigate whether antenatal steroids had an effect on the microvasculature independent of gestational age, I compared vessel density loss in a subgroup born late preterm who had received antenatal steroids to those who had not in order to compare like-sized groups (gestational age 35.9±0.5 vs 36.1±0.5 weeks, $p=0.14$; n= 20 vs 19 respectively). There were no significant differences between groups ($p=0.22$). I therefore took forward offspring blood pressure at birth, birthweight

z-score and gestational age into the multivariable model (Table 7.5). In this model, maternal hypertension remained a significant independent predictor of greater total vessel density reduction between birth and three months with more preterm birth also appearing to be associated independently with vessel density change.

Table 7.4: Bivariate regression coefficients for maternal and perinatal risk factors and reduction in total vessel density

	Change in TVD (%)		
	B	**95% CI**	***p*-value**
Maternal Factors			
Age at delivery, years	0.45	0.01 – 0.88	**0.04**
BMI at booking, kg/m^2	-0.009	-0.49 – 0.47	0.97
Maternal smoking during pregnancy	-9.51	-20.70 – 1.69	0.10
Booking sBP, mmHg	-0.02	-0.15 – 0.11	0.74
Booking dBP, mmHg	0.04	-0.13 – 0.22	0.62
Maternal hypertension during pregnancy	-6.74	-11.40 - -2.07	**0.005**
Perinatal Factors			
Gestational age, weeks	1.38	0.61 – 2.14	**<0.001**
Caesarean section	-2.05	-6.82 – 2.73	0.40
Age at birth assessment, days	0.25	-0.17 – 0.67	0.24
Birthweight z-score	3.05	1.01 – 5.09	**0.004**
Sex	0.41	-4.30 – 5.11	0.87
Apgar score (5mins)	-0.53	-3.56 – 2.50	0.73
Antenatal steroid exposure	-9.48	-14.33 - -4.64	**<0.001**
Birth sBP, mmHg	0.21	0.05- 0.37	**0.01**
Birth dBP, mmHg	0.22	-0.03 – 0.46	0.08
Postnatal infections	-0.48	-8.65 – 7.69	0.91
Days of oxygen	-0.17	-0.70 – 0.37	0.55
Age at follow up, days	0.14	-0.02 – 0.31	0.09
Three month sBP, mmHg	0.01	-0.18 – 0.21	0.91
Three month dBP, mmHg	0.04	-0.15 – 0.23	0.70

sBP indicates systolic blood pressure; dBP diastolic blood pressure.

Table 7.5: Multivariable regression coefficient for maternal and perinatal characteristics and reduction in total vessel density between birth and 3 months

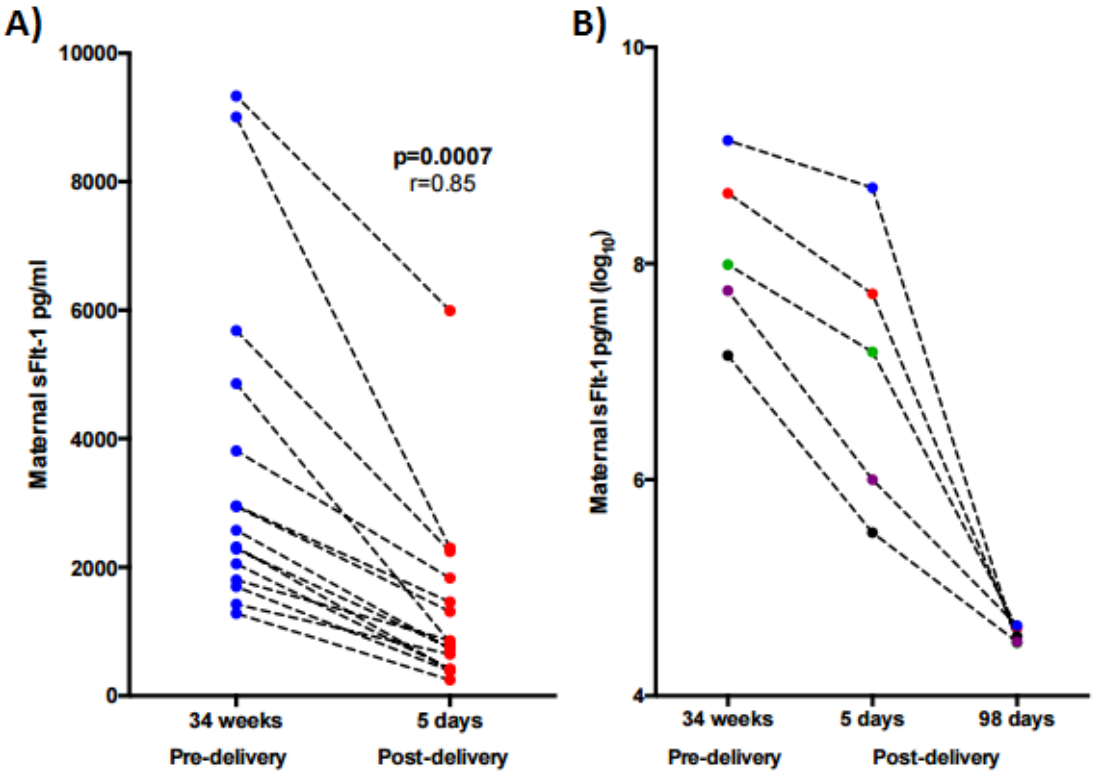
	Change in TVD (%)		
	B	**95% CI**	***p*-value**
Maternal age at delivery	0.43	-0.03 – 0.88	0.07
Smoking	-3.73	-15.30 – 7.85	0.53
Maternal hypertension during pregnancy	-6.46	-11.03 - -1.88	**0.006**
Gestational Age, weeks	1.07	0.28 – 1.86	**0.008**
Birthweight z-score	1.61	-0.45 – 3.67	0.13
Birth sBP , mmHg	0.11	-0.05 – 0.27	0.19
Age at follow up, days	0.13	-0.03 – 0.29	0.12

sBP indicates systolic blood pressure.

7.4.6 Maternal angiogenic profile and offspring microvascular development

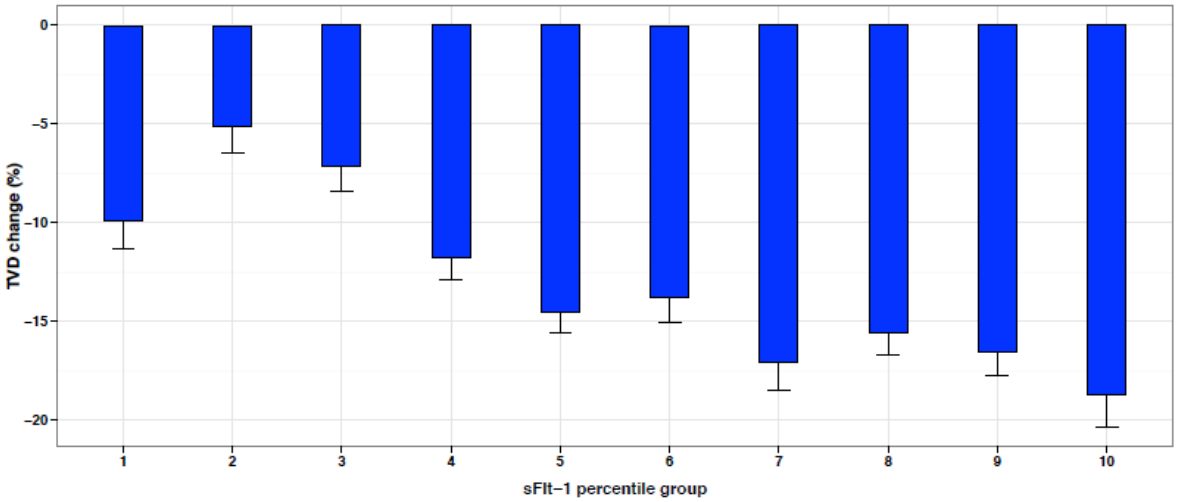
Maternal postnatal blood samples were available for 107 mother and offspring dyads. Samples were collected on average five days after birth, at which point, maternal sENG levels were still significantly higher in hypertensive mothers compared to normotensive mothers with a trend for higher sFlt-1 and lower VEGF (Table 7.1). In a subgroup of women, sFlt-1 measures in the last trimester were compared to early postnatal and three month postnatal values in the same patient. Measures at five days postnatally strongly correlate to late gestation values (r=0.85, *p*=0.007; Figure 7.7A). By three months, levels had fallen to very low values and no longer reflected either late gestation or early postnatal values (Figure 7.7B). Therefore associations identified with measures at 5 days are likely to relate to variation during pregnancy.

Figure 7.7: (A) Maternal sFlt-1 level is reduced from pre-delivery (34 weeks gestation, dots in blue) to post-delivery (average of five days, dots in red); and there is a significant correlation of maternal sFlt-1 measured at the two time points. (B) Change of maternal sFlt-1 levels from pre-delivery (at 34 weeks gestation), post-delivery (average of five days) and three months post-partum (average of 98 days). Each data point represents individual ELISA measurements. Dots with connection lines on Panel A and matching colour on B indicate paired maternal sample collected at each time point. sFlt-1 indicates soluble fms-like tyrosine kinase-1.



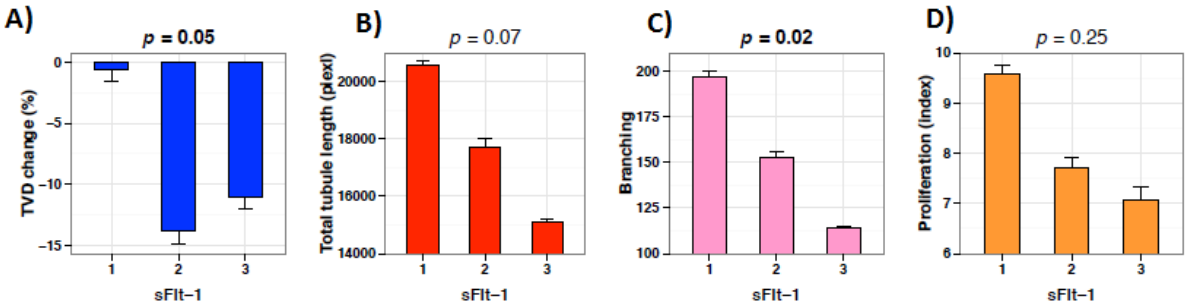
There was a graded relation between the level of sFlt-1 in the maternal blood sample and the loss of microvasculature in the offspring between birth and three months of age (Figure 7.8) with higher levels of sFlt-1, a predictor of greater vessel density loss (p=0.05). Although similar patterns were seen with levels of sENG and total vessel density reduction, the association was not significant. PlGF and VEGF levels were not related to microvascular loss.

Figure 7.8: Association between maternal sFlt-1 after delivery with total vessel density percentage change from birth to three months. Maternal sFlt-1 is presented in ten percentile groups. sFlt-1 indicates soluble fms-like tyrosine kinase-1.



To study whether this may represent a co-association with other clinical factors associated with hypertensive pregnancy, I performed a sensitivity analysis in normotensive pregnancies. Interestingly, significant differences across the tertiles of sFlt-1 were evident in offspring of normotensive mothers for TVD change (p=0.01; Figure 7.9A) and trends with respect to *in vitro* tube formation were also seen, although not all reached statistical significance (total tubule length p=0.07; branching p=0.02; proliferation p=0.25; Figure 7.9B, C and D). However, offspring of hypertensive pregnancies appeared to have increased microvessel density loss at all levels of angiogenic factors in their mother.

Figure 7.9: Comparison of maternal sFlt-1 tertiles with total vessel density percentage loss (A), total tubule length (B), branching (C) and proliferation (D) in normotensive pregnancies. Bar plots are presented as Mean±Standard Error of the Mean. *P*-values represent the result of a one-way ANOVA test between groups. TVD indicates total vessel density; sFlt-1 soluble fms-like tyrosine kinase-1.



7.5 Discussion

This study provides the first evidence of an almost two fold greater postnatal reduction in microvascular density in neonates born following a pregnancy complicated by hypertension compared to a normotensive pregnancy. The degree of postnatal microvessel loss in the infant is predicted by the vasculogenic capacity of their endothelial cells at birth, which, in turn, relates to levels of angiogenic factors in the maternal circulation around the time of birth. This phenomenon was evident across the spectrum of hypertensive pregnancy disorders and degree of growth restriction, and not attenuated by the increased vascular density seen at birth in those born preterm. Intriguingly, the associations were present in clinically normal pregnancies but in which anti-angiogenic factors were higher in the maternal circulation around the time of delivery. These findings identify a critical postnatal microvascular

developmental window and provide a potential explanation for how pregnancy pathophysiology links with later microvascular rarefaction in the offspring.

