MONASH University

The Effect of Moderate Preterm Birth on an Immature Cardiovascular System

Vivian B Nguyen

Bachelor of Biotechnology with Honours

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

Department of Anatomy and Developmental Biology

Faculty of Medicine, Nursing and Health Sciences

Monash University, Clayton, Victoria, Australia

2016

Copyright

Table of Contents

Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I, Vivian Nguyen, hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Where others have contributed to my studies (particularly in relation to help with the animal studies) their contributions have been duly acknowledged in the thesis.

Vivian Nguyen

Acknowledgments

Firstly, I would like to thank my supervisors, Prof. Jane Black and Dr. Graeme Polglase. Thank you Jane for allowing me to work in your lab since my Honours year and throughout my PhD; I greatly appreciate your invaluable support, assistance, effort, patience, tolerance and guidance. I also thank you for assistance in the writing up of my thesis. To Graeme, thankyou also for all your support and guidance in introducing and teaching me all about sheep; I appreciate the time you set aside from your busy schedule to help me with all the animal work.

A big thankyou to the past and present members (Joon Lim, Kom Yin, Shanti Diwakarla, Tracey Flores) of the Black Laboratory in the Department of Anatomy and Developmental Biology for your advice and help throughout the years. In particular, I would especially like to thank Jonathan Bensley for his advice, teaching me the techniques for all my heart and blood vessel analyses and his willingness to assist me with anything I needed whenever I needed it.

I would like to thank all the members of the Hooper Laboratory at the Ritchie Centre (part of Graeme's group) for all their assistance in all animal work. This was a major part of my PhD and I am deeply grateful for all the help provided in every lamb delivery, sheep surgery, animal experiment and post-mortems; most particularly, Dr. Beth Allison, Natasha Blasch, and Dalibor Stanojkovic.

I would like to acknowledge Dr. Robert De Matteo and Prof. Richard Harding from the Department of Anatomy and Developmental Biology for their advice about the sheep model used in my study. I also express my gratitude to A/Prof. Chrishan Samuel from the Department of Pharmacology for teaching and assisting me in the hydroxyproline assays and also Dr. Aneta Stefanidis from the Department of Physiology for assistance in performing the DEXA scans presented in this thesis.

To the entire animal house staff on the Clayton and Gippsland campuses involved with caring for my animals, I am grateful for your help.

To Monash University histology staff I would like to thank Camilla Cohen, Stef Tombs, Jonathan Bensley, Julie Hickey and Sue Connell for all histological advice and assistance.

To my fellow PhD students to whom I have had the pleasure of sharing this experience with, especially Dana Ryan, Stacey Hokke, Amanda Vrselja, Ryan Wood-Bradley, Ashlee Clarke and Richard Rebello. Thank you for the lunches, the drinks, and chats which have made these years so much more enjoyable.

Lastly, I would like to thank my family and friends. To my parents, Myly and Bau, and my brother, Dan, for all your love, support and home cooked meals. To my other half, Michael – thank you for all your endless love and constant encouragement; also thank you to the Gillett family, especially Greg for proof reading. To my friends I could not live without, Zoe, Willow, Michelle – I greatly appreciate your support and your friendship means the world to me.

I am extremely grateful for this opportunity to do my PhD and acknowledge the support and advice of all the wonderful people I have met along the way. It was the most gratifying experience; I have learnt so much and hope to make you all very proud.

Publications

Published abstracts

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black (2013). Moderate preterm birth in a sheep model: effects on postnatal body growth and arterial pressure (2013), *8th World Congress on Developmental Origins of Health and Disease, Journal of Developmental Origins of Health and Disease, 4, pp S51-S339. doi: 10.1017/S2040174413000421.*

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black. Differences in postnatal body growth, body composition, arterial pressure and heart rate between moderately preterm and term lambs up to 12 months of age (2014), *Poster Abstracts. Journal of Paediatrics and Child Health, 50: 65–116. doi: 10.1111/jpc.12528_3.*

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black. Arterial structure and composition is different between preterm and term lambs (2015), *Poster Presentations: Tuesday 21 – Wednesday 22 April. Journal of Paediatrics and Child Health, 51: 106–138. doi: 10.1111/jpc.12884_7*

Conference Presentations

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black. Comparison of the structure of blood vessels after preterm birth between males and females. *The Australian Early Origins of Hypertension Workshop, 2012,* Adelaide, South Australia, Australia – Poster presentation

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black. The effect of preterm birth on the structure of the aorta and carotid arteries. *The 26th Fetal and Neonatal Workshop of Australia and New Zealand (FNWANZ), 2012,* Port Stephens, New South Wales, Australia – Oral presentation

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black. Moderate preterm birth in a sheep model: effects on postnatal body growth and arterial pressure. *The 8th World Congress on Developmental Origins of Health and Disease (DOHaD), 2013,* Suntec, Singapore – Poster presentation

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black. The effect of moderate preterm birth on postnatal growth, heart rate and blood pressure. *The 27th Fetal and Neonatal Workshop of Australia and New Zealand (FNWANZ), 2013,* Barossa Valley, South Australia, Australia – Oral presentation

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black. Does postnatal growth, arterial pressure and heart rate differ between lambs born at term and those born moderately preterm? *The Australia Society of Medical Research (ASMR) VIC Student Symposium, 2013,* Melbourne, Victoria, Australia – Oral presentation

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black. Differences in heart structure of lambs immediately after preterm and term birth. *The 41st Fetal and Neonatal Physiological Society Meeting (FNPS), 2014*, Saint Vincent, Italy – Oral presentation **(Awarded Student Travel Grant)**

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black. The effect of moderate preterm birth on the hearts of lambs 2 day after birth. *The 28th Fetal and Neonatal Workshop of Australia and New Zealand (FNWANZ), 2014,* Yanchep, Western Australia, Australia – Oral presentation

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black. Differences in postnatal body growth, body composition, arterial pressure and heart rate between moderately preterm and term lambs up to 12 months of age. *The 18th Perinatal Society of Australia and New Zealand Annual Congress (PSANZ), 2014,* Perth, Western Australia, Australia – Poster presentation **(Awarded New Investigator Award for Basic Science)**

- **Vivian Nguyen**, Robert De Matteo, Richard Harding, Graeme Polglase and M. Jane Black. Arterial structure and composition is different between preterm and term lambs. *The 19th Perinatal Society of Australia and New Zealand Annual Congress (PSANZ), 2015,* Melbourne, Victoria, Australia – Poster presentation

Abbreviations

α	alpha
ANOVA	analysis of variance
ART	assisted reproductive technology
AV	atrioventricular
BMI	body mass index
bpm	beats per minute
Caspase-3	Cysteine-aspartic proteases 3
C.A.S.T	Computer Aided Stereological Toolbox
cGlu	glucose levels
cLac	lactate levels
cm	centimetre
CrL	crown-rump length
ctHb	haemoglobin levels
DAB	diaminobenzidine
DEXA	Dual-energy X-ray absorptiometry
dL	decilitre
DNA	deoxyribonucleic acid
DPX	Di-n-butyl phthalate in Xylene
EtOH	ethanol
FLL	front limb length
g	gram

HBSS	Hank's Balanced Salt Solution
HCl	hydrochloric acid
HLL	hind limb length
IL-1β	Interleukin-1 beta
i.m.	intramuscular
IU	international units
IUGR	intrauterine growth restriction
i.v.	intravenous
IV	interventricular
kg	kilogram
LV	left ventricle or left ventricular
LV+S	left ventricle plus septum
M	molar
MAS	Monash Animal Services at Monash University
MMCAF	Monash Medical Centre Animal Facility
mg	milligram
ml	millilitre
mm	millimetre
mmHg	millimetres of mercury
mmol	millimole
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
n	number of samples

nm	nanometre
NS	not significant
O_2	oxygen
OCT	optimal cutting temperature
pCO_2	partial pressure of carbon dioxide
P_G	P value for gestational age (preterm vs. term)
pO_2	partial pressure of oxygen
P_S	P value for sex (female vs. male)
P_{G*T}	P value for interaction between gestational age and time
P-selectin	platelet selectin
P_{S*G}	P value for interaction between gestational age and sex
P_{S*T}	P value for interaction between sex and time
P_{S*G*T}	P value for interaction between gestational age, sex and time
P_T	P value for time
PTEA	post-term equivalent age
RDS	respiratory distress syndrome
RV	right ventricle or right ventricular
SEM	standard error of mean
sO_2	oxygen saturation levels
TEA	term equivalent age
TG	thoracic girth
µL	microlitre
µm	micrometre

VEGF	vascular endothelial growth factor
vs.	versus
WGA-HRP	wheat germ agglutinin-horseradish peroxidase
3D	three dimensional
%	percentage
=	equals
/	divide/per/or
<	less than
>	greater than
≤	less than or equal to
≥	greater than or equal to
±	plus minus
^{o}C	degree Celsius

Summary

Preterm birth (<37 weeks completed weeks of gestation) affects 9 - 12% of all live births; with the majority being moderately preterm birth (32 - 36 weeks gestation). It is the leading cause of neonatal mortality and morbidity and males have been reported to be at greater risk than females. In this thesis, I have examined the effect of moderate preterm birth, using an ovine model, on the structure of the heart and cardiomyocyte growth, and on the structure and composition of the large conduit arteries (thoracic aorta and left carotid artery).

Pregnant ewes were induced to deliver vaginally using epostane (50 mg in 2 ml ethanol) at 0.9 of term (132 ± 1 days of gestation) or at term (147 ± 1 days of gestation). Clinically relevant doses of Betamethasone (11.4 mg) were also administered 24 hours and 48 hours before birth to ewes delivering preterm. Offspring were euthanised 2 days after birth or at 14.5 months of age.

During the study period, preterm sheep were significantly lighter and smaller compared to term controls, with survival rates 75% for both preterm male and preterm female lambs after the first 2 weeks of life. At 12 months of age, body composition was not different between preterm and term sheep. By 14 months of age, preterm female sheep had caught up in body weight to terms; however preterm males were still lighter than term males. At this time, no detectable differences in arterial pressure and heart rate were observed between preterm and term sheep.

In the immediate period after birth, preterm hearts were lighter and smaller compared to terms; however, relative to body weight, only right ventricular (RV) chamber volume remained smaller and left ventricle (LV) and RV walls were thicker. Total number of cardiomyocytes within the LV plus septum (LV+S) was not significantly different between preterm and term lambs; however relative to body weight, preterm lambs exhibited significantly more cardiomyocytes. Preterm lambs also had a significantly higher proportion of mononucleated cardiomyocytes in the LV+S but significantly lower proportion of binucleated cardiomyocytes compared to term lambs; indicative of an immature heart. In adulthood, preterm sheep had smaller RV and LV wall volumes, but when adjusted to body weight, there were no longer any differences. Interestingly, there was sexual dimorphism in the long-term effects on the LV+S; preterm male sheep exhibited significantly reduced wall volume, wall

thickness and cardiomyocyte number compared to term male sheep, whereas no differences were observed between preterm and term females.

In the immediate period after birth, there was no evidence of arterial injury in the thoracic aorta or left carotid artery. At this time, preterm lambs had narrower lumen areas in both blood vessels and thinner walls were observed in the left carotid artery. When adjusted for body weight, lumen area and wall thickness actually increased in the aorta of preterm lambs compared to term controls. Collagen deposition within the aortic wall was significantly reduced in preterm male lambs compared to terms. In adulthood, the number of elastin layers and elastin content was significantly reduced, whereas smooth muscle content increased in the left carotid artery of preterm sheep compared to term sheep. Interestingly, there was sexual dimorphism observed in the effects of preterm birth on aortic structure, such that preterm male sheep exhibited significant reductions in lumen size, medial area, intima-media thickness and layers of elastin compared to term male sheep, however no differences were observed in females.

In my studies, moderate preterm birth after antenatal corticosteroid exposure has resulted in significant alterations in cardiovascular structure, predominantly in males. Even though, these differences are likely attributed to the smaller body size of the preterm male sheep, the reduced complement of cardiomyocytes in the LV+S and thinner walls with fewer layers of elastin in the aorta are likely to render the adult preterm male cardiovascular system particularly vulnerable to secondary postnatal insults and increase the risk of adverse cardiovascular consequences in later life.

In conclusion, this thesis provides valuable insight into the effects of moderate preterm birth on the cardiovascular system in the immediate period after birth and in early adulthood. Overall, this study is clinically important given the large proportion of individuals born moderately preterm. The findings highlight the cardiovascular vulnerability of those born moderately preterm, particularly in males, and these adverse effects are expected to be greater with increasing severity of prematurity.