7.5.1 Previous studies

A reduction in total vessel density during the first three months of postnatal life[362, 363] [360] occurs as the disorderly fetal capillary network remodels into a horizontal subpapillary plexus and papillary loops structure. This postnatal phenomenon was demonstrated in my study and results from my normotensive cohort were in good agreement with previously reported findings.[362, 363] However, despite equivalent vessel density at birth, those born to a hypertensive pregnancy had a much greater change in the first three months of life. A previous study of infants born at term after preeclampsia found subtle reductions in vessel density measured with capillaroscopy soon after delivery and it is possible the process starts very early in some.[349] My recruitment strategy, sample size and perinatal data allowed me to include the full range of severities of hypertensive pregnancy and also a range of gestational ages within our normotensive group. The increased vessel density in those born preterm was a feature of prematurity, independent of the hypertensive disorder, with similar density at birth between hypertensive and normotensive pregnancies across the range of gestations. This is consistent with a previous study which demonstrated that offspring born preterm had higher skin capillary density at birth but which the authors put down to low birthweight rather than preterm birth per se.[358] However, although birthweight z-score was related to microvessel change in my study, this was accounted for by co-association with hypertensive pregnancy and preterm birth.

In modelling, gestational age was an independent predictor of a postnatal microvessel loss, along with maternal hypertension, consistent with our previous observation of capillary rarefaction in adults born preterm.[145] Low systolic blood pressure at birth was also a predictor and other complications that result in a low cardiac output or peripheral vasodilataion, for example in response to hypoxia or infection, may be worth investigation as influences on postnatal microvascular development.

7.5.2 Angiogenic profile and offspring microvasculature

Postnatal loss of vessel density was seen in hypertensive pregnancies across the spectrum of hypertensive pregnancies when severity was defined by clinical markers, such as gestation of diagnosis. I hypothesised that circulating biomarkers which reflect the maternal biological response to reduced placental perfusion[364] may more closely reflect the real potential impact of hypertensive pregnancy on fetal development. Strikingly, maternal levels of sFlt-1 predicted the postnatal offspring vascular postnatal changes across the range of pregnancy syndromes and still associated with vascular phenotype when analysis was restricted to normotensive, full term and appropriate for gestational age pregnancy groups. It may be that these mothers would have gone on to develop hypertension were it not for delivery as sFlt-1 levels have been shown to rise two to three months before clinical onset[348] although they did not correlate with risk factors for preeclampsia in my normotensive cohort. It may be that clinically diagnosed hypertensive pregnancy syndromes may sit at one end of a spectrum,[365] with subclinical endothelial activation, low grade inflammation and cardiovascular dysfunction[366] evident in a broader group of apparently normal pregnancies.[348] In

mothers with risk factors for hypertension, but who have normotensive pregnancies, a tendency towards an anti-angiogenic profile during pregnancy has been noted.[367, 368] Therefore, my findings of links between the maternal biological response to pregnancy and offspring postnatal vascular development may have relevance to hypertensive risk in a broader group of the population than is defined by hypertensive pregnancy alone.

Similar, but non-significant, patterns were observed with sENG despite it being the only biomarker that was significantly different between normotensive and hypertensive groups. However, sENG levels have been shown to fall more rapidly in preeclamptic women post-delivery,[369] which may explain the lack of significant associations.

VEGF levels were not associated with microvascular loss, although our group have demonstrated a positive correlation between VEGF levels and *in vitro* proliferation in this cohort.[238] Placental growth factor was also not associated with vascular phenotype in contrast to previous reports of associations with childhood vascular structure.[357, 367] These earlier findings were based on measures in mid pregnancy, when differences in PlGF are most evident[370] and, around the time of birth, levels have been shown to have decreased rapidly[370] so other factors may better reflect severity of the pregnancy syndrome.[348, 364, 367, 370-372]

After birth, the profile starts to normalise and my maternal samples had been collected at the postnatal infant assessment on average a week postpartum. The timing of measurement meant acute fluctuations related to labour and delivery were avoided but also means other biological reasons for persistent elevation of anti-

angiogenic markers in the mother post-delivery may need to be taken into consideration to understand why the offspring have these postnatal vascular changes. In addition, adults born to hypertensive pregnancies have differences in circulating levels of sIFlt-1 in later life[145] and persistent elevations in anti-angiogenic factors in the infant may be of relevance to their postnatal vascular remodelling. I did not have infant blood samples to test this hypothesis but studies have shown that, at birth, the offspring has changes in circulating factors that reflect those in the mother.[373]

7.5.3 Vasculogenic potential and offspring microvasculature

In my study I was also able to link for the first time multiple quantitative measures of *in vitro* cellular behaviour with *in vivo* vascular responses and maternal measures in the same cohort. Studies have shown that serum from preeclamptic women inhibits normal HUVEC microtubule formation, which can be rescued by exogenous VEGF and PlGF[374] and microtubule inhibition has been observed after the addition of recombinant sENG.[369] My results have demonstrated that there is not only a graded association between endothelial cell phenotype and maternal circulating factors but also that the vasculogenic capacity predicts subsequent loss of microvessels over the first three postnatal months. However, our group have previously demonstrated reduced microtubule formation ability in offspring-derived HUVECs taken from hypertensive pregnancies in control media[238] suggesting a specific persistent alteration in cellular phenotype seems more plausible.

Collectively, these findings raise the possibility that hypertensive pregnancies result in an impairment of vasculogenic potential of fetal endothelial cells resulting in an

endothelial cell dysfunctional phenotype. Similar changes have been shown in cord blood endothelial colony forming cells, which account for a proportion of HUVECs,[375] harvested after a preterm birth.[155] Therefore, changes in the vasculogenic potential of endothelial cells may underlie a common pathological pathway through which pregnancy complications result in capillary rarefaction. As a result, although microvascular density may be maintained in the offspring *in utero,* for example as a compensatory response to the relative hypoxia of the hypertensive pregnancy or through normal development in the run up to a preterm birth, on transition to the *ex utero* environment the infant may fail to remodel their vasculature. These changes may have long term relevance as those born preterm or to a hypertensive pregnancy have microvascular rarefaction throughout childhood and into adult life.[81, 145, 215, 216, 373, 376] By adulthood the offspring also have higher blood pressure and it has been suggested the microvascular changes may be secondary to the hypertension. In my neonatal cohort, blood pressures were similar at three months of age despite reductions in vessel density, consistent with microvascular changes being a primary event, similar to the pathophysiological pathways described in animal hypertensive models and other at risk populations.[152, 377]

7.5.4 Limitations

My study is associative and I did not aim to prove causality in this clinical observational study between the pregnancy maternal biomarkers, offspring vascular cell potential and postnatal vascular development. The total sample size is also relatively modest but I was able to undertake very detailed clinical, physiological and sample data collection

along with repeated measures over short time frames in order to investigate maternal hypertension and prematurity due to my stratified recruitment strategy. As such, the phenomenon I describe of a postnatal vascular loss in offspring of hypertensive pregnancies is striking, particularly in view of the links with multiple *in vitro* cellular assays and maternal blood samples. I feel these findings provide a robust basis for future intervention and experimental studies. It will be of interest to investigate whether the changes in dermal microvasculature reflect a generalised phenomenon involving other organ-specific vascular beds such as cardiac and pulmonary systems.

7.6 Conclusions

In conclusion, I have found offspring born to hypertensive pregnancies have reduced endothelial capacity for microtubulogenesis *in vitro* at birth. The degree of impairment predicts the degree of reduction in vascular density during the first three months of postnatal life, as the fetal microvasculature remodels into its postnatal *ex utero* structure. Intriguingly, these changes can also be predicted by the circulating biomarker profile of the mother around the time of birth, which suggests an association between the maternal biological state during pregnancy and offspring vascular development. Future studies will help define the mechanisms underlying the altered vasculogenic capacity and understand whether these changes are tractable to reduce the long term cardiovascular risk of the offspring evident in early life including hypertension and metabolic disease.

8: Cardiac Autonomic Function, Pregnancy Complications & Offspring Cardiovascular Development

In this chapter I will be investigating the effect of pregnancy complications on offspring heart rate variability at birth which provides a non-invasive measure of autonomic function. I will then explore the relationship between autonomic function at birth and cardiovascular development by looking for associations between changes in heart rate variability and the differences that I have found in cardiovascular structure and function that I have identified in previous chapters in infants exposed to pregnancy complications at birth and three months of age.

8.1 Abstract

Background – Heart rate variability (HRV) provides a non-invasive measure of autonomic function. I tested the hypothesis that pregnancy complications associate with HRV at birth and that disordered autonomic function identifies offspring with abnormal fetal and postnatal cardiovascular development.

Methods- 98 sleeping neonates had 5-minute electrocardiogram recordings at birth using a Shimmer® device connected via Bluetooth to an Android smartphone. Standard time (rMSSD, SDNN) and frequency (HF, LF, LF/HF ratio) domain parameters were calculated and associations with pregnancy complications identified. Autonomic function was then related to blood pressure, heart rate and cardiac structure and function, as well as microvascular and macrovascular measures at birth, to understand relevance to *in utero* development, and with measures at three months of age, to determine whether HRV parameters predicted abnormal postnatal developmental patterns.

Results- Increasing prematurity, but not maternal hypertension or growth restriction, associated with more deranged autonomic function at birth characterised by decreased HRV (B coefficient for gestational age at birth and rMSSD 0.99 week^{-1}, 95%CI 0.43 to 1.54, p<0.001; LF 23.50 week^{-1}, 95% CI 6.02 to 40.98, p=0.009; HF 19.25 week^{-1}, 95%CI 5.51 to 33.00, p=0.007) and a relative imbalance between sympathetic and parasympathetic tone (B coefficient for gestational age at birth and LF/HF ratio -0.20 week^{-1}, 95%CI -0.34 to -0.06, p=0.005). However, autonomic dysfunction did not predict differences in cardiovascular structural and functional measures at birth or three months.

Conclusions- Altered cardiac autonomic function at birth relates to gestational age rather than other pregnancy complications and does not predict cardiovascular developmental patterns. Longer term studies will be needed to understand relevance to cardiovascular risk.

8.2 Introduction

Heart rate variability (HRV) analysis provides a non-invasive measure of cardiac autonomic function based on variation in the QRS to QRS (RR or normal to normal NN interval) interval sequence of the electrocardiogram (ECG). The derived metrics of HRV allow evaluation of sympathetic and parasympathetic balance within the autonomic nervous system (ANS) and the ability of the sinoatrial node to adapt to extrinsic signals. In a multitude of well-designed studies decreased HRV has emerged as a strong predictor of cardiac risk in adults and death in patients at increased cardiovascular risk.[378-384] Interestingly, attenuation in HRV is also evident in infants born preterm[266, 267] with dysfunction being greater in those with higher clinical illness scales[265] or pathological problems such as respiratory distress syndrome,[251-256] birth asphyxia,[253, 257] intraventricular haemorrhage,[253, 258] small-for gestational age[259] and sudden infant death syndrome.[260-264] Pregnancy complications, in particular, preterm birth and maternal hypertension, have been found to associate with an increased risk of cardiovascular disease in later life[145] and the offspring display a distinct cardiovascular phenotype characterised by microvascular rarefaction and cardiac hypertrophy.[108, 109, 238]

These cardiac and vascular patterns become evident during the first three months of life when differences in autonomic function have been identified in preterm infants.[216, 238, 376] Therefore, I investigated, for the first time, using short ECG recordings in a large cohort of newborn infants, whether differences in neonatal HRV relates just to prematurity or are found in other pregnancy complications linked with later cardiovascular disease. Furthermore, I studied whether altered HRV may be a marker of abnormal *in utero* or postnatal cardiac and vascular development in these infants.

8.3 Methods

8.3.1 Study population

This chapter includes data from infants in whom heart rate variability measures were performed at birth. These infants also underwent detailed cardiovascular phenotyping at birth and three months of age. Inclusion and exclusion criteria and diagnostic definitions are detailed in Section 2.1. Details of these cohorts and how they were recruited are set out in Chapter 2.

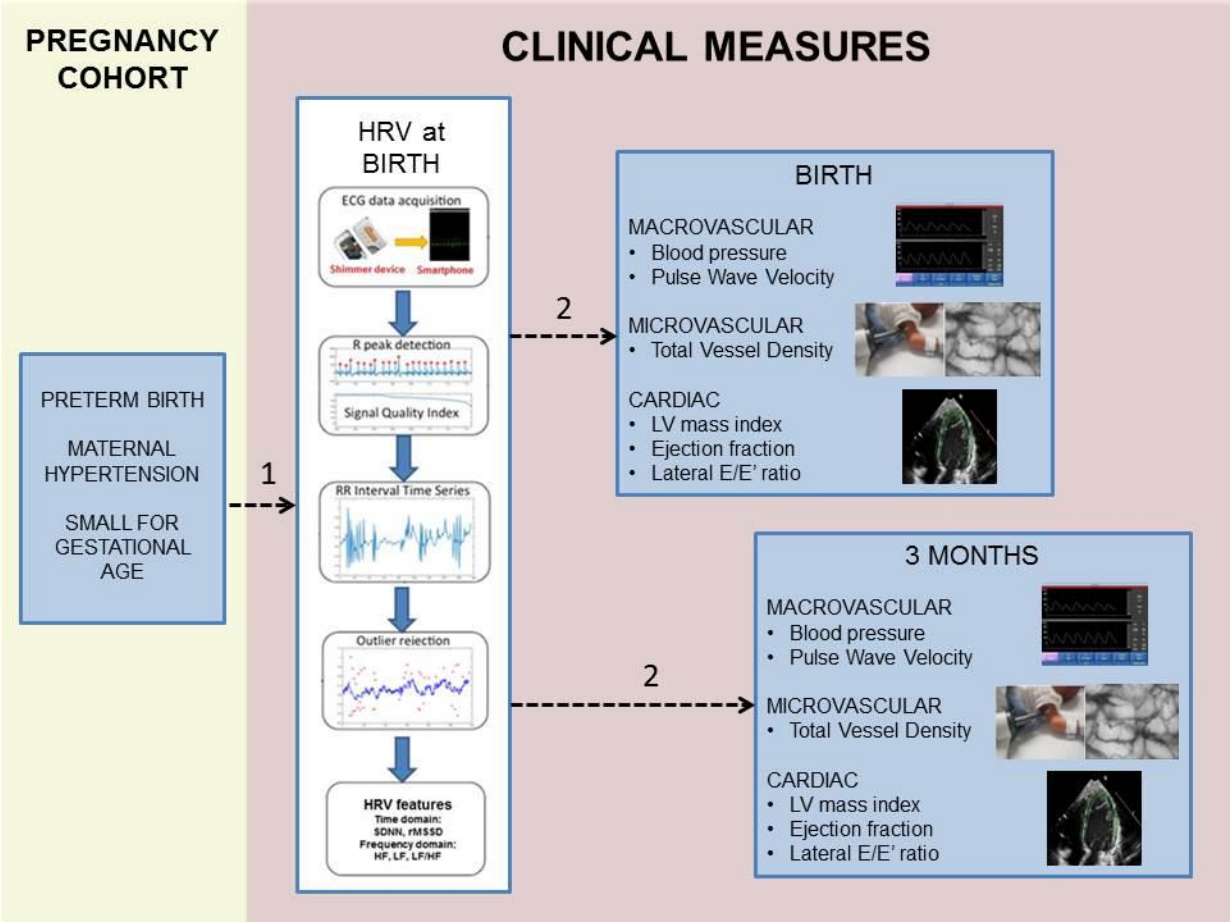
8.3.2 Study visit

Infants underwent the following assessments and analysis at birth and three months (Figure 8.1), which are described in detail in Chapter 3:

- Anthropometry (Section 3.3.2)
    - Weight

    - Head circumference
- Echocardiography (Section 3.4.1)
    - Cardiac structure
        - LV mass
    - Cardiac function
        - LV systolic and diastolic function
- *In vivo* microvascular imaging (Section 3.5.1)
    - Total vessel density
- Macrovascular measures
    - Blood pressure (Section 3.6.1)
    - Pulse Wave Velocity (Section 3.6.2)
- Autonomic function – at birth only (Section 3.7.1)
    - Heart rate variability
- Medical Records and Questionnaires (Section 3.9 and Appendix)

Figure 8.1: Overview of study design investigating whether (1) pregnancy complications had an effect on heart rate variability at birth and (2) if heart rate variability at birth predicted cardiovascular development at birth or at 3 months of age as measured by macrovascular, microvascular and cardiac assessments in the offspring. HRV indicates heart rate variability.



8.3.2.1 Additional methods - ECG processing and analysis

After the ECG recordings were acquired (Section 3.7.1), the data was processed and analysed by Dr Julien Oster in the Institute of Biomedical Engineering and the acquired HRV measures returned for further interpretation.

Data processing – The ECG signals were processed in order to extract the RR interval time-series, but also a Signal Quality Index (SQI). These features were extracted using

previously published techniques.[385] The SQI was used in order to select the "best" five minute segment, and the RR interval time-series from this segment was kept for heart rate variability (HRV) analysis.

Heart Rate Variability analysis – The five minute RR interval segment was used to extract Heart Rate Variability (HRV) features. These features were extracted using the HRV toolkit which has been validated and is freely available online.[386, 387] Processing included detection and extraction of the normal to normal (NN) interval time-series and automated outlier removal for rejection of artefactual RR points. Calculated HRV features were based on basic time-domain HRV statistics used in the literature, specifically, the standard deviation of the NN intervals (SDNN) and root mean square of the difference between adjacent NN intervals (rMSSD). The frequency-domain features were extracted using the Lomb periodogram, eliminating the need for evenly sampled data in contrast to the traditional Fast Fourier Transformation. The benefit of this is that sections of the recordings in which there are gaps or extreme noise in the data can be omitted. Parameters included the total spectral power of all NN intervals between 0.05 and 0.2 Hz (low frequency, LF), the total spectral power of all NN intervals between 0.2 and 1 Hz (high frequency, HF) and the ratio of low to high frequency power (LF/HF ratio) using cut offs previously suggested in the literature for the neonatal population.[388-391] In order to be able to standardise these measures, only recordings during which the babies were asleep throughout were included in analysis.

8.3.3 Statistics

Statistical analysis is described in detail in Section 3.10.

8.3.3.1 Power calculation

The sample size n=33 preterm and n=65 term offspring provided me with 80% power at a significance level of α=0.05 to detect a difference of at least 0.85 standard deviations (SDs) between groups in rMSSD.

8.4 Results

8.4.1 Study population characteristics

The technology for assessment of HRV became available during the course of recruitment to the EPOCH study and therefore, out of the 255 infants in the full neonatal cohort, 140 had an ECG taken at birth. Of these, 3 were unanalysable due to poor signal quality and 40 were excluded as the infant was awake or restless during acquisition. Maternal and offspring demographic and anthropometric characteristics in the cohort are presented in Table 8.1 with subgroup characteristics available in Table 8.2.

Table 8.1: Cohort characteristics

	***n* = 98**
Maternal Demographics & Anthropometrics	
Maternal age at delivery, years	33.0±4.6
BMI at booking, kg/m^2	25.4±5.1
Smokers, n (%)	2 (2)
Maternal hypertension during pregnancy, n (%)	46 (47)
Offspring Birth Characteristics	
Gestational age at delivery, weeks	37.9±2.9
Males, n (%)	48 (49)
Birth order$^{\Psi}$	1±1
Caesarean section, n (%)	36 (37)
Antenatal steroids, n (%)	28 (29)
Offspring Physiological Measures at Birth	
Head circumference, cms	33.5±2.2
Birthweight, grams	2964±754
Birthweight z-score	0.10±1.0
sBP, mmHg	81±15
dBP, mmHg	44±10
Pulse Wave Velocity, (m/sec)	5.5±1.5
Offspring Physiological Measures at 3 months	
Weight, grams	5763±950
Head circumference, cms	40.5±1.6
sBP, mmHg	97±12
dBP, mmHg	53±13
Pulse Wave Velocity, m/sec	6.5±1.7

Values as Mean±Standard Deviation unless stated otherwise
$^{\Psi}$ Median±Interquartile range

Table 8.2: Subgroup cohort characteristics

	Preterm-NT (n=14)	Preterm-HTN (n=19)	Term-NT (n=32)	Term-HTN (n=33)
Maternal Demographics & Anthropometrics				
Maternal age at delivery, years	32.4±2.9	35.0±5.5	33.1±3.3	31.9±5.0
BMI at booking, kg/m^2	24.1±4.2	27.7±7.1	23.6±3.5	26.5±4.8
Smokers, n (%)	0(0)	1(5)	0(0)	1(3)
Offspring Birth Characteristics				
Gestational age at delivery, weeks	34.4±1.1	34.4±1.8	40.0±1.3	39.6±1.4
Males, n (%)	7(50)	9(47)	19(59)	13(39)
Birth order$^{\psi}$	1(1)	1(0)	1(1)	1(0)
Caesarean section, n (%)	4(29)	13(68)	8(25)	11(33)
Patent ductus arteriosus, n (%)	0	0	0	0
Antenatal steroids, n (%)	11(79)	17(89)	0	0
Offspring Physiological Measures at Birth				
Age at assessment, days	4.8±3.8	5.1±3.4	3.4±4.2	3.2±3.7
Head circumference, cms	31.0±1.3	31.6±1.6	35.1±1.4	34.4±1.7
Birthweight, grams	2245±372	2227±640	3562±477	3283±585
Birthweight z-score	-0.05±0.83	-0.15±1.25	0.55±0.91	0.08±1.16
sBP, mmHg	79±18	81±17	82±13	81±12
dBP, mmHg	42±13	45±10	44±9	44±9

8.4.2 Pregnancy complications and heart rate variability

Those born preterm had a higher heart rate and lower heart rate variability than those born term (Figure 8.2A). There was a positive association between SDNN, rMSSD, LF and HF and gestational age at birth; although the association with SDNN failed to reach significance after adjusting for postnatal age at assessment and offspring sex (Table 8.3). There was a negative correlation between LF/HF ratio and gestational age at birth even after adjustment. There was no significant difference in heart rate or heart rate variability parameters between those born to mothers with or without maternal hypertension (Figure 8.2B and Table 8.3). I separately analysed those whose mothers had a more severe hypertensive disorder of pregnancy, classified as preeclampsia, and found no differences in neonatal heart rate variability in this group (Figure 8.2C). Furthermore, there were no significant associations between birthweight z-score and any HRV parameter (Table 8.3), or any difference between those classified as small or appropriate for gestational age (Figure 8.2D). I additionally studied whether particular perinatal clinical features, including days of oxygen or APGAR score, predicted autonomic dysfunction in those born preterm but were not able to identify specific markers of more deranged function except for a borderline association between caesarean delivery and a greater rMSSD (Table 8.4).

Figure 8.2: Boxplots demonstrating (A) a significantly decreased rMSSD and increased heart rate in offspring born preterm but no significant difference in those exposed to maternal hypertension (B), preeclamspia (C) or those born small for gestational age (D). rMSSD indicates root mean squares of successive NN differences; HTN exposure to maternal hypertension; PET preeclamptic pregnancy; SGA small for gestational age; AGA appropriate for gestational age.

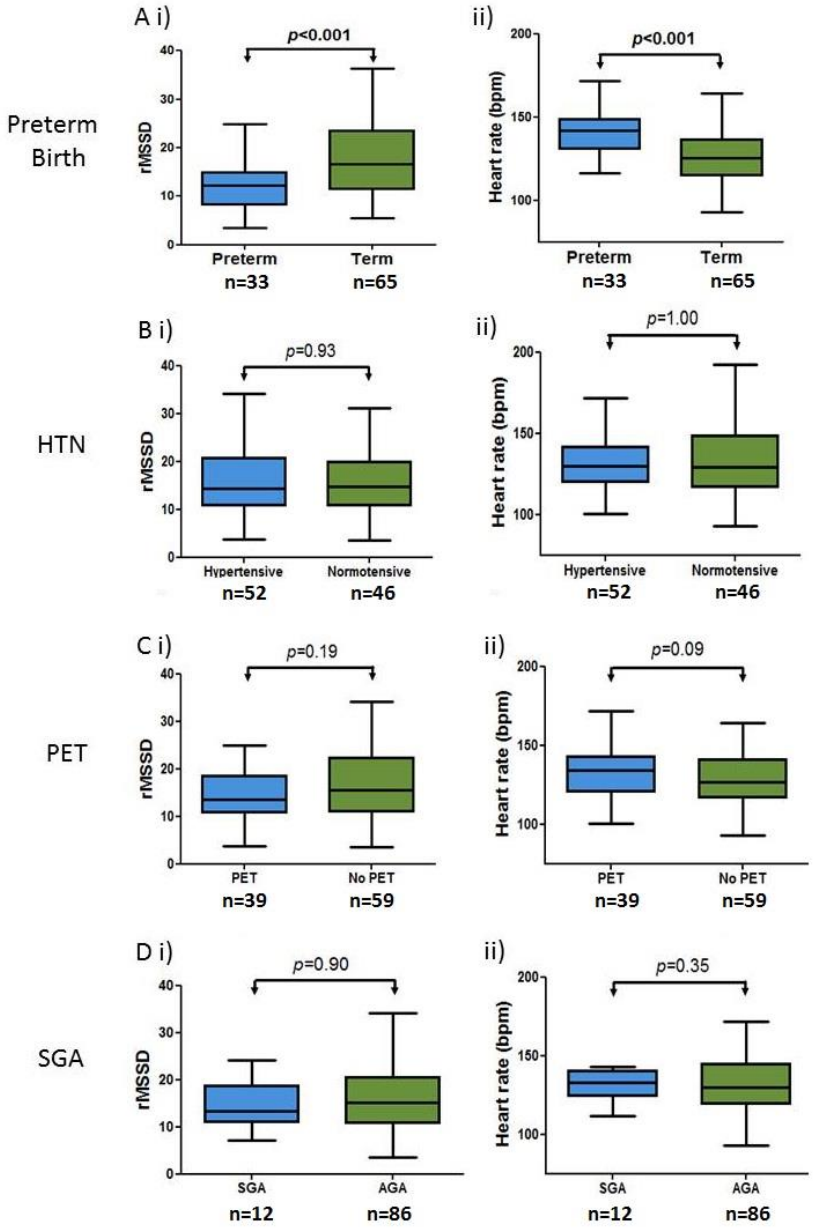


Table 8.3: Bivariate regression coefficients for heart rate variability parameters at birth and pregnancy complications