Chapter 1:

Literature Review

1.1 Introduction

Preterm birth (birth before 37 completed weeks of gestation) is one of the leading causes of perinatal mortality and morbidity. It occurs in 9 - 12% of all live births worldwide; with the majority categorised as moderate preterm birth (32 - <37 weeks gestation). Infants born preterm are more likely to be at risk of infections, neuro-developmental problems, respiratory complications and chronic health issues; with males reported to be at greater risk than females (termed 'male disadvantage'). Recent epidemiological studies have reported a link between being born premature and development of hypertension in adulthood. This is concerning as hypertension is a major risk factor for cardiovascular disease and is likely to lead to lifelong adverse consequences to cardiovascular health in individuals who were born preterm. It is therefore essential to determine the underlying cause of hypertension in subjects born preterm. In order to do this, it is important to gain an understanding of how preterm birth affects the structure of the heart and blood vessels in the early neonatal period and also in the long-term (adulthood).

1.2 Preterm birth

1.2.1 Definitions

In human pregnancies, preterm birth is defined as delivery of an infant before 37 completed weeks of gestation; term is birth between ≥37 - 42 weeks gestation (Martin et al., 2005). Preterm birth can be further subdivided according to gestational age at birth and severity: extremely preterm (birth before 28 weeks of gestation), very preterm (birth before 32 weeks of gestation) and moderate preterm (birth between 32 to <37 weeks of gestation) (Figure 1.1) (Tucker and McGuire, 2004, Goldenberg et al., 2008). Birth before 22 weeks of gestation often results in fetal loss. Of all preterm births, the majority (approximately 80 - 90%) are born moderate preterm (Raju, 2006, Cheong and Doyle, 2012, Shapiro-Mendoza and Lackritz, 2012), with numbers decreasing as gestational age becomes lower; for instance, only 5% of preterm births are extremely preterm (Goldenberg et al., 2008).

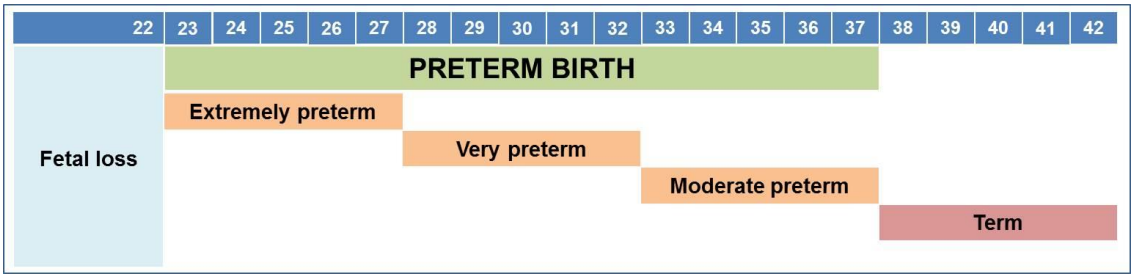

***Figure 1.1**. Subcategories of preterm birth (modified from Tucker and McGuire (2004)).*

1.2.2 Incidence

Preterm birth occurs in 9 - 12% of all live births (Tracy et al., 2007, Beck et al., 2010, Heron et al., 2010) and contributes to more than two-thirds of all perinatal deaths (Lumley, 2003). The World Health Organisation (2014b) has reported that every year, 15 million babies are estimated to be born preterm and this number is increasing. In general, industrialised countries have shown a steady increase in the incidence of all births classified as preterm. Recent reports suggest that this is mainly due to the significant increase in preterm births between 34 - 36 weeks of gestation (Martin et al., 2009).

Over the span of a decade in Australia, it was reported by Tracy et al. (2007) that the overall proportion of babies born preterm, at greater than or equal to 22 weeks of gestation, had risen by 12.1%, from 5.9% in 1994 to 6.6% in 2003. Similarly, a 10-year study in Denmark, showed a marked increase in preterm births with the overall proportion of preterm deliveries being 22% higher in 2004 than 1995 (Langhoff-Roos et al., 2006); when subdivided further into extremely preterm, very preterm and moderately preterm, the increases in incidence were 41%, 22% and 22%, respectively. Additionally, the United States Centers for Disease Control and Prevention reported that the preterm birth rate in 2006 in the United States had risen to 12.8%, rising more than 20% since 1990 (10.6%) (Hamilton et al., 2007a) and 36% since the early 1980s (Martin et al., 2009). Overall, from 1990 to 2010, the absolute numbers of preterm births for 65 countries in Europe, the Americas and Australasia has increased from 2 million to approximately 2.2 million, despite the reduction of live births (Blencowe et al., 2012, Blencowe et al., 2013).

The incidence of preterm births can differ considerably between certain racial/social groups (Beck et al., 2010); for example, there is a high prevalence in subpopulations such as African Americans (Savitz et al., 1991, Zhang and Savitz, 1992, Collins et al., 2007), Canadian

Aboriginals (Heaman et al., 2005, Auger et al., 2012) and Australian Aboriginals (Westenberg et al., 2002, Leeds et al., 2007, Smylie et al., 2010), in contrast to other ethnic groups residing in the same countries. In 2003 in the US, the rate for preterm birth in African American women was 17.8%, whereas in Asian and Pacific Islander women, and Caucasian women, it was 10.5% and 11.5%, respectively (Menon, 2008). Hispanic Americans have a similar prevalence of premature birth as Caucasians at about 12% (Hamilton et al., 2007a, Heron et al., 2010). In Canada, a three-year study observed that 7.7% of all live births were preterm in Aboriginal women compared to 6.4% in non-Aboriginal women (Heaman et al., 2005). Of concern, in Australia between the years 2001 to 2004, preterm babies born to Indigenous women represented 14.6% of all live births, which was almost double the incidence of preterm births of non-Indigenous women (7.7 %) (Leeds et al., 2007).

Worldwide, preterm births occur at a higher frequency in teenage pregnancies. Table 1.1 shows the percentages of preterm birth in the US between 1990 and 1996 in teenage mothers (aged 13 to 20 years) compared to mothers at 25 years of age. It is apparent that the incidence of preterm birth declines with increasing childbearing age and also racial disparities are evident; additionally, those women that deliver moderately preterm make up the majority of all preterm births, regardless of age and race. In a more recent study in the US, there was a further 5% increase in teenage birth rates from 2005 to 2007, with the majority of the increase occurring between 2005 and 2006 (Heron et al., 2010). A study in South Australia from 1995 to 1999, found that 17.5% of Australian Aboriginal teenagers gave birth to preterm babies, while non-Aboriginal teenagers and Aboriginal women over the age of 20 had lower rates: 9.3% and 16.3%, respectively (Westenberg et al., 2002).

Race/Ethnicity and Birth Status	Age groups				
	13 and 14	15 to 17	18 and 19	20	25
White non-Hispanic					
Preterm	19.6	12.5	9.7	8.2	5.6
Moderate preterm	13.4	9.9	8.1	6.9	0.7
Black non-Hispanic					
Preterm	26.8	20.4	16.7	14.9	12.1
Moderate preterm	18.4	8.4	13.3	12.0	9.5
Hispanic					
Preterm	16.6	13.4	10.4	8.9	6.5
Moderate preterm	12.3	11.3	9.0	7.8	5.7

Table 1.1. *Percentages of preterm births (before 37 completed weeks of gestation) in multiparous mothers (given birth two or more times) in the US between 1990 and 1996 (adapted from Akinbami et al. (2000)). In this study, preterm indicates all births less than 37 weeks of gestation whilst moderate preterm was defined as birth between 33-36 weeks of gestation.*

Overall, it is of concern that the incidence of preterm birth continues to rise despite increases in medical knowledge and advancement in the understanding of the risk factors associated with preterm birth. Investigations into this alarming trend clearly demonstrate strong links between preterm birth and certain sub-populations (described previously), with many studies now focussing on the aetiology of preterm birth.

1.3 Factors associated with preterm birth

In order to address the increasing rates of preterm births, it is imperative to develop an understanding of the underlying mechanisms leading to premature delivery. In the many women who spontaneously deliver preterm, the exact cause leading to the premature birth cannot be conclusively established, as the aetiology of preterm birth in most cases is

multifactorial (Goffinet, 2005, Leitich, 2005, Savitz et al., 2005, Goldenberg and Culhane, 2007). In this regard, a number of risk factors have been associated with risk of preterm delivery as shown in Figure 1.2. The major risk factors include: intrauterine infection or inflammation (such as chorioamnionitis, bacterial vaginosis and sexually transmitted infections) and maternal risk factors (reproductive history, genetics, maternal health, demographic and behavioural factors and maternal age), intrauterine growth restriction (IUGR), multiple births and assisted reproductive technologies (Goffinet, 2005, Hamilton et al., 2012). These are discussed in further detail below.

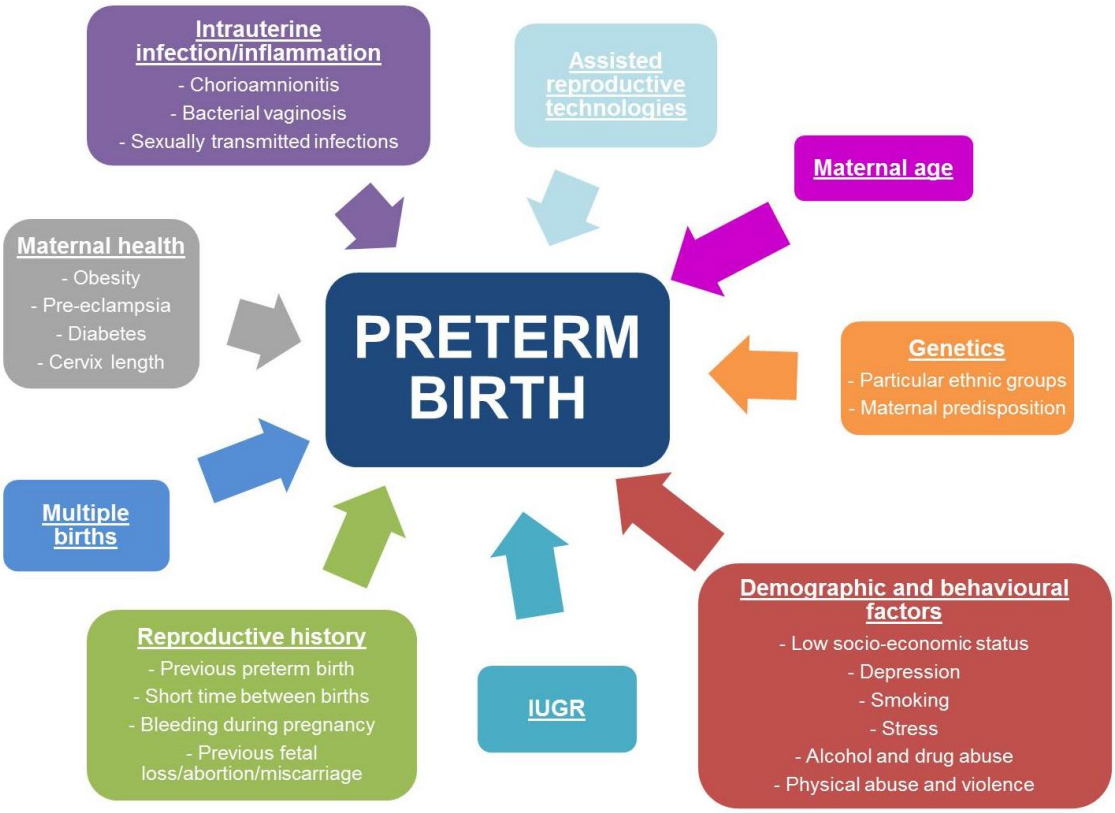

***Figure 1.2**. Factors associated with preterm birth.*

1.3.1 Intrauterine infection and inflammation

Intrauterine infection is one of the most frequent complications of preterm delivery and is thought to be the underlying cause of 25 - 40% of preterm births (Lettieri et al., 1993, Cram et al., 2002). These infections occur when microorganisms invade the amniotic cavity by:

ascending from the cervical/vaginal area, from needle contamination during amniocentesis, or by migration from the abdominal cavity (Minkoff, 1983, DiGiulio et al., 2010, Romero et al., 2014). They multiply in the placenta, decidua and membranes, releasing endotoxins and cytokines that prompt the production of prostaglandins, which in turn stimulate uterine contractions leading to preterm delivery (Goldenberg et al., 2000, Waites et al., 2009).