	Gestational age at birth (weeks)			**Maternal Hypertension**			**Birthweight z-score**		
	B	**95% CI**	***p*-value**	**B**	**95% CI**	***p*-value**	**B**	**95% CI**	***p*-value**
SDNN	0.76	-0.13-1.66	0.09	2.30	-2.92-7.53	0.38	-1.92	-4.32-0.47	0.11
rMSSD	0.99	0.43-1.54	**0.001**	0.24	-3.17-3.65	0.89	-0.41	-1.98-1.17	0.61
LF	23.50	6.02-40.98	**0.009**	61.16	-42.84-165.16	0.25	-41.59	-89.25-6.07	0.09
HF	19.25	5.51-33.00	**0.007**	9.28	-73.32-91.87	0.82	-10.85	-48.96-27.27	0.57
LF/HF ratio	-0.20	-0.34- -0.06	**0.005**	0.34	-0.49-1.17	0.42	0.15	-0.23-0.53	0.44

B unstandardized coefficient presented with 95% confidence intervals after correcting for postnatal age at assessment and offspring sex
SDNN standard deviation of NN interval; rMSSD root mean squares of successive NN differences;
LF low frequency spectral power; HF high frequency spectral power.

Table 8.4: Bivariate regression coefficients for perinatal clinical features and rMSSD at birth

	rMSSD		
	B	**95% CI**	***p*-value**
Maternal smoking	-3.29	-14.98-8.39	0.58
Antenatal steroids	0.11	-0.02-0.24	0.58
Caesarean section	3.63	-7.16- -0.10	**0.04**
APGAR score at 5 mins	0.20	-2.06-2.46	0.86
Days of oxygen	0.16	-1.04-1.36	0.80
Postnatal infection	-1.28	-7.69-5.13	0.69

B unstandardized coefficient presented with 95% confidence intervals after correcting for postmenstrual age at assessment and offspring sex
rMSSD indicates root mean squares of successive NN differences.

8.4.3 Relationship with cardiovascular structure and function at birth and three months of age

I studied whether heart rate variability was a predictor of other cardiovascular developmental differences at birth or related to those known to be found in preterm offspring, specifically postnatal cardiac hypertrophy and microvascular rarefaction. I based analysis on associations with rMSSD at birth, as the HRV parameter with the strongest association with gestational age. However, there were no association with these parameters (Table 8.5) even when the cohort was split into preterm and term groups (data not shown).

Table 8.5: Bivariate regression coefficients for cardiovascular development in early postnatal life and rMSSD at birth

	Birth			3 Months		
	B	**95% CI**	***p*-value**	**B**	**95% CI**	***p*-value**
Macrovascular						
sBP, mmHg	-0.11	-0.47-0.25	0.55	0.07	-0.23-0.38	0.63
dBP, mmHg	-0.03	-0.27-0.20	0.78	-0.03	-0.34-0.28	0.85
PWV, m/s	-0.01	-0.05-0.03	0.63	0.01	-0.03-0.05	0.60
Microvascular						
TVD, mm/mm^2	-0.04	-0.17-0.09	0.56	0.02	-0.08-0.11	0.75
Cardiac						
LVMI, g/m^2	0.00	-0.09-0.09	1.00	-0.04	-0.18-0.12	0.60
Ejection fraction, %	0.06	-0.23-0.34	0.69	0.20	-0.05-0.46	0.11
Lateral E/E' ratio	0.002	-0.07-0.07	0.96	-0.06	-0.11-0.01	0.07

B unstandardized coefficient presented with 95% confidence intervals after correcting for postnatal age at assessment and offspring sex

sBP indicates systolic blood pressure; dBP diastolic blood pressure; PWV pulse wave velocity; TVD total vessel density and LVMI left ventricular mass index.

8.5 Discussion

In this study I have demonstrated that heart rate variability parameters derived from short length ECG recordings in the first week of life are significantly associated with gestational age at birth. HRV is decreased in preterm infants compared to term counterparts with reduced parasympathetic activity[392-394] and a relative imbalance between sympathetic and parasympathetic tone.[250] In contrast, I found no association between HRV and exposure to maternal hypertension or fetal growth restriction within our cohort. I have also found no evidence that HRV at birth associates with patterns of cardiovascular development in the early postnatal period that I have previously reported in those born preterm in Chapters 5 and 7.[238]

8.5.1 Heart rate variability measurements

HRV is controlled by the autonomic nervous system and information on the functionality of the homeostatic mechanisms of the heart via the vagus and sympathetic nerves may be derived by measuring HRV indices. HF power, like the time-domain parameter rMSSD, is thought to be dependent on vagal tone, whereas LF is influenced by both sympathetic and parasympathetic arms of the autonomic system. [392-394] Consequently, LF/HF ratio reflects the balance between the two arms of the ANS and is an indicator of sympathetic influences on heart rate.[250] In general, the lower the frequency of interest, the longer the duration of recording [250] and therefore the very low frequency (VLF) component of the spectrum (<0.05Hz in this study) was not analysed due to the short length of my ECG recordings.

8.5.2 Heart rate variability and pregnancy complications

Preterm birth has been associated with altered autonomic control characterised by an increased sympathoadrenal activity.[395, 396] My findings are consistent with several previous studies that have also observed reduced HRV at birth in those born preterm.[266, 267, 397] The changes observed could have been established in response to specific *in utero* stressors linked with the preterm birth. Alterations in maternal heart rate variability are seen in pathological pregnancies; with preeclampsia having been associated with reduced maternal heart rate variability, which worsens as the pregnancy progresses.[380-382] Interestingly, there is evidence that this is of relevance to the child as maternal autonomic heart rate modulation relates to fetal heart rate patterns in hypertensive pregnancies.[380, 398] However, my data suggests that any links between maternal and fetal heart rate do not persist after delivery in those born to hypertensive pregnancies, irrespective of the severity or classification of the hypertensive disorder. Furthermore, even when there is evidence of fetal compromise, with a reduced birthweight z-score or classification as small for gestational age, if there are any *in utero* differences in autonomic function, they are not evident after birth although, interestingly, adults born small for gestational age have been shown to have sympathetic nerve hyperactivity.[399]

8.5.3 HRV abnormalities in preterm infants

An alternative explanation for my, and others, findings in those born preterm is that they merely reflect a relative functional immaturity in autonomic system (ANS) activity. The fetal ANS develops progressively throughout pregnancy,[400-404] with more rapid

development of the parasympathetic branch,[405, 406] and therefore differences in ANS function at birth would be expected, proportional to gestational age.

Nevertheless, this functional immaturity at birth could still have pathological significance. Previous studies have demonstrated deficits in HRV parameters in the preterm population may persist after birth up to term equivalent age,[407-412] which suggests normal development may require the fetus to remain *in utero* until term.[259, 405, 406] In one longer term study of preterm offspring HRV, any differences observed seem to have attenuated by two years of age and the two groups were still similar at six to seven years old.[410] Another potential hypothesis for this delayed or arrested maturation is disordered anatomical and cellular development of the nervous system[413] or disruption of neuropeptide synthesis caused by inflammatory events[414] which are more common in the preterm population. HRV has been shown to be altered in conditions such as intraventricular haemorrhage[258] and sudden infant death syndrome[261-264] as well as being an indicator of the severity of clinical conditions such as respiratory distress syndrome,[252, 253, 255, 256] and clinical illness scales.[265] Nutritional,[415] environmental[416, 417] or iatrogenic stress[418-422] in the *ex utero* environment has also been potentially linked with abnormal ANS development, although it is unclear as to whether ANS immaturity is actually the primary insult which results in inappropriate adaptation to these stressors causing decreased HRV. However, previous studies looking at the beneficial effect of skin-to-skin contact on preterm HRV seem to suggest the former explanation.[423, 424] In addition, emotional stress and anxiety in adults has also been shown to inhibit parasympathetic activity.[425]

In my cohort, perinatal conditions were not related to HRV and measures at birth were not predictive of cardiac and vascular structure and function at birth or the postnatal changes in these parameters I have reported in this preterm group. Therefore, altered ANS function is unlikely to explain these cardiovascular developmental differences in those born preterm. The lack of association between HRV measures and cardiovascular development may be because my preterm subgroup had an average gestational age of 34.4 weeks which is later than some of the other studies that show a residual deficit at term equivalent age[408, 409] and the frequency of severe clinical postnatal conditions was low. In addition, another study only showed differences in HRV in babies born between 27-33 weeks with no difference in their late preterm group (34-36 weeks) compared to term infants in the first week of life which implies that the ANS in my cohort may already be mature.[397]

An alternative explanation might be that HRV, although being a sensitive measure of overall autonomic imbalance which also allows evaluation of cardiovagal function to a certain degree, does not allow sufficient assessment of sympathetic function and therefore predominance of pathology in one of the ANS branches might have been obscured by compensatory interactions with the other ANS branch that were not captured by HRV analysis.[426, 427]

8.5.4 Future work

Since my data demonstrate that overall cardiac autonomic dysfunction relates to increasing prematurity but appear not to associate with altered cardiovascular development it clearly highlights the necessity of follow up research to elucidate

whether separate assessment of sympathetic and parasympathetic functional integrity (including non-cardiac measures) might provide additional insights into the mechanisms whereby birth complications such as preterm birth affect the development of the cardiovascular system.

The ANS of late preterm infants matures more quickly after birth[411] and previous studies that showed continued reduction in HRV at term equivalent age or later have tended to be in the more extreme preterm infants born prior to 32 weeks.[408, 409, 428] Therefore it remains possible a functional immaturity of the ANS has a greater impact on cardiovascular development for the more extreme preterm infant.

Whether the autonomic dysfunction I have observed at birth in those born preterm could be of relevance to the increased risk of hypertension in adulthood, independent of changes in cardiac and vascular phenotype,[429-431] remains to be seen in future studies. HRV attenuation has long been implicated in adult cardiovascular disease states such as acute myocardial infarction[378] and congestive heart failure.[379] Changes are also seen in other conditions in adults such as depression,[432] sepsis,[433] chronic obstructive pulmonary disease[434] and diabetes.[435] However, one longer term study of preterm offspring HRV found early differences were attenuated by two years of age and equivalent to term born offspring by six to seven years of age,[410] suggesting there would need to be a re-emergence of HRV differences in adult life.

8.5.5 Limitations

Studies of ANS function in neonatal populations need to confront several challenges. I used state-of-the art, small, remote monitoring technology to capture data but the analysis needed to be based on short length recordings of between 2 and 15 minutes. However, the measures that were derived from these short recordings have previously been shown to correlate well with parameters measured from longer recordings in adults.[436] Measurement of HRV is also only an indirect measurement of autonomic function but structural measures of the ANS, such as skin nerve biopsies, are not feasible in the neonatal population. In addition, specific measurements of sympathetic function such as by measuring muscle sympathetic nerve activity by microneurography[437] would be technically challenging in this age group which might constitute one of the major challenges in future studies of the specific role of the integrity of both ANS branches in cardiovascular development. I used data on sleeping infants so as to control external conditions and stimulation to make it easier to interpret differences in HRV parameters between groups of subjects. However, I did not differentiate between active and quiet sleep states using a simultaneous electroencephalogram but instead stopped recordings during periods of observed unrest. Nevertheless, a previous study has suggested in healthy term neonates that there is no difference in HRV measures between groups when divided into behavioural states during sleep[401] with a close agreement between low mean heart rate and quiet sleep and high mean heart rate and active sleep in infants.[438] Mean respiration rate during the recording was also not recorded although, again, a previous study has suggested this may not correlate with HRV indices.[401]

There was also a significant difference in the postnatal age at which the ECG recordings were taken with the term infants being on average younger by two days. However, time and frequency domain HRV indices have been shown to increase over the first week of postnatal life in both in heathy term newborns [401, 439] and either increase [253, 255, 390, 440] or stay the same[266] in preterm infants during this time period and therefore differences between groups may again have been underestimated. I also did not adjust for confounding factors such as maternal medication or diseases other than cardiovascular which may have affected their offspring.