1.3.1.1 Chorioamnionitis

Chorioamnionitis is a polymicrobial infection, generally involving two or more microbes, which results in inflammation of the placenta and membranes, the amnion and chorion (Czikk et al., 2011). There are two types of chorioamnionitis: (1) histologic, when there are no indicators of infection, however, after examining the placenta, micro-organisms are present; and (2) clinical, which is when overt symptoms can be recognised during pregnancy. Clinical chorioamnionitis with overt symptoms is only present in 5 - 10% of preterm births, whereas histologic chorioamnionitis is identified in more than 50% of preterm births (Edwards, 2005, Tita and Andrews, 2010).

The most commonly detected microbes found in amniotic fluid and placental cultures of women who have delivered prematurely are *Ureaplasma urealyticum* and *Mycoplasma hominis* (Hillier et al., 1988, Gibbs et al., 1992, Andrews et al., 1995, Krohn et al., 1995, Waites et al., 2009, Tita and Andrews, 2010). Other microorganisms commonly isolated include *Gardnerella vaginalis*, *Group B streptococcus* and *Escherichia coli*. A study by Anderson et al. (2007) showed that the severity of histological chorioamnionitis directly correlated to the colony count of *Group B streptococcus* bacteria. In another study, untreated *Group B streptococcus* was found to be associated with chorioamnionitis and treated *Group B streptococcus* was shown to decrease the risk of preterm delivery (Thomsen et al., 1987, Kataoka et al., 2006).

1.3.1.2 Bacterial vaginosis and sexually transmitted infections

Bacterial vaginosis is the overgrowth of microorganisms such as Bacteroides species, Peptostreptococcus species and *Gardnerella vaginalis*, with an accompanying decrease in other normally occurring lactobacillus species (Leitich et al., 2003). On examination, diagnosis is accurate if three of four findings are present: (1) a thin homogeneous vaginal discharge, (2)

high vaginal pH levels (pH < 4.5, as normal vaginal pH is 3.8 to 4.5), (3) positive Whiff test (where potassium hydroxide solution is added to the sample of vaginal discharge to see if a "fishy" odour is produced) and (4) presence of "clue cells" (vaginal discharge is mixed with sodium chloride and checked for epithelial cells that are coated with bacteria nicknamed 'clue cells') (Eschenbach et al., 1988, Money, 2005).

Approximately 40% of pregnant women have bacterial vaginosis (Glantz, 1997) and this infection has been associated with doubling the risk of spontaneous preterm birth (Gravett et al., 1986, Holst et al., 1994, Hillier et al., 1995b, Leitich and Kiss, 2007). However, even though vaginal infection has been found to be associated with preterm birth, its role is unclear. Alternatively, it is suggested that diagnosis of bacterial vaginosis is actually a marker for the colonisation of similar organisms that may lead to chorioamnionitis and eventually preterm birth (Silver et al., 1989, Hillier et al., 1995a, Leitich, 2005).

Sexually transmitted infections can also be detected amongst pregnant women and are linked to preterm delivery. *Neisseria gonorrhoea* and *Chlamydia trachomatis* are two of the most commonly isolated sexually transmitted organisms, which infect the genital tract and can potentially migrate to other organs. Risk factors for sexually transmitted infections include having a history of multiple sexual partners, unprotected sex or illicit drug use. *N. gonorrhoea* cervicitis is known to be strongly associated with preterm delivery (Romero et al., 1991) and studies have shown that treatment of gonococcal cervicitis is linked to decreased rate of preterm birth (Gibbs et al., 1992). Furthermore, co-infection with *C. trachomatis* is very common and pharmacological treatments are available to concomitantly treat both infections. By itself, the incidence of *C. trachomatis* in pregnant women can range from 5 to 26% and patients infected are mostly asymptomatic (Cram et al., 2002).

1.3.2 Maternal risk factors

There are many maternal factors that may contribute to the increased risk of delivery before term; these include: reproductive history, genetics, health, demographic and behavioural factors and lifestyle choices. Hence, an understanding of maternal history and situations can help improve the precision of diagnosing women 'at risk' of preterm labour and the effectiveness of managing preterm labour.

1.3.2.1 Reproductive history

Previous pregnancy history is an important predictor in assessing the risk of delivering prematurely. If clinicians are aware of pregnancy history, this enables appropriate management of future pregnancies, especially if the mother has been identified as being 'at risk'. One of the strongest prognostic indicators of preterm birth is if a woman already has a history of preterm birth. Studies have shown that both spontaneous and indicated preterm births are highly repetitive, with the second birth being classified more likely to deliver preterm after preterm delivery in the first pregnancy; the earlier the first birth the more likely the second will also be born early (Mercer et al., 1999, Adams et al., 2000, Goldenberg et al., 2006).

Other factors in maternal pregnancy history associated with an increased risk of preterm birth include: previous fetal loss/abortions/miscarriages, short periods of time between pregnancies, and bleeding during pregnancy (Mueller-Heubach and Guzick, 1989, Owen et al., 1990, Voigt et al., 2008). Previous abortions and miscarriages have been reported to be the most significant risk factor for subsequent preterm births (Ekwo et al., 1993, Goldenberg et al., 1993, Buchmayer et al., 2004, Brown et al., 2008). Health data studied from 600,000 Scottish women (whose second pregnancy was after an induced abortion, fetal loss after live birth, or a miscarriage) found that women who had a previous induced abortion were 37% more likely to have a spontaneous preterm birth compared to women who had never been pregnant before (Bhattacharya et al., 2012).

Furthermore, a short interval between two pregnancies has also been associated with preterm birth. A study by Zhu et al. (1999) reported that infants conceived 18 to 23 months after the previous birth had the lowest risk of being premature, whereas an inter-pregnancy interval of less than six months doubled the risk of preterm birth and other complications (Smith et al., 2003). The exact mechanisms leading to the increased risk of preterm delivery are unknown. It has been speculated that a short period of time between pregnancies may restrict the uterus from returning to its original state, thereby preventing the replenishment of maternal stores of essential nutrients, vitamins and energy in time for the subsequent pregnancy (Goldenberg et al., 2008).

Lastly, vaginal bleeding episodes during pregnancy have been reported to complicate up to a quarter of all preterm pregnancies (Ananth and Savitz, 1994, Axelsen et al., 1995, Sharami et al., 2013). In approximately half of these cases the cause is unknown; however, approximately

50% of cases are caused by placental abruption, placenta preavia, general inflammation or infection of the uterus (French et al., 1999, Lockwood and Kuczynski, 1999). Thrombin generation, which is a consequence of bleeding, is a potential mediator of the induction of preterm delivery as it is known to stimulate contractions and therefore promote preterm labour (Lockwood and Kuczynski, 1999, Rosen et al., 2001). Studies have found that bleeding in both first and second trimesters are highly correlated with preterm birth and this is influenced by heaviness of bleeding, recurrent bleeding and a higher total blood loss and longer duration of bleeding (Strobino and Pantel-Silverman, 1989, Yang et al., 2004).

1.3.2.2 Genetics

There is an emerging body of evidence linking genetic susceptibility and gene-environment interactions with the aetiology of preterm birth. Indeed, there is evidence to suggest that there may be a maternal genetic predisposition to spontaneous preterm delivery. If a women herself was born prematurely, she exhibits an increased risk of delivering preterm (Porter et al., 1997, Bhattacharya et al., 2010). However, there seems to be a lack of paternal influence as this predisposition did not carry over to preterm pregnancies fathered by males born preterm (Wilcox et al., 2008). Familial patterns of preterm birth have also been reported whereby pregnant women who have had an older sister deliver preterm have an 80% higher risk of also delivering a preterm infant (Winkvist et al., 1998).

In recent years, there have been a number of genetic studies examining both maternal and fetal genes in an attempt to identify specific genotypes which may directly contribute to the risk of preterm delivery or lead to vulnerability to deliver prematurely when exposed to risk factors associated with preterm birth. For example, inflammatory genes (particularly tumor necrosis factor-α, interleukins and their respective receptors), have been targeted given that maternal infection is a common cause of preterm delivery and these genes are mediators of the inflammatory response (Lockwood et al., 2006, Menon et al., 2006, Fortunato et al., 2008). However, to date there have been mixed findings and hence, further investigation is required.

As mentioned previously, the incidence of preterm birth is high in women from particular ethnic groups, whereas within the same community (country/city) women of different ethnicities exhibit a much lower risk. Hence, these epidemiological studies support the idea that there is a genetic predisposition to preterm birth (Varner and Esplin, 2005). However, it is

important to note that the findings of these studies can often be confounded by maternal health complications, socio-economic status and education influences. Interestingly, a number of epidemiological studies have controlled for many of these confounding factors, yet the increased propensity for preterm birth has remained (for example, in the African American population), thus supporting a genetic predisposition to preterm birth. In relation to this, it has been shown that African American women compared to non-African American women are more likely to carry allele variants which upregulate the cytokines that can promote inflammation (Hassan et al., 2003); thus these women may be more vulnerable to infections during pregnancy.

1.3.2.3 Maternal health

Good maternal health during pregnancy is imperative in order to maintain an optimal intrauterine environment for the baby's development. Hence, factors that adversely impact on maternal health can also impact on pregnancy outcome.

Maternal obesity

Maternal obesity is a growing concern globally and as the average body mass index (BMI) of the world's population increases, there is a concomitant rise in the weight of women when they become pregnant (Siega-Riz et al., 2006). Of concern, a study of pregnant women in Sweden has found that overweight and obese pregnant women are at increased risk of delivering preterm, most particularly extremely preterm delivery (Cnattingius et al., 2013). Another recent study has found that infants born prematurely were more likely to have obese mothers, with the risk increasing if other medical conditions were also present (Madan et al., 2010). However, not all studies have replicated these findings (Torloni et al., 2009); hence the inconsistency in findings makes it difficult to definitively conclude that obesity during pregnancy is directly linked to prematurity. Alternatively, it may be the medical complications that arise and occur frequently in obese women that are the contributing factors leading to preterm delivery (Aly et al., 2010).

Pre-eclampsia

Maternal medical conditions and disorders that exist pre-pregnancy or during pregnancy can be major risk factors for preterm birth; these include hypertension and diabetes mellitus (Lepercq et al., 2004, Zhang et al., 2012). Maternal illnesses can affect the delivery of nutrients and oxygen to the developing fetus, thus hindering its growth and increasing the risk of preeclampsia (Committee on Understanding Premature Birth Assuring Healthy Outcomes, 2007). Pre-eclampsia is a common maternal complication in which hypertension arises during pregnancy and it has been strongly linked to high BMI pre-pregnancy and the result is normally medically-induced preterm birth (Ananth et al., 1997, Duley, 2009). The incidence of preeclampsia is approximately 3 - 6% of all pregnancies, with an overall rate of 3.3% in singleton births in Australia between 2000 and 2008 (Thornton et al., 2013). There is no effective cure of preeclampsia, other than delivery of the fetus, which depends on the gestational age of the fetus and the severity of the gestational hypertension.

Diabetes

Importantly, correlating with the rise in maternal weight, the prevalence of diabetes is also increasing and is therefore a common complication of many pregnancies (Rosenberg et al., 2005). Both type 1 and type 2 diabetes before pregnancy and gestational diabetes are known to increase the chance of preterm deliveries (El Mallah et al., 1997, Ray et al., 2001, Vangen et al., 2003). Sometimes, gestational diabetes is due to pre-existing type 2 diabetes that has failed to be diagnosed before the mother becomes pregnant. In other cases, especially in obese women, pregnancy can lead to glucose intolerance, and subsequently to gestational diabetes (Waller and Dawson, 2005, Retnakaran et al., 2008).

Cervix length

Interestingly, there have been a number of studies reporting an association between the length of the cervix during pregnancy and spontaneous preterm birth. It has been reported that the relative risk of preterm delivery increases as the length of the cervix shortens (Iams et al., 1996, Stevens-Simon et al., 2000, Welsh and Nicolaides, 2002). Between 20 to 24 weeks of gestation, ultrasonographic measurement of the cervix can predict the risk of preterm birth.

Asymptomatic pregnant women measured at this time who were found to have a cervix length of 15 mm or less had a greater chance of delivering preterm (Fonseca et al., 2007).