8.5.6 Conclusions

In summary, heart rate variability at birth is significantly associated with gestational age at birth with increasing prematurity resulting in increased autonomic dysfunction as suggested by reduced time and frequency domain heart rate variability parameters. No associations between HRV and maternal hypertension or fetal growth restriction were found. In addition, I found no evidence that autonomic function at birth had an impact on cardiovascular development in the early postnatal period, but whether it in part explains the long-term risk of hypertension in offspring exposed to pregnancy complications remains to be seen.

9: CONCLUSIONS

In conclusion, this thesis aimed to characterise the cardiovascular phenotype of infants born to complicated pregnancies over the first three months of postnatal life. The objective was to identify differences in development that may be of relevance to long-term cardiovascular sequelae associated with pregnancy complications in these cohorts. This body of work has provided a better understanding of the effects of preterm birth and hypertensive disorders of pregnancy on cardiac, macrovascular and microvascular structure and function. Key perinatal factors were identified and potential mechanisms for these changes were explored, such as changes in vasculogenic potential, maternal angiogenic profile and autonomic dysfunction.

This was achieved through conducting detailed cardiovascular investigations at birth and again at three months of life on a cohort of babies born preterm and/or to hypertensive pregnancies with comparison to a control group born term to a normotensive pregnancy. The three month time point was specifically selected as the phenotypic switch in cardiomyocytes from hyperplastic to hypertrophic pattern and the restructuring of the microvascular system which is known to take place after birth

would have been completed. Fetal echocardiography was also performed in a cohort of infants in order to explore *in utero* cardiac changes. Information was collected from medical and obstetric records in addition to patient questionnaires. Maternal biomarkers and umbilical cords were also collected to give further insights into potential mechanisms underlying any differences observed.

The first objective was to create nomograms of fetal cardiac structure using two dimensional echocardiography as none currently exist in the literature. This was so I could, in subsequent chapters, create trajectories of cardiac development from fetal through to postnatal life with which to compare data from infants born to complicated pregnancies. Therefore, in Chapter 4, I used a state-of-the art automated software to derive off-line measurements of cardiac mass and volume from a single-plane four chamber view in a cohort of fetuses from 15 weeks through to 42 weeks gestation. These measurements were found to be feasible and reproducible and when compared to previously published results using more novel techniques, were in good agreement especially up to 28 weeks gestation. In late third trimester, my estimates were in better agreement with previous neonatal studies, suggesting that, at extremes of gestation, the two dimensional technique may actually be the method of choice.

In Chapter 5, I then went on to quantify changes in left and right ventricular structure, function and shape between preterm and term born offspring using two dimensional echocardiography. Infants born preterm had a structurally normal but smaller heart at birth. Fetal to neonatal cardiac structure trajectories confirmed similar *in utero* cardiac development in the preterm group compared to term born counterparts. Over the first

three months of life, there was a disproportionate ventricular hypertrophy in infants born preterm, graded with the degree of prematurity, associated with diastolic dysfunction. Maternal hypertension was a predictor of right ventricular mass change but was not significant in multivariable models. Shape differences in the left ventricle in the preterm group had normalised by three months of age. This pattern of ventricular hypertrophy was similar to one that has been observed in preterm born young adults in previous studies published by our group[108, 109] and supports the hypothesis of a premature switch in cardiomyocytic phenotype in this cohort.

I then wanted to further explore the effect of maternal hypertension in the absence of preterm birth. In Chapter 6, I excluded all of the preterm born infants in my cohort and compared the ventricular structure and function of those born to hypertensive and normotensive pregnancies. At three months, left ventricular mass indexed to body surface area and ventricular volume was increased in those born to hypertensive pregnancies, which was correlated with capillary rarefaction over the first three months of life. Intriguingly, this relationship was not seen in the preterm hypertensive cohort, supporting the idea that the two pregnancy complications may cause ventricular hypertrophy by different mechanisms and that prematurity may outweigh any effects from maternal hypertension. Right ventricular mass indexed to body size at three months was correlated with markers of hypertensive disease severity and may be linked to pulmonary vascular dysfunction observed in offspring of preeclamptic pregnancies. No differences in cardiac function were observed.

In Chapter 7, I then sought to establish changes in *in vivo* microvascular structure between birth and three months of age in offspring exposed to maternal hypertension

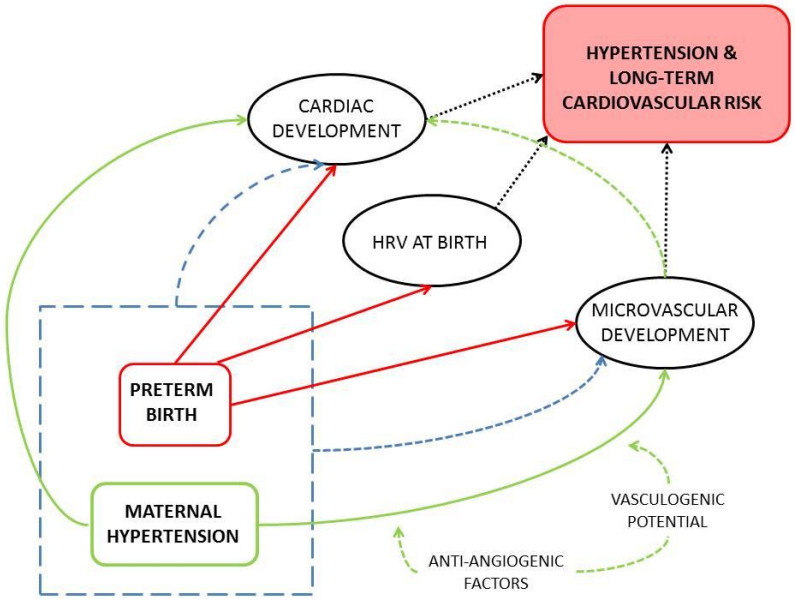
and their association with *in vitro* vasculogenic potential, maternal angiogenic profile and preterm birth. I found that infants exposed to a hypertensive pregnancy had an increased capillary density loss which was correlated with maternal anti-angiogenic profile at birth and with vasculogenic potential in their human umbilical vein endothelial cells.[238] Interestingly, the preterm normotensive group also had an increased capillary density loss which was not significantly different to the preterm hypertensive group suggesting that prematurity also has an effect on microvascular structure which needs to be further explored.

My final results chapter (Chapter 8) aimed to quantify changes in heart rate variability parameters at birth in babies born to pregnancy complications and investigate their relationship with any cardiovascular changes observed in these individuals from previous chapters. At birth, infants born preterm showed signs of autonomic dysfunction which was graded with degree of prematurity, but these were not related to cardiovascular changes seen over the first three months of life despite increased long term risk of hypertension in this population. Infants exposed to hypertensive disorders of pregnancy and those born small for gestational age did not demonstrate any differences in heart rate variability compared to controls.

The results from my thesis (Figure 9.1) have strengthened our understanding of the cardiovascular changes that occur in the early postnatal period in infants born either preterm or to a hypertensive pregnancy. As 10% of infants are born preterm and 8% of pregnancies are affected by hypertensive disorders of pregnancy, any findings are relevant to a growing population of individuals. My findings point towards a distinct cardiovascular phenotype in those born to a complicated pregnancy which is not

present at birth, but emerges over the early postnatal period. It is not yet possible to attribute the changes seen to the increased risk of hypertension and cardiovascular disease observed in these individuals in later life. However, as my findings of increased ventricular mass, decreased capillary density and autonomic dysfunction are in other populations related to increased cardiovascular risk, one can only postulate that the differences found are likely relevant to long term sequelae. It is interesting to note that these differences occur in the absence of blood pressure differences between groups, suggesting that developmental changes are likely to be the primary event rather than a downstream effect of hypertension. As such, those born to complicated pregnancies require counselling to reduce modifiable risks and close monitoring and follow up throughout life for early diagnosis and treatment of cardiovascular disease.

Figure 9.1: Summary of findings from the thesis. Dashed lines indicate results that require further investigation.



9.1 Future questions

This work has enabled the identification of key differences in cardiovascular development in infants born preterm and/or to a hypertensive pregnancy, potentially leading to an increased risk of cardiovascular disease in later life. Future work needs to address three questions:

1) What is the driving force behind these cardiovascular changes?

Although this body of work has identified differences in cardiovascular development in infants born to complicated pregnancies with some hypotheses and associations put forward, further work needs to be carried out in order to elucidate the underlying key pathophysiological mechanisms behind these changes. In addition, it would be interesting to investigate other perinatal exposures and interventions that may be relevant.

Firstly, it would be fascinating to further study the effects on cardiovascular development in the fetal period. Although I created preterm trajectories of cardiovascular development, numbers were small and it would have also been of value to create similar trajectories of babies exposed to hypertensive pregnancies. In addition, gathering information on indices of placental resistance such as umbilical artery doppler measurements may have given insights into the mechanism behind ventricular hypertrophy in hypertensive pregnancy offspring. Collecting cord blood at birth would have also given more information on circulating angiogenic factors in the fetus rather than sampling maternal blood which is not in direct contact with the fetal

circulation. It may also be interesting to look at blood markers which are elevated in both preterm birth and hypertensive disorders of pregnancy such as corticotropin-releasing hormone (CRH).[441, 442]

As the mean gestational age of the preterm group was 34 weeks, most of the babies did not spend a prolonged amount of time in the neonatal unit and did not require therapeutic interventions during the postnatal period. It was therefore not possible to investigate effects of many postnatal exposures on cardiovascular development such as ventilation which has been shown to account for some of the variation in right ventricular mass in young adults born preterm.[108] It is also difficult to ascertain whether my findings can be extrapolated to infants born very preterm, although previous studies suggest that similar patterns exist[108, 109] and the results from my thesis are relevant to a large population given that 80% of preterm births occur between 32 and 36 weeks gestation.[7] Collection of detailed data on nutrition during the postnatal period may also have been of interest as previous studies have shown that intravenous lipids[158] and breast milk[160] have a long-term effect on the cardiovascular system of the offspring.

In addition, it would be of great interest to investigate the effects of preterm birth based on its aetiology. Does a baby that is born spontaneously preterm due to cervical incompetence have the same cardiovascular changes as one that is induced early due to being small for gestational age? Some of my results addressed the interaction between prematurity and maternal hypertension, although this could be explored further with greater numbers. In addition, it would be fascinating to further characterise infants born to preeclampsia versus those born to pregnancy induced

hypertension. Clinical data on these subgroups from my cohort suggest that they have distinct phenotypes and long term data from other cohorts support this.[216] However, any differences in the cardiovascular development were not significant, although this could be due to insufficient numbers or reflect the difficulty in clinically distinguishing between the two conditions.

2) ***What is the long-term relevance of these changes seen in the early postnatal period?***

In order to understand the long term relevance of my findings, tracking this cohort into later life is required. Currently, I am collaborating with Dr Adam Lewandowski, a postdoctoral research fellow in our group, to design a study to follow up my cohort. We are aiming to perform echocardiography in order to investigate whether the disproportionate increase in left and right ventricular mass continues into childhood. This will allow for the extension of cardiac growth trajectories which I created in collaboration with Dr Eric Ohuma. Measures of function and shape will also be performed using the same methods as in this thesis. Furthermore, more detailed cardiac imaging using cardiac magnetic resonance imaging will be performed in order to investigate whether the altered cardiac morphology relates to altered cardiac flow changes and dynamics. We are also proposing to take blood samples from this cohort in order to measure the anti-angiogenic profile in the children and performing retinal vascular imaging in order to study whether microvascular structural changes persist.

3) Are there any therapeutic strategies that can be employed to prevent the progression of cardiovascular disease in individuals born to complicated pregnancies?

There are three ways in which therapeutic strategies may prevent cardiovascular disease in the offspring. The first is through prevention of the complication itself. A huge body of work is currently taking place in order to try and prevent preterm birth. However, most of the focus is in preventing extremely or very preterm births. My thesis has identified potential long term effects on the cardiovascular system of being born late preterm. These births, therefore, also need to be prevented if possible. For example, traditionally, women who have spontaneously ruptured their membranes prematurely are induced at 34 weeks gestation as at this point it is felt that the risk of infection outweighs the risk of prematurity. However, if it is known that at 34 weeks there are may be long term cardiovascular implications for the baby, would this alter decision making? Education of women hoping to conceive may also help prevent pregnancy complications. For example lifestyle factors such as optimising BMI and smoking cessation may help in addition to the use of low dose aspirin to minimise the risk of hypertensive disorders of pregnancy.

Where complications cannot be prevented, the second therapeutic strategy is to optimise the management of pregnancy complications in order to help to reduce long term sequelae in the offspring. Strict and early treatment of hypertension during pregnancy may be warranted as blood pressure levels were found to be correlated with right ventricular mass at three months in the offspring. The choice of antihypertensive agents may also have an effect. First line treatment for hypertension

in pregnancy is traditionally either a beta blocker such as labetalol or a calcium channel antagonist such as nifedipine. As calcium channel antagonists have been shown to increase capillary density,[341] it may be that this is the treatment of choice in order to try to minimise microvascular density loss in the offspring.

The third and final strategy is the treatment of individuals born to pregnancy complications. My thesis has identified the early postnatal period as a critical time for cardiovascular development and one that can be targeted in future interventional studies. Therapeutic interventions such as ACE-inhibitors which have been shown to reverse cardiovascular changes in preterm animal models[342] and also be beneficial in left ventricular hypertrophy and heart failure[443, 444] may be worth considering. Further work in elucidating the effects of perinatal interventions on offspring cardiovascular development may also help optimise the care of babies born to complicated pregnancies.

Longer term, it is important to monitor individuals born to a complicated pregnancy. Education in order to optimise lifestyle and regular monitoring for the early identification and treatment of cardiovascular disease is essential. Other therapeutic interventions such as the modification of cardiovascular physiology for example through exercise training programmes are currently being evaluated by our research group in the form of a randomised control trial.

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Appendices

Participant invitation letter and information sheet

Participant questionnaires

Endothelial cell isolation, processing and analysis

DIVISION OF CARDIOVASCULAR MEDICINE, RADCLIFFE DEPARTMENT OF MEDICINE
University of Oxford
Cardiovascular Clinical Research Facility
Level 1 Oxford Heart Centre, John Radcliffe Hospital, Oxford OX3 9DU

ANTENATAL Invitation Letter- Prematurity/Hypertension

Effect of Prematurity and hypertensive disorders of pregnancy on Offspring Cardiovascular Health (EPOCH) Study

Dear Parent

The University of Oxford Division of Cardiovascular Medicine and Departments of Obstetrics and Paediatrics are doing a joint research project to understand the potential long term effects of complications during pregnancy such as raised blood pressure and premature birth on the health of infants. You and your baby are eligible to participate in this study because you have developed high blood pressure or preeclampsia (high blood pressure and protein in the urine) during your pregnancy and/or will give birth prematurely. We would like to provide you with some brief information about the study to see if you would be interested in participating.

What is the EPOCH Study? The purpose of the EPOCH study is to find out whether babies born to a mother who had complications during pregnancy, in particular raised blood pressure and premature delivery, have differences in how their heart and blood vessels look and work before birth or immediately after they are born.

Why is this being studied? Previous research suggests that children have slightly higher blood pressure if their mothers had preeclampsia or they were born prematurely. Although the children's increase in blood pressure is not high enough to need treatment during childhood, it is possible that these babies have changes in the way their heart and blood vessels function after birth and we want to investigate if this is the case. We are specifically looking at changes in the few days before and after birth because this is known to be a time of rapid changes and development in the blood vessels of infants. We also want to find out whether this process is different in infants after one of these complications. This knowledge could help to develop ways to help prevent heart disease later in life in these children.

Do we have to participate? It is completely up to you whether or not you and your baby want to participate.

What will happen if we do participate? Participating in our study involves having an extra ultrasound scan of your baby's heart before it is born (Visit 0) and then taking measurements at birth (Visit 1) and when your baby is 3 months old (Visit 2).

Visit 1 will happen in the first few days after your baby is born usually whilst you are still in hospital.

> For you it involves a blood test and a questionnaire asking questions about your pregnancy and some lifestyle factors
> For your baby it involves taking special scans of the heart, blood vessels and arm, taking blood pressure readings and measuring their size and weight. An additional blood sample will be taken at the same time (and by the same person) as blood samples being taken as requested by your baby's doctor – if your baby does not need a blood test as part of their medical care they will NOT receive one as part of this study.
> We will also review you and your baby's medical notes for detailed information about the pregnancy, delivery and medical treatment your baby has received since birth.

Visit 2 will be when your baby is 3 months old, and involves exactly the same tests for you and your baby – except that there will be NO further blood tests for your baby. All of the scans on your baby's heart and blood vessels are safe and non-invasive and have been performed before on infants the same age as your baby.

Are there any benefits for me and my baby? There is no direct benefit for you or your baby as individuals taking part in this study. We hope that this information may one day help to identify babies who are at higher risk of developing heart disease later in life and subsequently develop ways to prevent or reduce the risk of heart disease in these infants.

What happens now? If you are interested in learning more about the study let one of the medical team looking after you know and we will organise for you to meet one of the researchers and receive some more detailed information on this study and what it would involve for you and your baby.

Thank you for taking the time to read this.

Yours Sincerely

Professor Paul Leeson
Honorary Consultant Cardiologist
Department of Cardiovascular Medicine
Clinical Cardiovascular Research Facility
University of Oxford

Dr Brenda Kelly
Consultant Obstetrician
Department of Obstetrics and Gynaecology
John Radcliffe Hospital

Dr Kenny McCormick

Consultant Neonatologist
Department of Paediatrics
John Radcliffe Hospital

DIVISION OF CARDIOVASCULAR MEDICINE
UNIVERSITY OF OXFORD
LEVEL 6 WEST WING, JOHN RADCLIFFE HOSPITAL, OXFORD OX3 9DU
Tel: 01865 234679 Fax: 01865 221111

PARTICIPANT INFORMATION SHEET

Effect of Prematurity and hypertensive disorders of pregnancy on Offspring Cardiovascular Health (EPOCH) Study

ANTENATAL - Prematurity/Hypertension

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study.
Feel free to ask any question if there is anything that is not clear or if you would like more information. Thank you for reading this.

PART 1

What is the purpose of the study?
The purpose of the study is to find out whether babies of mothers who had complications during pregnancy, in particular raised blood pressure (hypertension) during pregnancy or a preterm delivery (before 37 weeks), have any differences in the structure and function of their heart and surrounding vessels immediately before or after they are born.

Studies have shown that children whose mothers had these complications during their pregnancy tend to have slightly higher blood pressure in childhood and early adult life. Although the increase in blood pressure is not sufficient to need treatment during childhood we wish to establish why this may be the case. In the future, this information may allow us to develop better ways to prevent heart disease risk in later life.

To do this, we need to compare the way the heart and blood vessels look and function in babies whose mothers had these problems to babies whose mothers did not. We also want to compare babies born prematurely to those born at term.

Why have I been invited?
You and your baby are being invited to take part in the study because you have either experienced high blood pressure during your pregnancy (isolated or in the form of preeclampsia) or you will give birth to your baby prematurely.

Do we have to take part?
It is up to you to decide whether or not you are happy to take part. If you decide to take part, you are free to change your mind at any time without giving a reason. This would not affect the standard of care you or your baby receive. If you decide that you and your baby no longer wish to continue with the study, we would still retain any data already obtained unless you request otherwise

What would happen if we take part?

If you agree to take part there will be three assessments. The first will involve an ultrasound scan of your baby's heart before it is born, just like at your 20 week scan, which will take about 30 minutes (VISIT 0). This will happen just before your baby is born, usually at the same time as your last antenatal clinic appointment or after you have been admitted to hospital for the delivery of your baby. The next assessment will happen in the few days after your baby's birth usually while you and your baby are still in hospital which should take less than an hour and can be divided into two, shorter visits if that is more convenient for you. This visit includes two non-invasive scans of your baby's heart and blood vessels. These scans are all safe and suitable for infants from birth onwards, including those cared for in Neonatal Units. The final visit (VISIT 2) will occur when your baby is around 3 months old.

VISIT 0 (before birth)

1. *Obstetric ultrasound scan (30 minutes)*
   We will look at the structure and function of your baby's heart and big blood vessels.

VISIT 1 (after birth, usually during hospital stay)

Information from you:

1. *Questionnaire (10 minutes):*
   Ask you questions related to the current and (any) previous pregnancies and your and your family's medical history.
2. *Blood tests (10 minutes):*
   Take a blood sample from your arm (about 20mls), to check levels of glucose and cholesterol as well as measure substances released by blood vessels that may tell us about their function. These can be done at the same time as any tests that your doctors may want you to have.

Information about your baby:

1. *Scan of the Small Vessels in the skin (10 minutes)*
   We will take a movie of the blood vessels under the baby's arm to see how fast the blood cells are flowing and how many small blood vessels there are.
2. *Ultrasound Scan including heart beat recording (echocardiogram and electrocardiogram) (30 minutes)*
   We will look at the structure and function of your baby's heart and big blood vessels as well as looking your baby's body composition by measuring the fat on your baby's arm using an ultrasound scan. We will also record your baby's heart beat.
3. *Measurements of weight and length (5 minutes)*
   We will measure your baby's weight and length (just like they were measured and weighed after birth).
4. *Blood Pressure: (5 minutes)*
   Your baby's blood pressure will be measured using a special cuff designed for infants.

5. *Blood Tests (5 minutes)*
   A small additional sample (2-3 ml) to test for substances released by blood vessels that may tell us about their function will be taken by staff specially trained in taking blood from your baby. This will be done at the same time as routine clinical blood tests requested by your doctor. If your baby does not need any blood tests then we will NOT take any samples.

All of these measurements would be undertaken while you are still in hospital. Neither you, nor your baby would need to stay in hospital for any longer because of the study. You will be present with your baby at all times, and if your baby becomes distressed for any reason we can stop at any point.

We will also review the medical records of both you and your baby to gather information about your health, pregnancy, labour and delivery and any treatment that your baby has received since birth.

VISIT 2 (follow up visit)

After you are discharged from hospital we will ask you and your baby to come back to a follow up appointment about 3 months later. This follow up will take place at the Cardiovascular Clinical Research Facility at the John Radcliffe Hospital or the Day Assessment Unit at the Horton General Hospital. You will need to arrive to the appointment fasted (nothing to eat or drink) for 4-6 hours – your baby can feed normally right up to the appointment. Travel expenses will be reimbursed by filling a claim form. At this appointment we will:

1. Ask you to complete another questionnaire related to your lifestyle and your baby's feeding and growth.
2. Again look at your baby's medical records to record any medical treatment they have received since the last time we saw them.
3. Take a second blood sample from you but NOT from your baby

Repeat the measurements of small blood vessels, the ultrasound scan, blood pressure readings and the measurements of weight and length on your baby in exactly the same way as Visit 1.

After this your participation in our study will be finished. We do however ask whether you would mind us contacting you in the future about similar studies relating to cardiovascular health.

What is being tested?
We are testing the structure and function of the heart and blood vessels which may help understand how and why certain groups of babies develop higher blood pressure later in life.

Are there any other possible risks from taking part?
It is not felt that there are any significant disadvantages or risks for you or your baby in taking part. Some people find drawing the blood samples (venepuncture) transiently uncomfortable and may develop slight bruising at the site of needle entry. Our staff are highly competent in venepuncture and will make sure you are as comfortable as possible. All of the other tests are non-invasive and all of them have been used before on young children and newborns.

What are the possible benefits?
There is no benefit for you or your baby as individuals taking part in this study. We hope that by studying the impact of high blood pressure in pregnancy and premature delivery, it may help to improve the understanding of the development of heart disease risk in certain babies later in life. Eventually, we hope this information will identify ways of detecting the babies at greatest risk in order to develop ways of monitoring, preventing and treating heart disease early. If we did identify that you or your baby has any evidence that you are at greater risk of heart disease, such as very high blood pressure, with your permission, we would inform your GP who could undertake appropriate follow up and treatment.

What happens when the research study stops?
You will officially end participation in the study. Copies of any publications connected to this study will be available on request from Professor Paul Leeson (paul.leeson@cardiov.ox.ac.uk).

Will my taking part in the study be kept confidential?
Yes. We will follow ethical and legal practice and all information about you will be handled in confidence.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

PART 2

Will my/my baby's taking part in the study be kept confidential?
If you take part in the study, this will be indicated on your hospital medical records [and your GP would also be informed]. Your baby's medical records would also include that they have participated in this study. Some parts of your medical records and the data collected from the study would only be looked at by authorised persons from the University of Oxford, to check that the study is being carried out correctly. They may also be looked at by representatives of the regulatory authority or authorised persons from the NHS Trust. All investigators have a duty of confidentiality to you and your baby as research participants and nothing that could reveal your identity would be disclosed outside the research site. The data collected from the study will be recorded anonymously and you would not be identifiable from this.