In clinical practice, it is important to develop biochemical markers to predict impending preterm birth. In this regard, the presence of fetal fibronectin in the cervix and vagina of women has been discovered as an important biochemical marker in better identifying and managing women with the risk of preterm birth (Lockwood et al., 1991, Goldenberg et al., 1996, Peaceman et al., 1997, Berghella et al., 2008). The predictive accuracy of the fetal fibronectin screening test is apparently improved in women with a cervix length of more than 15 mm but less than 30 mm (Ness, 2009).

1.3.2.4 Maternal demographic and behavioural factors

Maternal age and ethnicity can play a crucial role in predicting the likelihood of preterm events. As mentioned previously, it is currently well-known that young mothers and mothers of African descent are at higher risk of experiencing preterm delivery (Scholl et al., 1988, Scholl et al., 1989, DuPlessis et al., 1997, Collins et al., 2007).

Other socio-economic factors and maternal lifestyle choices that have also been associated with increased risk of preterm birth are presented in Table 1.2; some of these factors are linked (Escriba-Aguir et al., 2001a, Moutquin, 2003, Beeckman et al., 2009, Delnord et al., 2015). It is important to note some of these factors have been more strongly associated with the risk of preterm delivery at earlier gestational ages than late gestational ages (Smith et al., 2006). For instance, reports have found that social disadvantage is more highly associated with very preterm birth compared to moderate preterm birth (Donoghue et al., 2013, Auger et al., 2014).

Maternal Factors	References
Low socio-economic status – based on earnings and education (socially disadvantaged)	(Ahern et al., 2003, Savitz et al., 2004, Hidalgo et al., 2005, Delpisheh et al., 2006, Smith et al., 2007)
Demanding jobs and hard working conditions	(Mamelle and Munoz, 1987, Berkowitz and Papiernik, 1995, Escriba-Aguir et al., 2001b, Saurel-Cubizolles et al., 2004)
Marital status – single mothers	(Zeitlin et al., 2002b, Luo et al., 2004, Raatikainen et al., 2005)
Depression	(Peacock et al., 1995, Alder et al., 2007, Gavin et al., 2009, Poehlmann et al., 2009)
Stress	(Copper et al., 1996, Wadhwa et al., 2001, Whitehead et al., 2002, Pike, 2005, Nkansah-Amankra et al., 2010)
Maternal/paternal smoking	(Burguet et al., 2004, Triche and Hossain, 2007, Jaddoe et al., 2008)
Alcohol consumption	(Lazzaroni et al., 1993, Sokol et al., 2007, O'Leary et al., 2009)
Lack of education	(Peacock et al., 1995, Astolfi and Zonta, 1999, Grjibovski et al., 2005, Luo et al., 2006)
Illicit drug abuse	(Gillogley et al., 1990, Spence et al., 1991, Kliegman et al., 1994, Burns et al., 2006)
Physical abuse and violence	(Heaman, 2005, Rodrigues et al., 2007, Chambliss, 2008)
Lack of prenatal care	(Hulsey et al., 1991, Orvos et al., 2002, Maupin et al., 2004, Taylor et al., 2005)

Table 1.2. *Maternal demographic and lifestyle factors associated with preterm birth*

1.3.3 Intrauterine growth restriction (IUGR)

Intrauterine growth restriction (IUGR) is a common complication of pregnancies (Rosenberg, 2008). In the clinics, the classification of IUGR is assigned to infants that are small for gestational age, with birth weight and/or length below the 10th percentile for that gestational age (Resnik, 2002). IUGR infants can exhibit symmetrical growth, where there is low weight, length and reduced body length and head circumference. Alternatively, asymmetrical growth restriction occurs when there is disproportionate growth of the head (increased head circumference), relative to body size (Lockwood and Weiner, 1986, Bryan and Hindmarsh, 2006). Importantly, IUGR is strongly associated with preterm birth, such that there is extensive evidence reporting that pregnancies complicated by IUGR also have a high incidence of preterm delivery (Zeitlin et al., 2000, Bukowski et al., 2001, Das and Sysyn, 2004). It has been suggested that the susceptibility for preterm birth in IUGR pregnancies is due to the factors that restrict appropriate fetal growth (Palliser et al., 2014). Furthermore, preterm birth when combined with IUGR, leads to an even greater risk of adverse health outcomes (McMillen et al., 2001); such that extremely and very preterm infants born small for gestational age are at increased risk of death and disability compared to preterm infants born appropriate for gestational age (Regev et al., 2003). Interestingly, IUGR independent of preterm birth, has also been associated with an increased risk of developing cardiovascular disease in later life (Barker et al., 1989, Barker, 1995, Barker et al., 2005, Demicheva and Crispi, 2014); and this is linked to structural and functional adaptations of the cardiovascular system (Hocher, 2007).

1.3.4 Multiple births

Being pregnant with twins, triplets or more is associated with an increased risk of preterm birth, with studies reporting that twin pregnancies account for a higher proportion of preterm births compared to singleton pregnancies (Gardner et al., 1995, Kurdi et al., 2004, Chauhan et al., 2010). Recent birth data in the US, reported that 57% of twins born were delivered prematurely, 93% of triplets were born preterm, 96% of quadruplets and 100% for higher order multiple births, compared to 10% of singletons (Martin et al., 2015). Overall, twin pregnancies make up the majority of multiple pregnancies and the incidence of twin rates has been increasing in recent years due to the use of assisted reproductive technologies (which

will be described later), maternal age, ethnicity and the decreasing numbers of triplets and higher multiple gestations (Eriksson and Fellman, 2007, Martin et al., 2009).

1.3.5 Assisted reproductive technologies

Assisted reproductive technologies (ART) are fast becoming one of the major contributors to increasing the risk of preterm birth. ARTs encompasses a number of techniques such as the use of donor sperm, embryo transfer, cryopreservation of sperm or ova, use of hormones to help induction and control of ovulation, micromanipulation of oocytes and embryos *in vitro* and many more (Barad and Witt, 2000). With infertility being a problem for at least one in six couples (Evers, 2002), the chance of conceiving for infertile couples has more than doubled with the development of ART, since the first birth by *in vitro* fertilisation in 1978. However, women who do conceive through ART are at higher risk of preterm birth, mostly because there are likely to be pregnant with multiple fetuses (Aboulghar, 2005) and it is known that the risk of preterm delivery increases with the number of fetuses being carried as mentioned previously (Barlow et al., 1988, Hill et al., 1990).

There are now laws to regulate the number of embryos transferred in ARTs (Alvero, 2002, Practice Committee of American Society for Reproductive Medicine and Practice Committee of Society for Assisted Reproductive Technology, 2013). For instance, Germany allows only 1 - 3 fertilised embryos to be transferred into the uterus (Beier and Beckman, 1991, van der Ven et al., 2002), whereas in America the number of embryos transferred depends on the likelihood of the mother having successful implantation; for a favourable prognosis, no more than 2 embryos are normally transferred, but for below average chances, no more than 5 embryos should be transferred (Dickey, 2007). This can help reduce the number of multiple gestations and increase the chances of survival and full-term delivery due to the lower number of fetuses. However, it is important to note that even in singleton pregnancies, the risk of preterm birth is higher compared to naturally conceived singleton pregnancies (McGovern et al., 2004); such that a population-based study in Australia of mothers with singleton gestations (born between 2007 - 2009) concluded that preterm birth was approximately 1.5 times higher in ART mothers compared to non-ART mothers (Xu et al., 2014).

1.4 Preterm birth survival

Over recent decades there have been ongoing advances in the neonatal care of preterm infants, leading to marked improvements in survival rates. A number of these strategies lead to accelerated organ maturation (particularly of the lungs) in the preterm infant (World Health Organisation, 2014a), thus facilitating postnatal organ function, as well as the realisation by the clinicians that preterm infants require different approaches to care after birth, compared to full-term babies (Yu and Doyle, 2004). If preterm birth cannot be prevented, these advances in technology have allowed for babies as early as 22 weeks of gestation to have an improved chance of surviving infancy (Allen et al., 1993, El-Metwally et al., 2000, Vohr and Allen, 2005, Goldenberg et al., 2008). The chance of survival amongst neonates born at 25 weeks of gestation is now as high as 79% (Kutz et al., 2009).

1.4.1 Antenatal corticosteroids and postnatal surfactant treatment

One of the major advancements leading to improved survival of preterm infants has been the introduction of antenatal administration of corticosteroids to women who are 'at risk' of preterm labour (Mwansa-Kambafwile et al., 2010, Roberts and Dalziel, 2013). This leads to accelerated maturation of the lungs of the infant, which facilitates survival if the baby is prematurely delivered. Immaturity of the lungs in preterm infants often leads to respiratory distress syndrome (RDS), which challenges breathing in the immediate period after birth and this is mainly due to the lack of surfactant production in the immature lungs (Smith et al., 2010). Importantly, administration of corticosteroids such as betamethasone or dexamethasone to the mother before birth can advance maturation of the respiratory epithelium by accelerating surfactant production to prevent the air sacs from collapsing and sticking together when breathing. When babies are born at term, the respiratory epithelium is sufficiently mature to naturally produce surfactant within the lungs, to lubricate the lining of the air sacs during breathing.

The current standard protocol of antenatal corticosteroids, involves administering betamethasone 12 mg intramuscularly, in 2 doses 24 hours apart (Surbek et al., 2012). Administration is normally between 24 and 34 weeks of gestation; however, under some circumstances it can be beneficial at 23 weeks or between 35 - 36 weeks of gestation.

Alternatively, surfactant (120 mg/1.5 ml vial; Curosurf, Chiesi USA, Inc., North Carolina, USA) can be administered directly to the preterm neonate via an endotracheal tube, by instilling the surfactant into liquid form. Currently, the treatment involves an initial dose of 1.25 - 2.5 ml/kg administered within 15 minutes of birth; subsequent doses of 1.25 ml/kg may be given 6 to 12 hours after the first dose and then 12 hours later in neonates who remain ventilator dependent, with a maximum total dose of 300 - 400 mg/ml. While the efficacy of surfactant in preventing and treating of RDS has been well-established, there are still concerns raised regarding the criteria, mode, timing, surfactant type and ventilatory management after administration (Ma and Ma, 2012). The most common and standard protocol currently used is early high doses of surfactant administration via an endotracheal tube during mechanical ventilation with subsequent extubation; this has been shown to reduce the time of oxygen therapy and mechanical intervention in preterm infants (Dani et al., 2004). However, a disadvantage to this method is that not all preterm infants will suffer from RDS and thus, some infants are unnecessarily exposed to intubation and surfactant administration.

1.4.2 Male disadvantage

With the improved survival following preterm birth, it has become apparent that there is a significant 'male disadvantage' associated with preterm birth (Zeitlin et al., 2002a, Roy et al., 2014). Males are more vulnerable to preterm birth than females, exhibiting higher rates of neonatal mortality and long-term morbidity (Kent et al., 2012). National figures in Sweden have shown that 55 - 60% of infants born between 23 and 32 weeks of gestation were boys (Ingemarsson, 2003). Furthermore, preterm male deaths were found to be more prominent in the first week of life; the difference in infant mortality for extremely and very preterm infants was 60% in males compared to 30% in females (Ingemarsson, 2003). It is generally understood that females are less vulnerable and have better outcomes after preterm birth, as a higher number of males develop RDS and chronic lung disease (Elsmén et al., 2004b). Therefore, males often do require more initial respiratory and circulatory support and neonatal care which can result in other long-term consequences (Elsmén et al., 2004a)

A number of studies have suggested that the potential mechanism for this vulnerability and increases of morbidity and mortality in males is their delayed development of organ systems (Peacock et al., 2012). In particular, it has been reported that there is slower lung and cardiac development in males. Fleisher et al. (1985) have shown that females have more advanced

lung maturation during late gestation, exhibiting lung maturation one week ahead of males in the last two months of gestation. This is likely to be the major contributing factor to gender differences in mortality and the increased risk of preterm males developing RDS (Perelman et al., 1986). Similarly, for the cardiovascular system, studies in sheep have reported a higher proportion of binucleated (therefore mature) cardiomyocytes within the hearts of female sheep fetuses compared to males of the same gestational age; thus suggesting cardiomyocyte maturation and thereby cardiac maturation occurs earlier in females compared to males (Lumbers et al., 2009).

1.5 Development of the cardiovascular system and the haemodynamic transition at birth

To date, much of the research in relation to preterm infants has focussed on the effects of being born early on an immature respiratory system. This has allowed for improvements in the care of preterm infants in the neonatal intensive care unit to facilitate their survival. However, at present very few studies have examined the effects on the immature cardiovascular system. This forms the focus of this thesis. In order to investigate the effects of preterm birth on the immature cardiovascular system, it is important to have an understanding of cardiovascular development and of the haemodynamic transition that takes place at birth.