What will happen to any samples I/ my baby give?
All samples will be retained in a secure environment for future analysis and will be stored in an anonymous format at the John Radcliffe Hospital and Wellcome Trust Centre for Human Genetics, University of Oxford under the custodianship of the Department of Cardiovascular Medicine. The samples of serum and DNA will be stored for up to 10 years and may be used in future research as our understanding of blood vessel function grows. Future research may include genetic research (see below). Any other samples will be destroyed at the end of the study period.

Will any genetic tests be done?
It is possible that some samples will be used for genetic research. This research may be conducted by the study research team or collaborating research teams. The samples will be stored in an anonymous format but a record of who donated the samples will be kept so that we can relate any findings to your medical history. Keeping these records ensures that if you decide to withdraw your consent for us to keep your or your baby's data, we will be able to destroy your samples. The genetic tests would involve looking at common variations in genes that affect how blood vessels work. We do not propose to test for inherited genetic diseases, or for conditions that will involve any other members of your family. There is no evidence to suggest that the results of these genetic studies are likely to have significant implications for you personally.

What will happen to the results of the research study?
We anticipate that the results will be published in a scientific journal for the benefit of the wider medical community. However, individual patients will not be identified in any publication and your personal and clinical details will remain strictly confidential. Any scientific publications arising from the study will be available on request to all participants. You would have no legal right to a share of any profits that may arise from the research.

Who is organising and funding the research and who has reviewed the study?
The research is funded by the British Heart Foundation. It has been organised by researchers at the University of Oxford, and the University of Oxford will act as research sponsor for this study.

This study was given a favourable ethical opinion for conduct by the Berkshire Research Ethics Committee.

Participation in future research

We will ask if we can contact you about future studies. If you consent, we will keep your contact details separately from research data you have provided. Both your details and data will carry the same unique ID. This means your data is anonymised but that we can "link" details to data. In this way we can approach subjects about studies relevant to their particular history. You can withdraw your consent for future contact at any time.

Unexpected findings on your/ or your baby's tests

In the unlikely event the blood tests or scans show any significant abnormality, a designated clinical specialist will discuss the implications with you, your GP or treating Doctor and may arrange for further investigations as necessary. However, it is important to note that we do not carry out tests for diagnostic purposes, and therefore these tests are not a substitute for a clinical appointment. Rather, our tests are intended for research purposes only. So if we find anything unusual, it would be appropriate for us to contact your GP so that they can arrange ongoing clinical care for you. But we would only do this after we and the specialist had discussed your options and gained your permission.

Involvement of the general practitioner

Your general practitioner (GP) will be informed of your participation in the study.

What will happen if I don't want my baby to carry on with the study?

You are free to withdraw your baby from the study at any time. If you wish, we can then just make use of the information we already have. Alternatively, we can ensure if your samples and information are used for future research it is entirely anonymously, or we can destroy any identifiable samples or information.

What if something goes wrong or I have a complaint?

Any problems would be dealt with initially by the researchers conducting the study. For complaints that have not been resolved by this, the departmental manager for cardiovascular medicine, Mrs. Lynn Clee, can be contacted *(lynn.clee@cardiov.ox.ac.uk)*. The University has arrangements in place to provide for harm arising from participation in the study for which the University is the Research Sponsor. NHS indemnity operates in respect of the clinical treatment with which you are provided. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you in addition to those of the University of Oxford detailed above.

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (contact Professor Paul Leeson) or you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 572224 or the head of CTRG, email ctrg@admin.ox.ac.uk .

Further information and contact details

If you wish to know more about any aspect of the study, please contact the Cardiovascular Clinical Research Facility on (01865) 572 832, Dr Christina Aye (christina.aye@cardiov.ox.ac.uk) or Professor Paul Leeson (paul.leeson@cardiov.ox.ac.uk).

EPOCH Pilot Study (Lifestyle Questionnaire 1)

Subject's Study Number .....

Date of Birth.....

Study Date……..

Questions about medical history

1 Have you ever been told that you have/had any of the following and year of diagnosis:

High blood pressure	YES	NO	(Year……...)
Diabetes	YES	NO	(Year……...)
High cholesterol	YES	NO	(Year……...)
Other long term illness	YES	NO	(Year……...)

Comments…….....

2 Current medication list and duration of treatment

3. Medications taken prior to pregnancy (and duration of treatment)

4 Were you born prematurely? YES NO Don't Know

If so how prematurely (weeks) ……..

5 Did your mother have preeclampsia or problems with blood pressure when she was pregnant with you?
YES NO Don't Know

6 What was your weight at birth (if known) …….

Questions about pregnancy (please circle as appropriate)

1 Total number of pregnancies 1 2 3 4 more

	1st pregnancy	2nd pregnancy	3rd pregnancy	4th pregnancy
Year of Pregnancy and Month of LMP				
Diagnosis of preeclampsia				
What stage of pregnancy was this diagnosed (weeks)				
What treatment was received?				
Diagnosis of High Blood pressure during pregnancy				
What stage of pregnancy was this diagnosed				
Other problems during pregnancy				

What stage of pregnancy				
Treatment?				
Length of pregnancy (weeks)				
Mode of delivery				
Did the baby have medical complications? Comment				
Any speacial baby care or treatment				

	5th pregnancy	6th pregnancy	7th pregnancy	8th pregnancy
Year of Pregnancy and Month of LMP				
Diagnosis of preeclampsia				
What stage of pregnancy was this diagnosed (weeks)				
What treatment was received?				
Diagnosis of High Blood pressure during pregnancy				
What stage of pregnancy was this diagnosed				
Other problems during pregnancy				
What stage of pregnancy				
Treatment?				
Length of pregnancy (weeks)				

Mode of delivery				
Did the baby have medical complications? Comment				
Any speacial baby care or treatment				

Other Comments……

Questions about your family medical history

Have any members of your family ever suffered from (if more than 2 affected siblings continue on blank paper): Please tick all that apply

	mother	m age of onset	father	f age of onset	sibling1	s1 age of onset	sibling2	s2 age of onset
1 Angina	☐	☐☐	☐	☐☐	☐	☐☐	☐	☐☐
2 Heart attack	☐	☐☐	☐	☐☐	☐	☐☐	☐	☐☐
3 High blood pressure	☐	☐☐	☐	☐☐	☐	☐☐	☐	☐☐
4 Stroke	☐	☐☐	☐	☐☐	☐	☐☐	☐	☐☐
5 Diabetes	☐	☐☐	☐	☐☐	☐	☐☐	☐	☐☐
6 High blood cholesterol	☐	☐☐	☐	☐☐	☐	☐☐	☐	☐☐

7 Pre-eclampsia ☐ ☐☐ ☐ ☐☐ ☐

8 Restricted growth at birth ☐ ☐☐ ☐ ☐☐ ☐ ☐☐ ☐ ☐☐

9 Is there an illness which runs in your family? YES NO Don't know

9b If **yes**, what is the illness .....

Questions about the baby's father's health and family history

1 Has he ever been told that he has/had any of the following and year of diagnosis:

High blood pressure	YES	NO	(Year……...)
Diabetes	YES	NO	(Year……...)
High cholesterol	YES	NO	(Year……...)
Other long term illness	YES	NO	(Year……...)

Comments…….....

2 Current medication list and duration of treatment

3. Was he born prematurely? YES NO Don't Know

If so how prematurely (weeks) ……..

5 Did his mother have preeclampsia or problems with blood pressure when she was pregnant with him?
YES NO Don't Know

6 What was his weight at birth (if known) …….

Have any members of his family ever suffered from (if more than 2 affected siblings continue on blank paper): Please tick all that apply

	mother	m age of onset	father	f age of onset	sibling1	s1 age of onset	sibling2	s2 age of onset
1 Angina	☐	☐☐	☐	☐☐	☐	☐☐	☐	☐☐
2 Heart attack	☐	☐☐	☐	☐☐	☐	☐☐	☐	☐☐
3 High blood pressure	☐	☐☐	☐	☐☐	☐	☐☐	☐	☐☐

4 Stroke ☐ ☐☐ ☐ ☐☐ ☐ ☐☐ ☐ ☐☐

5 Diabetes ☐ ☐☐ ☐ ☐☐ ☐ ☐☐ ☐ ☐☐

6 High blood cholesterol ☐ ☐☐ ☐ ☐☐ ☐ ☐☐ ☐ ☐☐

7 Pre-eclampsia ☐ ☐☐ ☐☐ ☐ ☐☐ ☐ ☐☐

8 Restricted growth at birth ☐ ☐☐ ☐ ☐☐ ☐ ☐☐ ☐ ☐☐

9 Is there an illness which runs in his family? YES NO Don't know

9b If **yes**, what is the illness…….

Questions on sociodemographics

1 Are you currently....

Married / in a stable relationship

single, divorced, widowed, separated

2 What is the highest educational qualification/training you have obtained?

None GCSE or equivalent A-levels or equivalent Degree or higher

3 Are you ...caring for the home or children full time?

...in full-time paid employment?

...in part-time paid employment?

...a student/apprentice

4 What is/was the name of your most recent job/occupation? ……..

5 Are you ...a manager working for an employer?

...a foreman or supervisor working for an employer?

...working for an employer/apprentice/student?

...self employed with employees?

...self employed without employees?

6. Are there currently other wage earners in the home? YES NO

If so what is the name of their most recent job/occupation? ……..

Questions about Alcohol intake and Diet During Pregnancy

1 During your pregnancy, what was your normal weekly intake of?

1a Beer, lager or cider:	Peri-conception	.....	pints	During Pregnancy	..... pints
1b Red Wine	Peri-conception	.....	wineglasses	During Pregnancy	..... wineglasses
1c White Wine	Peri-conception	.....	wineglasses	During Pregnancy	..... wineglasses
1d Sherry, Port and aperitifs	Peri-conception	.....	glasses	During Pregnancy	..... glasses
1e Spirits	Peri-conception	.....	pub measures	During Pregnancy	..... pub measures
1f Tea	Peri-conception	.....	cups	During Pregnancy	..... cups
1g Coffee	Peri-conception	.....	cups	During Pregnancy	..... cups

2. How often do you eat the following foods? (tick)

	Never	Less than once a week	One or two days a week	Most days	Once a day	More than once a day
Fresh fruit in the summer	.....	.....	.....	.....	.....	.....
Fresh fruit in the winter	.....	.....	.....	.....	.....	.....
Salads in summer	.....	.....	.....	.....	.....	.....
Salads in winter	.....	.....	.....	.....	.....	.....

	Never	Less than once a week	One or two days a week	Most days	Once a day	More than once a day
Green vegetables	.....	.....	.....	.....	.....	.....
Fish (all kinds)	.....	.....	.....	.....	.....	.....
Poultry (chicken, turkey)	.....	.....	.....	.....	.....	.....
Red meat (beef, lamb, pork, ham, bacon)	.....	.....	.....	.....	.....	.....
Processed meat (burgers, sausages, pies, pasties, tinned meat, pate)	.....	.....	.....	.....	.....	.....
Cheese	.....	.....	.....	.....	.....	.....

Questions about Exercise During Pregnancy

1a During your pregnancy did you participate in any regular activity designed to improve or maintain your physical fitness?

YES NO

1b For how many hours a week did you take part in these activities? …….

2a Prior to your pregnancy did you participate in any regular activity designed to improve or maintain your physical fitness?

YES NO

2b For how many hours a week did you take part in these activities? …….

Questions about Supplements During Pregnancy

1a During your pregnancy did you take any dietary, nutritional or vitamin supplements?

YES NO

1b. What supplements were these …….

1c If you took folic acid during pregnancy

What Brand of Folic Acid was it? …….

What dose of Folic Acid did you take ……

1d When during Pregnancy did you take supplements

Supplement	Peri-conception	1st Trimester	2nd Trimester	3rd Trimester
Folic Acid				

Iron				
Other (Please Specify)				

Questions about smoking

At present:

Do you currently smoke cigarettes, cigars or a pipe regularly YES NO

If **yes**, how many cigarettes do you smoke per day? …….. cigarettes
how many cigars do you smoke per day? ……. cigars
how many ounces of tobacco do you smoke per day? …….. ounces
At what age did you start smoking? ……. years

If **no**, have you ever smoked? YES NO
from what age did you smoke? ……..years
at what age did you stop smoking?.....years
how much did you smoke on average per day? ……

During pregnancy:

Did you smoke when pregnant? YES NO

If **yes**, at what stages of pregnancy did you smoke? ` Peri-conception 1st Trimester 2nd Trimester 3rd Trimester
(please circle to indicate which stages and how many cigarettes a day per stage)

Did you quit smoking for any length of time during the pregnancy? YES NO

If **yes**, was nicotine replacement therapy used? YES NO

Did smoking take place inside or outside of the home? YES NO

Smoking of a partner

Does your partner smoke currently? YES NO

If **yes**, how many cigarettes are smoked per day? …….. cigarettes

how many cigars are smoked per day? ……. cigars

how many ounces of tobacco are smoked per day? …….. ounces

At what age did they start smoking? ……. Years

If **no**, has your partner ever smoked? YES NO

from what age did your partner smoke? ……..years

at what age did they stop smoking?.....years

how much was smoked on average per day? ……

During pregnancy:

Did your partner smoke at all during the pregnancy? YES NO

If **yes**, at what stages of pregnancy did your partner smoke? (please circle to indicate which stages and how many cigarettes a day per stage) ` Peri-conception 1st Trimester 2nd Trimester 3rd Trimester

Did smoking take place inside or outside of the home? ……

Other family members?

Does any other family member smoke within the home at present? YES NO

If **yes**, at what stages of pregnancy was a smoker present in the home? `Peri-conception 1st Trimester 2nd Trimester 3rd Trimester
(please circle to indicate which stages and how many cigarettes
a day per stage)

EPOCH Pilot (Lifestyle Questionnaire 2)

Subject's Study Number .....

Date of Birth .....

Study Date……..

Questions about your health since the birth of your baby

1. Since your baby was born have you developed any of the following

High Blood Pressure	YES	NO	(Date……..)
Diabetes	YES	NO	(Date……..)
High Cholesterol	YES	NO	(Date……..)
Other Illness	YES	NO	(Date……..)

Comments ……

Are your currently on any medications for any of the above conditions YES NO
Details ……..

2. Since your baby was born have you been on any **NEW** medications (including hormonal contraception)

3. *At present*:

Do you currently smoke cigarettes, cigars or a pipe regularly? YES NO

If **yes**, how many cigarettes do you smoke per day? …….. cigarettes

how many cigars do you smoke per day? ……. Cigars

how many ounces of tobacco do you smoke per day? …….. ounces

If **no**, have your smoked at all since the birth of your baby? YES NO

what age was your baby when you were smoking ? ……

how much did you smoke on average per day? ……

If you have **stopped** smoking since the birth of your baby was nicotine replacement therapy used?

4. At present what is your normal weekly intake of?

   Beer, lager or cider ..... pints

   Red Wine …..……wineglasses White Wine …..……wineglasses

   Sherry, Port and aperitifs..... glasses

   Spirits..... pub measures

   Tea ..... cups

   Coffee ..... cups

5. At present how often do you eat the following foods? (tick)

	Never	Less than once a week	One or two days a week	Most days	Once a day	More than once a day
Fresh fruit in the summer	.....	.....	.....	.....	.....	.....
Fresh fruit in the winter	.....	.....	.....	.....	.....	.....
Salads in summer	.....	.....	.....	.....	.....	.....
Salads in winter	.....	.....	.....	.....	.....	.....
Green vegetables	.....	.....	.....	.....	.....	.....
Fish (all kinds)	.....	.....	.....	.....	.....	.....
Poultry (chicken, turkey)	.....	.....	.....	.....	.....	.....
Red meat (beef, lamb, pork, ham, bacon)	.....	.....	.....	.....	.....	.....

Processed meat (burgers, sausages, pies, pasties, tinned meat, pate)	.....	.....	.....	.....	.....	.....
Cheese	.....	.....	.....	.....	.....	.....

6. At present are you participate in any regular activity designed to improve or maintain your physical fitness?

YES NO

For how many hours a week do you take part in these activities? ……

7. At present are you taking any dietary, nutritional or vitamin supplements?

YES NO

What supplements were these ……

Questions about your Baby's growth and health since the last study visit?

1. How long was your baby a patient in the neonatal unit ……..(days)

2. Did they have any operations while they were an inpatient ? YES NO
   Comments ……

3. Has your baby had any health issues since discharge YES NO
   Comments ……

4. Where they discharged home on any medications (if so which medications and for what duration)

5. What was your Baby's weight when they were discharged home …….. kg/ lbs

6. What is your Baby's weight now ……..kg/lbs

7. What is your Baby's diet…… breast milk/ formula/ combination/other (please specify) ……..

If your baby has transitioned from breast milk to formula what age did this occur…….. days/weeks

If your baby is on formula, what brand of formula is it ?…….

How many times a day does your baby feed on average ……..

How many times at night does your baby feed on average? …….

If your baby recives a combination of breast milk and formula:

How many bottles of formula do they have per day (over 24 hours)? ……

How many times does your baby breast feed in 24 hours? ……..

Has your baby had any difficulties with feeding YES NO

Comments ……

8. Has your baby received their

2 month old Vaccines	YES	NO
3 month old Vaccines	YES	NO

9. Has your baby/ at what age did your baby

Turn Head while lying on their back	YES	NO	Age ……..
Hold Head erect and lift head	YES	NO	Age ……..
Prop themselves on forarms while lying on front	YES	NO	Age ……..
Turn from side to back	YES	NO	Age ……..
Bring an object to his/her mouth	YES	NO	Age ……..
Hold Hands together	YES	NO	Age ……..
Bat at objects	YES	NO	Age ……..

Follow an object with his/her eyes	YES	NO	Age ……..
Recognises You/ Your Partner	YES	NO	Age ……..
Reaches for faces	YES	NO	Age ……..
Startle at loud noise	YES	NO	Age ……..
Become alert in reponse to voices	YES	NO	Age ……..
Vocalise	YES	NO	Age ……..
Smile	YES	NO	Age ……..

Questions about other people in your family

1. *At present*:

   Does your Partner currently smoke cigarettes, cigars or a pipe regularly? YES NO

   If **yes**, how many cigarettes per day? …….. cigarettes

   how many cigars per day? ……. Cigars

   how many ounces of tobacco per day? …….. ounces

   Has your partners smoking pattern changed since the birth of your baby…

   increased smoking/ decreased smoking / no change

   Do they smoke inside or outside the house ……

If **no**, has your partner smoked at all since the birth of your baby? YES NO

what age was your baby when they were smoking ? ……

how much did they smoke on average per day? ……

2. Does any other member of your family smoke at home at present? YES NO

Approximately how many cigarettes are smoked per day?.....

Do they smoke inside or outside the house ……

Questions about your reasons for participating in Research

1. Have you ever previously participated in a research study YES NO

2. Have other members of your family ever participated in a research study YES NO

3. Do you or your partner work in a medical/ health care related field YES NO

4. Do you or your partner work in a research related field YES NO

5. Which of the following best describes your reasons for participating in this research study (Please circle)
    a) Percieved benefit for self/baby
    b) Interest in understanding more about preeclampsia
    c) Desire to help others
    d) Previous positive experience with participation in research
    e) Other (Please Describe below)

……
……
……
……..

Endothelial cell isolation, processing and analysis

All cords were processed within 12 hours of delivery by a dedicated team managed by Dr Grace Yu. A photograph was taken to record the physical state of the cord and then any damaged or clamped areas removed. Blood clots were removed by gentle massage. One end of the vein was cannulated and secured with surgical clamping scissors to allow perfusion of 20 mL of Hanks' Balanced Salt Solution (HBSS) to wash out any remaining blood. To detach the endothelial cells, the other end of the vein was cannulated and secured to allow the vein to be perfused with 20 mL of CollagenaseA (Sigma; C9722) solution (1mg/mL in HBSS with Calcium and Magnesium) and the whole cord to be incubated at 37˚C for 10 minutes. After incubation, one cannulated end of the vein was opened and the solution was collected. To improve endothelial cell yield, the vein was then perfused with an additional 30 mL HBSS, and was pooled with the previously collected 20ml of CollagenaseA solution. Cells were pelleted by centrifugation of the solution at 1,250 rpm for five minutes before seeding into a T75 culture flask in EGM-2 (Lonza) and incubated at 37˚C with 5% CO2. After 24 hours, fresh medium was replaced to remove any remaining non-adherent cells. EGM-2 medium was changed every two to three days until cell growth reached 70-80% confluence. Isolated cells were harvested using Accutase (PAA; L11-007) at 37˚C for five minutes and pelleted at 1,250 rpm for ten minutes. A proportion of samples were used for flow cytometry to demonstrate purity and the remainder transferred into aliquots containing 1x106 cells per well and transferred for storage in liquid nitrogen.

Flow cytometry was used to characterise cell surface markers. Isolated cells were washed by fluorescence-activated cell sorting (FACS) buffer (1% BSA in DPBS, Sigma)

mixing with FcR blocking buffer (BD Biosciences). Conjugated monoclonal antibodies or isotype-matched negative-controls were used for staining at a 1:10 dilution. The following monoclonal antibodies were used: PE-CD31 (mIgG1, BD; 555446), FITC-CD90 (BD; 555595), PE-Cy7-CD45 (mIgG1, BD; 345809) PE-mIgG1 isotype control, FITC-mIgG1 isotype control and PE-Cy7mIgG1 isotype control. Flow cytometry was performed using a BD LSRII flow cytometer with FACSDiva software (BD Biosciences). An unstained sample was included in each flow cytometry analysis as a negative control.

Matrigel assay - To assess microtubule formation ability of HUVECs, a 96-well plate was evenly coated with 50µl of growth factor-reduced Matrigel (BD Biosciences, UK) and HUVECs placed with EGM-2 (Lonza) at a density of $1x10^4$ cells per well. The plate was incubated at 37˚C for 16 hours before photomicroscopy. Each sample was replicated in triplicate and the image of each well was taken at x4 magnification using a Nikon Eclipse TE2000-U microscope (Nikon Ltd, London, UK).

Co-culture assay for detecting microtubule formation - To validate the Matrigel results we also co-cultured HUVECs with bone marrow stromal mesenchymal stem cells (MSCs) as a support for vessel maturation. Primary extracted bone marrow mesenchymal stem cells (BMMSCs) (Lonza, Cat. No. PT-2501) were cultured using mesenchymal growth media (Lonza) until reaching a sufficient number of cells for seeding a 48-well collagen coated plate, at a concentration of $2x10^4$ cells per well. The BMMSC-seeded plate was incubated at 37˚C for 24 hours before seeding HUVECs. HUVECs (either isolated from normotensive or hypertensive pregnancies) were then

co-cultured on top of the BMMSCs monolayer at a ratio of 1:5. Each sample was replicated in triplicate. The cells were gently swirled to ensure an even distribution on top of the BMMSCs monolayer. Cells were cultured in EGM-2 (Lonza) media for 14 days, and the media was replaced every two days. After 14 days incubation, the media was removed and cells were washed in PBS and fixed with ice cold 70% ethanol for one hour. BSA (5%) was used to block the cells for 30 minutes. The buffer was removed before adding mouse anti-hCD31 primary antibody (1:4000) in blocking buffer (AbD Serotec; Cat No. MCA1738) before incubation overnight at 4°C on a rocker. The antibody was completely removed by washing the cells three times with PBS. Biotinylated-goat anti-Mouse IgG (1:200) was added to each well (Vector Laboratories, Cat. No. BA-9200) and incubated at room temperature for one hour. The secondary antibody was removed and washed three times with PBS followed by incubation with Vectastain Elite ABC reagent (Vector Labs Cat. No. PK-6100 series). After tertiary antibody incubation, DAB Peroxidase Substrate working solution (Vector Labs Cat. No. SK-4100) was added and incubated for ten minutes. Subsequently the cells were washed three times with dH_2O for five minutes. The plate was air dried before images were taken using a Nikon Eclipse TS2000-U microscope.

Image processing and microtubule measurements - Images obtained from Matrigel and co-culture assays were adjusted for mean brightness using acquisition software to control the bright field illumination of the microscope (Simple PCI version 6.6.0.0; Hamamatsu corporation, Sewickley, PA). Images were saved as TIFF files, and microtubule formation analysed using AngioSys 1.0 (TCS Cell Works, UK). Image threshold was adjusted based on the intensity values of the monochrome image and

each image then skeletonized to reduce to one pixel wide. A line was drawn over each tubule and each branch point marked with a dot. The total length of lines was quantified in pixels and total number of branch points were recorded.

Proliferation assay- To assess the proliferation ability of HUVECs of hypertensive and normotensive cords, CyQUANT® NF Cell Proliferation Assay (Life Technologies, USA) was performed, based on measurements of cellular DNA content via fluorescent dye binding. Cells were plated in black 96-well plates (BD Biosciences, UK) at a density of 500 cells per well, and incubated in EGM-2 medium at 37°C in 5% CO_2 overnight for cellular attachment. A separate plate was prepared simultaneously as a baseline control. Fluorescence intensity values were obtained following the CyQUANT Cell Proliferation Assay kit protocol for attached cells. In brief, cells were incubated with 1x CyQUANT® NF dye binding solution for 60 minutes at 37°C. Fluorescence intensity was measured at excitation of ~485nm and emission of ~530nm using VICTORTM fluorescence microplate reader (Perkin Elmer, Vienna, Austria). Proliferation index was reported as fold change of averages of quadruplicate samples with baseline subtraction. Cells were used for the CyQUANT assay at passage two.