1.5.1 Development of the heart

The heart is normally one of the first organs to develop in vertebrates, including humans (Sucov, 1998), as the circulation of blood is imperative to provide oxygen and nutrients to the tissues of the developing embryo/fetus. The formation of the primitive heart tube occurs very early in gestation; this is followed by the processes of heart looping, subsequent partitioning of the primordial heart chambers and morphogenesis of valves (Figure 1.3).

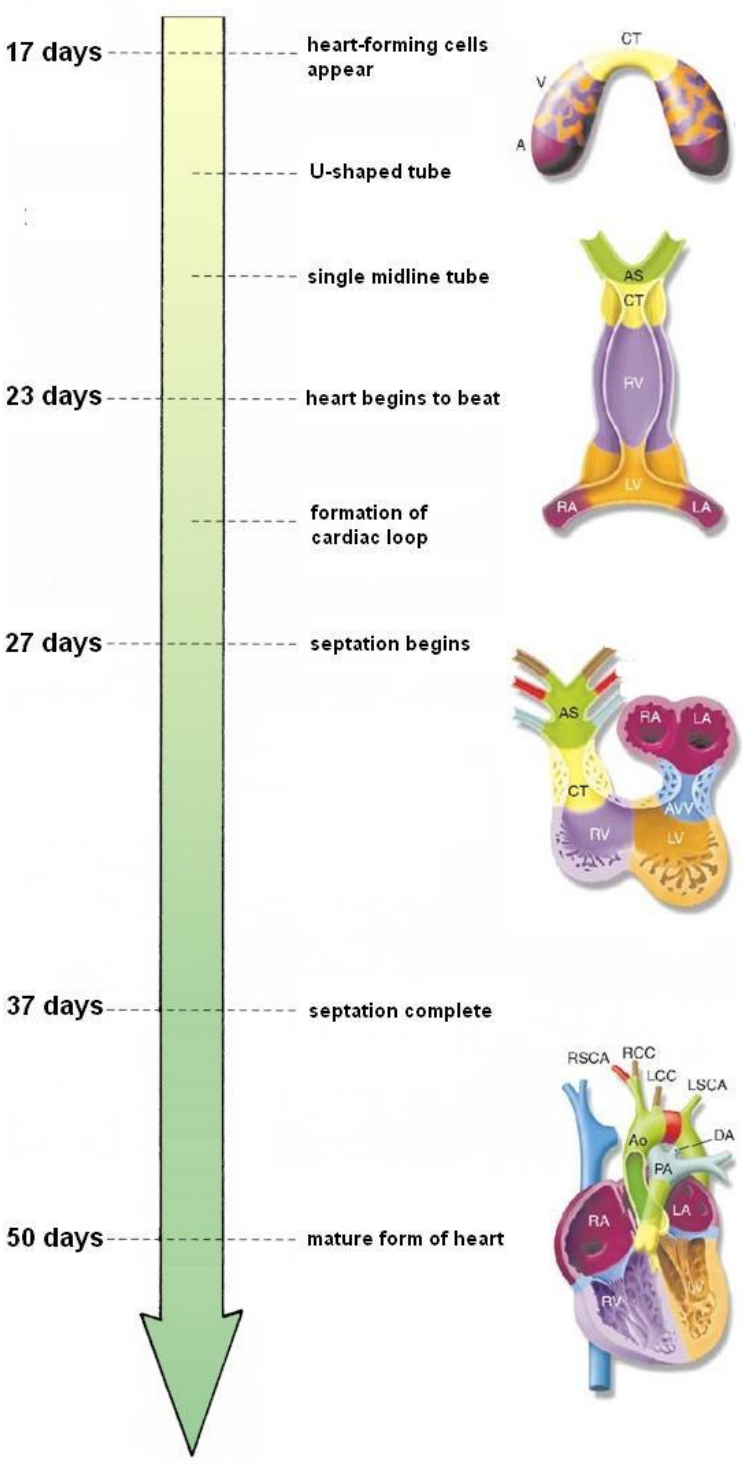

Figure 1.3. *Timeline for the development of the heart in humans. A-atrium, V-ventricle, CT-conotruncal, AS-aortic sac, RV-right ventricle, LV-left ventricle, RA-right atrium, LA-left atrium, Ao-aorta, DA-ductus arteriosus, RCC-right common carotid, LCC-left common carotid, PA-pulmonary artery, LSCA- left subclavian artery, RSCA-right subclavian artery (modified from Srivastava and Olson (2000)).*

Linear heart tube formation

Heart organogenesis begins very early in gestation when cells from the anterior lateral plate mesoderm in the primitive embryo are stimulated to form thin endocardial tubes from day 19 of gestation (Sedmera et al., 2000). Following cranial and lateral folding of the embryo, the endocardial tubes fuse in the midline to form the linear heart tube (Conway et al., 2001, Keating, 2004). By day 21 in gestation, constrictions and expansions divide the structure into segments to later give rise to the chambers of the heart (Fishman and Chien, 1997, Larsen, 2001, Carlson, 2009).

Heart looping

Between gestational days 23 and 28, the heart tube folds, bulges and loops rightwards to form an S-shaped heart. As looping progresses, this ultimately results in the correct spatial alignment of the chambers relative to each other as seen in the mature heart (Harvey and Rosenthal, 1999, Larsen, 2001, Carlson, 2009). The heart also begins beating in week 4 of gestation.

Chamber formation

Chamber formation begins in the middle of the 4th week with partitioning of the atrioventricular (AV) canal, primordial atrium and ventricle. These processes are simultaneously completed by the 8th week of gestation (Moore and Persaud, 2008).

- *Partitioning of the Atrioventricular Canal*

At the end of week 4, endocardial cushions protrude from the dorsal and ventral walls of the AV canal, to function as primitive valves. During the 5th week, the endocardial cushions fuse by the invasion of mesenchymal cells to separate the AV canal into left and right channels. Later in development, the valve in the right AV canal becomes the tricuspid valve, while the valve in the left AV canal becomes the bicuspid valve (Moore and Persaud, 2008, Carlson, 2009).

- *Partitioning of the Primordial Atrium*

The common atrium is divided into left and right atria by the formation of the septum primum and septum secundum. The septum primum extends from the superoposterior wall and grows towards the fusing endocardial cushions. As the septum grows, a large opening, the foramen primum, serves as a shunt for oxygenated blood to pass from the right to the left atrium. The foramen primum eventually disappears as the septum fuses with the endocardial cushions to form a primordial AV septum. However, another opening forms, the foramen secundum, which ensures continued shunting of oxygenated blood (Larsen, 2001, Moore and Persaud, 2008, Carlson, 2009).

While the septum primum is growing, another septum, the septum secundum, is also forming adjacent to it, posteroinferiorly from the ventrocranial wall of the atrium. This thick muscular fold eventually overlaps the foramen secundum but leaves an incomplete partition containing the foramen ovale. The cranial part of the septum primum slowly disappears and the remaining section forms the valve of the foramen ovale. The foramen ovale operates as the shunt between the right and left atria for the remainder of the gestational period. After birth, when the pressure in the left atria exceeds that in the right atria, the valve closes and subsequently fuses with the septum secundum to form the oval fossa (Larsen, 2001, Moore and Persaud, 2008, Carlson, 2009).

- *Partitioning of the Primordial Ventricle*

Partitioning of the primordial ventricle begins by a muscular interventricular (IV) septum growing from the floor of the ventricle near its apex. The IV septum gains height from dilation of the ventricles on each side and active proliferation of cardiomyocytes to increase its size. The IV foramen is originally present between the IV septum and the endocardial cushions, but after the 7^{th} week it closes due to further growth of the IV septum and a membranous component derived from the endocardial cushion connective tissue (Moore and Persaud, 2008, Carlson, 2009).

At the time when the IV septum is fully formed, the pulmonary trunk and aorta are in communication with the right ventricle (RV) and left ventricle (LV), respectively. This is brought about by division of the outflow tract and 180^{o} spiralling of the aorticopulmonary

septum. Finally, there is cavitation of the ventricular walls leading to the formation of the trabeculae carneae, papillary muscles and chordae tendinae (Moore and Persaud, 2008).

1.5.2 Cardiomyocyte growth and maturation

Cardiomyocytes are the cardiac muscle cells within the myocardium of the heart. They are present early in embryonic life and are responsible for heart morphogenesis (Bearzi et al., 2007). During early gestation, heart growth is predominantly achieved by hyperplasia (proliferation) of cardiomyocytes (Huttenbach et al., 2001, Yi et al., 2010, Mercola et al., 2011, Sedmera and Thompson, 2011) and as term approaches cardiomyocytes progressively undergo a process of maturation whereby they become terminally differentiated (Li et al., 1996). By term, the majority of the cardiomyocytes have ceased proliferation (Mayhew et al., 1997, Burrell et al., 2003, Bubb et al., 2007).

It has been long thought that the postnatal growth of the heart was almost exclusively by hypertrophy (enlargement) of cardiomyocytes and deposition of extracellular matrix, and that proliferation of cardiomyocytes was rare after birth (Oparil et al., 1984, Olivetti et al., 1996, Rudolph, 2000). However, recent new evidence in animal studies has shown that there remains the ability for cardiomyocytes to proliferate postnatally, albeit at a much lower rate, and this contributes to cardiac growth and regeneration between birth and adolescence (Mollova et al., 2013, Senyo et al., 2014, Ali et al., 2014). In particular, Mollova et al. (2013) reported a 3.4 fold increase in the number of cardiomyocytes within the LV from birth to 20 years of age in humans.

Fetal cardiomyocytes are morphologically different from cardiomyocytes in the mature heart. In their immature state, fetal cardiomyocytes are able to undergo cytokinesis (cellular division) whereas mature cardiomyocytes generally do not divide (Mayhew et al., 1997, Bubb et al., 2007). Furthermore, fetal cardiomyocytes are smaller in diameter and have fewer myofibrils within their cytoplasm when compared to cardiomyocytes in the mature state (Hoerter and Vassort, 1982, Rudolph, 2000).

Interestingly, cardiomyocytes often exhibit an increase in ploidy in the human heart (number of gene copies per cell) as they undergo hypertrophy (especially in adulthood), with a high proportion of cardiomyocytes becoming polypoid, where they contain tetraploid (4N) or greater DNA (Clubb and Bishop, 1984, Rudolph, 2000, Corstius et al., 2005). Alternatively, in most animal models (such as the sheep and rodents) the cardiomyocytes become binucleated

in their mature form. As seen in Figure 1.4, in the sheep, the timing of cardiomyocyte maturation in the developing heart is similar to the human with the majority of the cardiomyocytes mature (and binucleated) by birth (Burrell et al., 2003, Jonker et al., 2007). In rodent models, such as the mouse and rat, the cardiomyocytes are immature (mononucleated) and still proliferating at birth and they undergo a process of maturation within the first two weeks after birth whereby they become mature, differentiated and binucleated (Li et al., 1996, Soonpaa et al., 1996, Porrello et al., 2011).

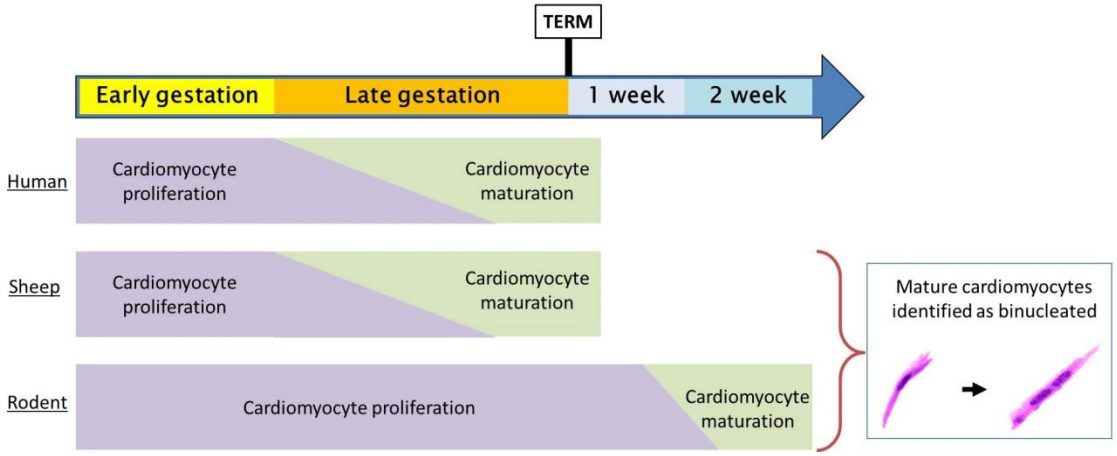

Figure 1.4. *Timeline of cardiomyocyte proliferation and maturation in the human, sheep and rodent.*

Since the proliferative capacity of cardiomyocytes markedly decreases around the time of birth, a reduced complement of cardiomyocytes in the heart at birth is likely to influence lifelong functional reserve and the adaptive capabilities of the heart postnatally (Anversa et al., 2007). Hence, factors in the early life environment, that lead to an accelerated transition from hyperplastic to hypertrophic growth in cardiomyocytes, are likely to adversely impact long-term postnatal growth of the heart; whereas a delayed transition from hyperplastic to hypertrophic growth in the early life environment, may adversely impact the contractility of the heart in the neonatal period.

In this regard, several studies have investigated the effects of perturbations in early life on the growth and maturation of cardiomyocytes (such as alcohol exposure, IUGR and preterm birth) (Stacy et al., 2009, Bensley et al., 2010, Black et al., 2012, Botting et al., 2012, Nguyen et al., 2014, Paradis et al., 2015). For example, the effect of maternal alcohol consumption during

late pregnancy has recently been examined in sheep (Goh et al., 2011). It was reported that ethanol exposure significantly advanced maturation of cardiomyocytes within the LV of fetal sheep, as the proportion of binucleated cells increased by 12% when compared to controls, and the proportion of mononucleated cells was decreased.

In other studies, the effect of IUGR on cardiomyocyte growth and maturation has been examined. Corstius et al. (2005) reported that IUGR in rat offspring, as a result of maternal protein restriction, resulted in a significant reduction in the number of cardiomyocytes in the heart. Furthermore, in IUGR lambs, it was reported that the incidence of binucleated cardiomyocytes was significantly lower in the LV compared to control lambs, which indicates delayed cardiomyocyte maturation (Bubb et al., 2007). Similarly, induction of umbilicoplacental embolization in a sheep model, to observe the effects of placental insufficiency and therefore restricted fetal growth, also led to a reduction of binucleated cardiomyocytes in the ventricles of these growth-restricted fetuses compared to control fetuses (Louey et al., 2007).

In addition, the effect of preterm birth on the growth and maturation of cardiomyocytes in the first weeks of life has recently been examined in an ovine model of moderate preterm birth (Bensley et al., 2010). Cardiomyocytes in both ventricles were found to be significantly larger in hearts of preterm lambs compared to term lambs and as expected, in term lambs most cardiomyocytes were binucleated. However, in the preterm lambs, there were significantly fewer binucleated cells and more mononucleated cardiomyocytes, indicating that the ventricular muscle was less mature in the preterm animals.

1.5.3 Development of blood vessels

Accompanying the formation of the heart, it is essential that the vascular system is concomitantly established to allow circulation of the blood throughout the developing embryo/fetus. At the beginning of gestation, embryonic growth occurs by simple diffusion of nutrients, but then in an ordered and sequential manner it becomes a highly vascular organism, relying on a complex array of blood vessels to ensure survival (Noden, 1989). The formation of blood vessels is a combination of two simultaneous processes: (1) vasculogenesis and (2) angiogenesis (Demir et al., 2006).

Vascular formation begins at week 4 in humans and development is fairly rapid to ensure the first circulatory loops are formed for the heart; blood supply to the heart muscle is essential in

order to pump the necessary oxygen and nutrients to the other developing tissues and organs (Figure 1.5) (Risau, 1997, Yancopoulos et al., 2000, Freedman and Isner, 2002, Llevadot and Asahara, 2002).

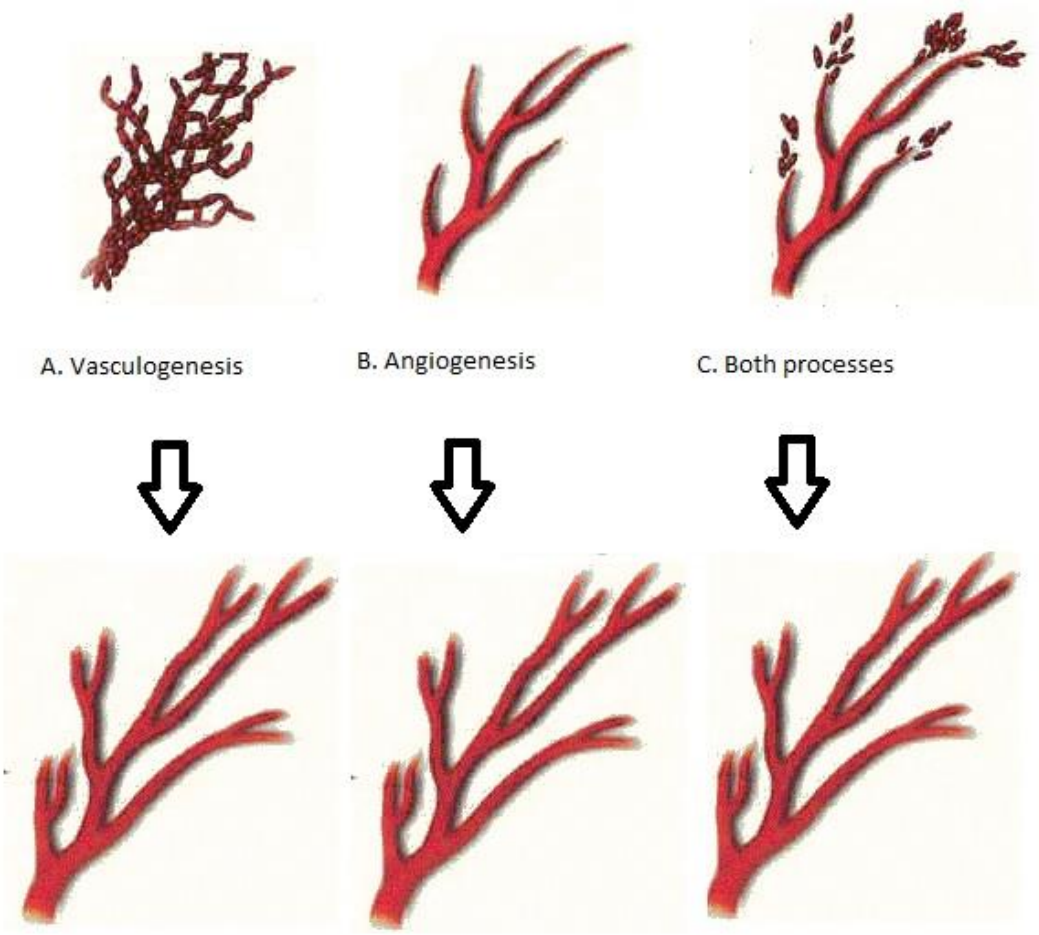

Figure 1.5. *A. Vasculogenesis, the aggregation of endothelial progenitor cells to form blood vessels. B. Angiogenesis, formation of new blood vessels from pre-existing ones by proliferation of differentiated endothelial cells or dividing of pre-existing vessels. C. The two processes can occur simultaneously (modified from Llevadot and Asahara (2002)).*

1.5.3.1 Vasculogenesis, angiogenesis and arteriogenesis

Vasculogenesis is defined as the spontaneous formation of blood vessels. It is the initial event which occurs when endothelial progenitor cells (angioblasts) migrate and fuse with others to differentiate into endothelial cells; these assemble into solid endothelial cords and then form a plexus of endothelial tubes (Conway et al., 2001). Vasculogenesis is induced by the mesoderm and endoderm and is regulated by the expression of vascular endothelial growth factor (VEGF) in the extra-embryonic visceral endoderm and mesoderm (Patan, 2000). It appears that a threshold level of VEGF is required for angioblasts to differentiate and subsequently generate the primary vascular plexus (Carmeliet et al., 1996, Ferrara et al., 1996).

Angiogenesis is the term for growth of new blood vessels from pre-existing vessels and this is mainly how vascular trees extend in the developing fetus and expand through budding capillaries (Carmeliet, 2005). With the primary vascular plexus formed, endothelial cells can form new capillaries by either one or a combination of two types of angiogenesis: (1) sprouting angiogenesis and (2) non-sprouting angiogenesis (also known as intussusception) (Logsdon et al., 2014). Sprouting angiogenesis involves the sprouting of new vessels from the sides and ends of pre-existing ones. This involves the proteolytic degradation of the extracellular matrix at the site of sprouting so that migration and proliferation of endothelial cells can occur to develop a lumen area and functional maturation of the endothelium. Sprouting angiogenesis is recognised by the 'sprout' which are long extensions of the endothelial cell at the tip reaching for VEGF-producing cells (Ucuzian et al., 2010). Alternatively, non-sprouting angiogenesis is characterised by the longitudinal division of pre-existing vessels (i.e. splitting of a pre-existing vessel into two). This can be initiated *in vivo* by the proliferation of endothelial cells within the vessel, which produces a larger lumen and consequently results in the fusion and splitting of capillaries or the division by pillars of periendothelial cells (Risau, 1997, Patan, 2000, Conway et al., 2001).

Arteriogenesis is the term for the maturation of arteries from pre-existing arterioles after an arterial occlusion by enlarging them via the recruitment of smooth muscle cells (Buschmann and Schaper, 1999, Carmeliet, 2000, Cai and Schaper, 2008). This process is generally linked to elevated pressure and flow, which increases oxidative stress and endothelial surface stress and so arteriogenesis occurs until the stress is normalised (Prior et al., 2004). In general, in the

adult, continuous growth of blood vessels occurs by angiogenesis and arteriogenesis (Heil et al., 2006).

1.5.3.2 Composition of the blood vessel wall and perinatal structural development

The blood vessel wall is primarily composed of three major components: (1) elastin, (2) collagen and (3) smooth muscle (Wells et al., 1999, Wagenseil and Mecham, 2012). Elastin and collagen make up what is known as the extracellular matrix. Elastin is thought to contribute most to the elastic properties of blood vessels during low to moderate arterial pressures with increasing collagen contributions at higher arterial pressures (Roach and Burton, 1957, Wolinsky and Glagov, 1964). The role of elastin is to assist with the expanding and relaxing of blood vessels during systole and diastole respectively, to allow the blood to move forward. In blood vessels, collagens type I and type III are the main collagen sub-types present. Collagen provides strength to the blood vessel wall and is also thought to help prevent elastic blood vessels from stretching during systole beyond their physiological limits (Ooshima et al., 1974). Vascular smooth muscle cells are responsible for contraction (vasoconstriction) or relaxation (vasodilation) of the blood vessel in response to autonomic nerve stimulation and external stimuli to control blood flow (Daubman, 2010).

Throughout the vasculature, the blood vessel wall is generally arranged into three layers: (1) the intima, (2) media and (3) adventitia. The intima is the thinnest and innermost layer bordering the lumen area of the vessel. It is usually comprised of a single layer of endothelial cells (simple squamous epithelium) and a subendothelial layer (which is large in arteries and thickens with age or disease), which is separated from the media by the internal elastic lamina (a dense elastic membrane). The medial layer is composed primarily of smooth muscle with varying amounts of elastin and collagen depending on the site within the vascular tree; for example, there are very high amounts of smooth muscle, elastin and collagen within the wall of conduit arteries but the relative proportions are reduced as the vascular tree branches. Overall, the structure of the blood vessel wall throughout the vasculature is site specific, with the structure reflecting the function and vice versa. The outermost layer of the blood vessel wall is the adventitia, which is predominantly composed of collagen (Fung and Liu, 1995, Patel et al., 2006). Separating the media from the adventitia, there is a dense elastic membrane called the external elastic lamina.

As the arteries are formed during development, it is the ratio of elastin, collagen and smooth muscle within the blood vessel wall that is thought to be important for long-term function. During the perinatal period, particularly immediately prior to birth, the large conduit arteries undergo much remodelling in dimensions and in the relative composition of the arterial wall. This is in preparation for the haemodynamic transition that takes place at birth (see later – Section 1.5.4), whereby there is a marked increase in arterial pressure. In particular, elastin and collagen are rapidly laid down during late gestation in conduit arteries to prepare these blood vessels for the large increases in postnatal arterial pressures which accompany the haemodynamic transition at birth (Langille et al., 1989, Di Stefano et al., 1998). It has been reported that experimentally induced alterations in blood flow rates after birth can modulate the growth of the arterial diameter (Coyle, 1985, Guyton and Hartley, 1985) as well as accumulation of wall constituents. A study by Bendeck and Langille (1991) showed in sheep studies that there was a dramatic accumulation of elastin and collagen in the aorta from 140 days of gestation to 3 days after birth (birth is normally at 147 days of gestation in sheep).

1.5.4 Haemodynamic transition at birth

The haemodynamic transition at birth is an extremely important process, where the cardiovascular system of the infant undergoes dramatic functional changes as the circulatory configuration changes from the fetal configuration to the postnatal configuration (Cuneo, 2013, Johnson et al., 2014). This occurs at birth when the infant breathes air for the first time and is therefore no longer receiving oxygen and nutrients from the placental vascular bed.

Prior to birth, the placenta is an essential organ that is the source of oxygen and nutrients to the fetus. It encompasses both maternal and fetal blood vessels in close apposition, which flow in separate vessels to prevent mixing, but still enabling the uptake of nutrients and oxygen and elimination of waste and gases in the fetal circulation. During gestation, as the fetus does not use its lungs for gas exchange, only a small amount of blood actually flows through the fetal lungs (11% of cardiac output) (Mielke and Benda, 2001, Kiserud, 2005). In addition, waste products from the fetal bloodstream are passed across the placenta and then eliminated via the maternal liver. Hence, in the fetal circulation there is a shunt (the ductus venosus) that allows blood to bypass the fetal liver and therefore, transporting the majority of the blood supply directly from the placenta through to the inferior vena cava and to the right

side of the fetal heart, with only a small proportion of placental blood flow directed to the fetal liver.

Within the heart, beginning in the right atrium, oxygenated blood from the placenta is mixed with the venous return; the majority of oxygenated flow is deflected through the foramen ovale into the left atrium, bypassing the pulmonary system. The foramen ovale (opening between the left and right atria) allows shunting of blood from the right atrium to the left atrium. In the fetal circulation, this opening is kept patent due to the higher pressure in the right atrium compared to the left atrium, but functionally closes within hours after birth as pressures within the heart change.

The blood that does not directly pass through the foramen ovale mixes with deoxygenated blood from the superior vena cava and flows into the RV and then exits the heart via the pulmonary trunk. There is another fetal shunt (the ductus arteriosus) which lies between the pulmonary trunk and the proximal descending aorta, which allows the majority of the blood leaving the RV to bypass the lungs and divert directly into the descending aorta (Walther et al., 1993). Blood flow preferentially flows through the ductus arteriosus due to the pressure gradient that exists between the high resistance low flow pulmonary circulation, and the low resistance high flow systemic circulation, which promotes blood flowing right-to-left, from the pulmonary to the systemic circulation (Crossley et al., 2009). Figure 1.6 illustrates the differences between fetal (A) and postnatal (B) circulation.

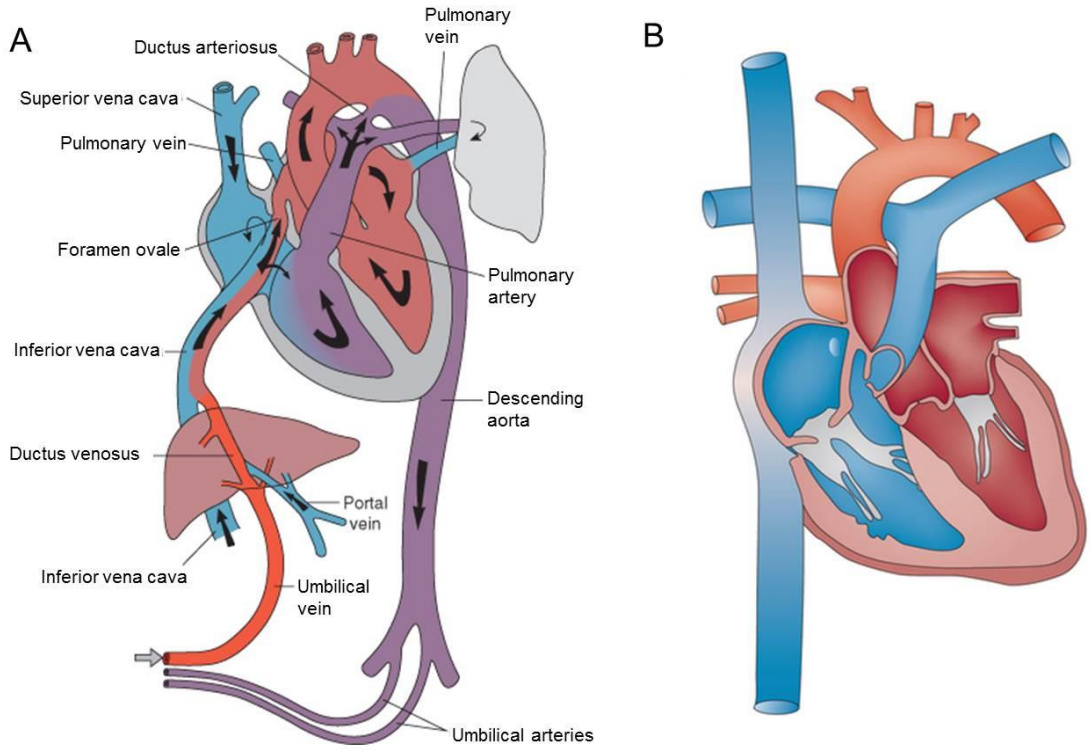

Figure 1.6. *(A) Fetal circulation: oxygenated blood from the placenta enters the right atrium via the umbilical vein, ductus venosus and inferior vena cava. Oxygenated blood (shaded red) passes through the right atrium to the left atrium via the foramen ovale to bypass the pulmonary circulation. A secondary shunt, the ductus arteriosus, is also present between the pulmonary trunk and the descending aorta to further allow mixed blood (shaded purple) that has entered the right ventricle to bypass the lungs. (B) Normal postnatal circulation: all shunts are closed after birth as the lungs are being utilised, with the right and left sides of the heart completely separated so that oxygenated and deoxygenated blood (shaded blue) no longer mix. Modified from Sinha et al. (2012) and Hunter and Simpson (2014).*

In utero, the RV is the dominant chamber; it ejects 66% of the combined ventricular output of the fetal heart and hence exhibits a thicker wall compared to the LV before birth (Rudolph, 1979). The RV receives the majority of the venous return. In contrast, because the pulmonary circulation is a low flow, high resistance circuit, there is less venous return to the left side of the heart. It can be elucidated from studies measuring pulmonary arterial pressure before and after birth, that there is a dramatic fall in pulmonary arterial (RV) pressure after birth (Wong et al., 1994, Crossley et al., 2007, Allison et al., 2010, Baron et al., 2012).

Hence, there are many changes that occur at birth to the cardiovascular circulation, including: loss of the low resistance placental vascular bed with cutting of the umbilical cord, alterations to venous return and subsequently cardiac output, the initiation of breathing of the newborn, exposure to atmospheric concentrations of oxygen and closure of the fetal shunts (foramen ovale, ductus arteriosus, ductus venosus) (Blackburn, 2006). The transition is initiated by clamping of the umbilical cord, which results in a sudden and rapid rise in systemic arterial pressure (Hooper et al., 2015). Heart rate is also markedly increased, as shown in Figure 1.7 where mean arterial pressure and heart rate of fetal sheep and postnatal lambs increased significantly after birth (Louey et al., 2000).

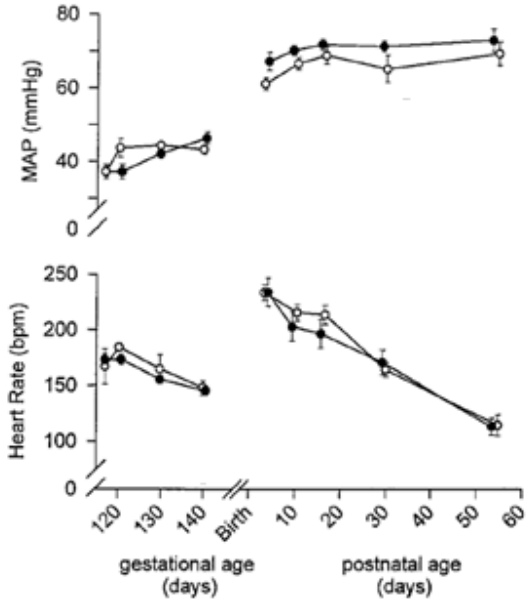

Figure 1.7. *Mean arterial pressure (MAP) and heart rate in IUGR (unfilled circles) and control (filled circles) fetuses and postnatal lambs until 8 weeks of age (reproduced with permission from Louey et al. (2000)).*

The initiation of breathing and removal of lung liquid results in a profound decrease in pulmonary vascular resistance, resulting in 100% of RV output to enter the lungs. The decrease in pulmonary vascular resistance combined with the increase in systemic arterial pressure results in a reversal of the pressure gradient across the ductus arteriosus, resulting in a rapid increase in pulmonary blood flow at birth (Crossley et al., 2009, Hooper et al., 2015). The increase in pulmonary blood flow allows for a large increase in venous return to the left

atrium and ventricle, causing an increase in LV output (Hooper et al., 2014). A study by van Vonderen et al. (2014), using echocardiography on newborns within the first 10 minutes of life, reported that LV dimensions (LV end-diastolic diameter and LV end-systolic diameter) and LV output significantly increased within the first 5 minutes after birth and stabilised after 10 minutes. These changes are due to the increased load on the ventricle resulting from pulmonary blood flow changes and ductal shunting as the LV is now pumping 100% of cardiac output compared to the 33% in the fetus.

1.6 Preterm birth and the heart

Babies born at term exhibit a relatively mature heart and vasculature, such that the cardiovascular system is structurally prepared for the haemodynamic transition at birth. However, when birth comes early, the cardiovascular system of the preterm infant is prematurely exposed to the haemodynamic transition at birth, at a time when the cardiovascular system is structurally immature. Indeed, during the last part of gestation, final cardiomyocyte number is established in the developing myocardium and it is known that increased systemic pressure impacts proliferation and terminal differentiation in immature cardiomyocytes (Barbera et al., 2000, Jonker et al., 2007). Also, there are many changes in circulating hormone levels (cortisol, angiotensin, thyroid hormone) in the lead up to parturition and in the 1-2 weeks after birth (Hillman et al., 2012); these hormones have known impacts on cardiomyocyte development (Thornburg et al., 2011). Hence, preterm birth in the absence of normal prepartum changes in these hormones and/or premature exposure to postnatal hormone levels can also impact development of the heart.

Early in life

Hypotension (low arterial pressure) is often observed in preterm infants in the immediate period after birth whilst in the neonatal intensive care unit (Georgieff et al., 1996, Dannevig et al., 2005). This is most common for preterm infants born very or extremely premature (Subhedar, 2003, Witcombe et al., 2008) and is thought to result from immature cardiovascular control of arterial pressure or a persistent ductus arteriosus (Harper, 2000, Sarkar et al., 2007). For instance, low superior vena cava flow in preterm infants born before 30 weeks gestation has been associated with higher upper body vascular resistance and larger

diameter ductal shunts which can result in an increased risk of developing of intraventricular haemorrhaging (Kluckow and Evans, 2000). Similarly, the delayed closure of the ductus arteriosus in preterm infants creates abnormal blood flow between the heart and lungs (Abdel-Hady et al., 2013); this is more evident in preterm infants born at earlier gestational ages as the incidence of patent ductus arteriosus increases with decreasing gestational age (Dagle et al., 2009). Hence, hypotension occurs when there is an inadequate rise of systemic arterial pressure in preterm infants due to a delayed decrease in pulmonary arterial pressure and therefore delayed increase of cardiac output after birth. These hypotensive babies generally require inotropic support, such as dopamine and/or dobutamine therapies, to normalise their arterial pressure and mitigate potential short-term and long-term consequences of inadequate pressures to organ systems (Ibrahim, 2008). However, during the first month of life, it has been reported that arterial pressure increases more rapidly in infants born preterm compared to those born at term (Pejovic et al., 2007). The initial hypotension in the immediate period following birth is in direct contrast to the hypertension often observed later in life in subjects born preterm; the mechanisms leading to this hypertension are poorly understood.

To date, despite the increasing incidence of preterm birth, the short-term and long-term effects on the heart and the growth of cardiomyocytes have not been extensively studied. Past studies have mostly used non-invasive methods, such as ultrasound, to examine the hearts of premature infants. These studies have shown that structural cardiovascular defects (patent ductus arteriosus and heart septal defects) were more likely to be found in preterm infants, but there is no evidence that preterm birth causes them (Tanner et al., 2005). Histological studies can only be undertaken in the hearts of deceased infants; this tissue is difficult to obtain and effects on the growth of the heart can be confounded in these analyses, by the fact that these have been very ill infants.

Animal models have allowed for carefully controlled studies on the effects of preterm birth in offspring that are in good health. In this regard, a study by Bensley et al. (2010) examined the effect of moderate preterm birth on the structure of the heart in 9-week old sheep that were born preterm. In this study, it was shown that the premature shift from a fetal to postnatal circulatory configuration leads to remodelling of the heart structure; collagen deposition was significantly greater, the size of cardiomyocytes was increased and there were changes in cardiomyocyte maturation (such that fewer cardiomyocytes were binucleated and there was an increase in the ploidy of mononucleated cardiomyocytes). These cardiac changes in the

myocardium have the potential to lead to long-term adverse consequences on cardiac structure and function.

Later in life

Based on these findings, clinical studies have recently been conducted in young adult subjects that were born preterm to look at the long-term impact of preterm birth on cardiac structure and function. In these studies, Lewandowski and Leeson (2014) performed cardiovascular phenotyping via magnetic resonance imaging (MRI) on young adults born preterm, to quantify ventricular mass, 3D geometric variation and myocardial function. Of concern, preterm birth was associated with increased ventricular mass (which was inversely related to gestational age) and abnormal ventricular shape; ventricles were shorter with smaller internal diameters and the apex was displaced in the LV. Ventricular function was also found to be significantly impaired, such that there was reduced longitudinal systolic (peak strain, strain rate and velocity) and diastolic (peak strain rate and velocity) function and rotational (apical and basal peak systolic rotation rate and net twist angle) movement in individuals born preterm compared to those born at term. Interestingly, the effects of preterm birth were more pronounced in the RV than the LV, in particular for systolic function where preterm subjects had significantly lower RV ejection fraction compared to term controls, however this was not the case in the LV (Lewandowski et al., 2013a, Lewandowski et al., 2013b).

1.7 Preterm birth and the vascular system

In addition, to adverse effects in the heart, there have been a number of reports in animal models and in human subjects demonstrating structural differences in blood vessels of preterm offspring when compared to term offspring.

Early in life

In recent studies, in an ovine model of moderate preterm birth, Bensley et al. (2012) have reported remodelling of the great vessels (aorta and pulmonary artery) following preterm birth. At 9 weeks of age, the aortas of preterm lambs exhibited thicker walls and smaller lumenal areas compared to term lambs, however, the pulmonary artery appeared unaffected.

Both blood vessels demonstrated a significant increase in elastin deposition, but only the aorta showed a marked decrease in smooth muscle content. Of concern, it was observed histologically in the aorta of four of the seven preterm lambs that there was severe injury to the arterial wall; this is likely related to the high increase in arterial pressure at birth in an immature aorta.

Later in life

There are a number of clinical studies that report altered cardiovascular structure in subjects born preterm. For example, Bonamy et al. (2005) using non-invasive imaging techniques, examined vascular function and structure in adolescent girls that were born at term or preterm (mean gestational age of 29 weeks at birth). Compared to terms, those born preterm were found to have significantly elevated brachial artery and aortic arterial pressures; they also exhibited a narrower lumen area in the abdominal aorta. A subsequent study by (Edstedt Bonamy et al., 2008) again reported narrowing of the aortic lumen (16% narrowing in the thoracic aorta and 19% narrowing in the abdominal aorta) in 15-year-old individuals born very preterm. In addition, 9-year-old children born very preterm were also found to have reduced capillary density compared to term subjects of the same age (Bonamy et al., 2007).

Increased arterial wall thickness has also been linked with preterm birth, with studies reporting a relationship between higher carotid artery intima-media thickness and cardiovascular risk factors in middle aged adults that were born preterm (Burke et al., 1995, Chambless et al., 1997, Chambless et al., 2000, Davis et al., 2001, Polak et al., 2011). Taken together, the findings collectively demonstrate that preterm birth can lead to persistent adverse effects on the growth of the vasculature, which have the potential to predispose to cardiovascular disease and to the development of hypertension. In this regard, there are now many studies that link preterm birth with the development of hypertension in later life.

1.8 Hypertension

Hypertension is a sustained elevation in arterial pressure and it is a major risk factor for cardiovascular disease (Chobanian et al., 2003, Aje and Miller, 2009). Over recent decades, preterm birth has been strongly linked to the development of hypertension by early adulthood; the risk is increased with the severity of prematurity (Bonamy et al., 2005,

Johansson et al., 2005, Doyle, 2008, Norman, 2010, Crump et al., 2011, Kerkhof et al., 2012, Sutherland et al., 2014). For instance, a study by Johansson et al. (2005) investigated the association between gestational age at birth and risk of high arterial pressure in young Swedish men. It was found that 20% of men born preterm had high systolic blood pressure (higher than 140 mmHg) and this was influenced by gestational age at birth, such that 32% of those born extremely preterm were hypertensive and 24% born moderately preterm were hypertensive. Overall, individuals that were born extremely preterm were reported to have almost a 2-fold increased risk of developing high systolic blood pressure compared to those born at term. Importantly, linear regression analyses from this study have illustrated that a 0.31 mmHg increase of systolic blood pressure was found for every week born less than 37 completed weeks of gestation. Similarly in another study, young adults born very preterm (<32 weeks of completed gestation), both small for gestational age and appropriately grown for gestation age, were found to have high daytime systolic blood pressure and higher risk of hypertension than those born at term (Keijzer-Veen et al., 2010).

The rise in arterial pressure in preterm individuals is clinically important given that hypertension is a major risk factor for cardiovascular disease. Indeed, only small changes in arterial pressure can have marked effects on cardiovascular disease risk; for example, a 4.7 mmHg reduction in diastolic blood pressure can lower the risk of stroke and ischaemic heart disease by 32% and 20%, respectively (Law et al., 2003). Hence, it is imperative to gain an understanding of the aetiology of hypertension in subjects that were born preterm. Indeed, it is likely that adaptive changes in the immature cardiovascular system following preterm birth are linked to the aetiology of hypertension and lead to vulnerability to cardiovascular disease.

1.9 Focus of this thesis

Preterm birth is a truncation of the *in utero* development period, resulting in early delivery to a very contrasting *ex utero* environment. For fetuses born at term, their organ systems are more mature and further developed compared to those born preterm; therefore, more "prepared" for the transition to *ex utero* life. Preterm infants experience the same changes with birth (i.e. increase in systemic pressure and decrease in pulmonary pressure); however, these sudden haemodynamic changes at birth will undoubtedly affect the underdeveloped cardiovascular system of these subjects. It is known that during late gestation that the total number of cardiomyocytes is set in the developing myocardium and that increased systemic

pressure can impact proliferation and terminal differentiation in immature cardiomyocytes. Similarly, cardiomyocyte development can be affected by the changes in circulating hormone levels (for example, cortisol, angiotensin, and thyroid hormone) in the lead up to parturition and in the first two weeks after birth. Hence, preterm birth in the absence of normal prepartum changes in these hormones and/or premature exposure to postnatal hormone levels can impact development and function of organ systems. Additionally, elastin is largely laid down in late gestation and so the truncation of normal deposition combined with the premature exposure to postnatal arterial pressures is likely to affect the vascular composition of large conduit arteries.

Therefore, it is imperative to gain an understanding of how preterm birth affects the heart and blood vessels in the immediate period after birth and in early adulthood; in particular, those born moderately preterm as they make up the majority of preterm births. In this thesis, I have examined the effect of moderate preterm birth, using an ovine model, on the structure of the heart and cardiomyocyte growth, and on the structure and composition of the large conduit arteries (thoracic aorta and left carotid artery).

1.10 Aims

In Chapter 2, I have established an ovine model of moderate preterm birth based on the previously published model of De Matteo et al. (2010) and administered a clinically relevant dose of betamethasone antenatally, that is routine in clinical practice. Using this ovine model of moderately preterm birth, the specific aims were to determine the effect of moderate preterm birth on:

- Survival, body growth and physiological parameters in the immediate period after birth (Chapter 2)
- Survival, body growth and physiological parameters from birth to early adulthood (Chapter 3)
- Heart structure and cardiomyocyte growth in the immediate period after birth and in early adulthood (Chapter 4)
- Structure and composition of the large conduit arteries (thoracic aorta and left carotid artery) in the immediate period after birth and in early adulthood (Chapter 5)

To address these aims, in Chapter 2, a sheep model of moderately preterm birth was developed with the first cohort of animals (short-term study) maintained for 2 days after birth. In these preterm and term lambs, percentage survival, body growth, arterial pressure, heart rate and organ weights at necropsy were examined.

In Chapter 3, using this model of moderate preterm birth established in Chapter 2, another cohort of preterm and term sheep (long-term study) were maintained until 14.5 months of age (early adulthood). Percentage survival, body growth, arterial pressure and heart rate were continuously measured over the experimental period; then in adulthood, body composition was measured using Dual-energy X-ray absorptiometry and organ weights recorded at necropsy.

In Chapter 4, with fixed and frozen heart tissues collected from both short-term and long-term cohorts of animals, heart size was examined using image analysis for ventricular wall thickness and the Cavalieri principle for ventricular wall and chamber volume. Cardiomyocyte number and cardiomyocyte nuclearity were determined using an optical disector/smooth fractionator approach and image analysis after immunohistochemistry, respectively. Collagen content in the ventricular walls was estimated using a hydroxyproline assay.

In Chapter 5, fixed and frozen segments of the thoracic aorta and the left carotid artery were excised from both short-term and long-term cohorts. To investigate potential differences in blood vessel structure, lumen area, medial area, wall thickness, number of elastin layers and biochemical composition were analysed with image analysis after staining with Verhoeff van Gieson's stain. Collagen content in the blood vessel wall was determined using a hydroxyproline assay.

Chapter 2:

The Effect of Moderate Preterm Birth on Survival, Body Growth, Arterial Pressure and Heart Rate in the Immediate Period after Birth

2.1 Introduction

Preterm birth is defined as birth that occurs before 37 completed weeks of gestation. It can be further subdivided according to gestational age at the time of delivery: extremely preterm (<28 weeks), very preterm (<32 weeks) and moderately preterm (32 to 37 weeks) (Tucker and McGuire, 2004, Goldenberg et al., 2008). Eighty to ninety percent of babies born preterm are in the moderately preterm subgroup (Raju, 2006).

It is well established that there is a 'male disadvantage' after preterm delivery, with preterm males being more vulnerable to cardiorespiratory illness than females (Elsmén et al., 2004a, Peacock et al., 2012). In addition, male infants are more likely to be delivered prematurely and deaths of preterm male infants are more common than female preterm infants (Cooperstock and Campbell, 1996, Ingemarsson, 2003, Lawn et al., 2013). The difference between sexes in infant mortality (within the first year) is most evident in those born extremely preterm; with 60% of preterm males dying compared to 38% for preterm females (Ingemarsson, 2003). Overall, the increased morbidity and mortality in preterm males has been linked to delayed development in males, with females exhibiting advanced lung (Fleisher et al., 1985) and cardiomyocyte maturation (Lumbers et al., 2009). Therefore, it is important in studies relating to preterm birth to not only compare differences between those born preterm and at term, but also the effects of preterm birth between males and females.

Preterm infants are born at a time when their organ systems are immature and so preterm birth can lead to injury of immature organs and/or adaptations in structure and function. Hence, it is imperative to gain an understanding of the impact of being born moderately preterm on organ development in the newborn and on long-term health outcomes. In this regard, the focus of this thesis was to examine the short-term and long-term effects of being born preterm on the cardiovascular system.

My PhD projects utilises an ovine model of moderately preterm birth. Sheep are an excellent animal model to investigate the effects of preterm birth on the cardiovascular system. Similar to humans, sheep have a long gestation period, they generally give birth to singletons or twins, and lambs are born at a similar size and weight to a newborn human baby. In addition, the ontogeny of vital organ systems is similar to that in the humans. Importantly, in relation to this PhD project, heart development and cardiomyocyte proliferation and maturation in the sheep fetus closely resembles that in humans (Huttenbach et al., 2001, Burrell et al., 2003).

Furthermore, sheep become sexually mature by 12 months of age and therefore it is possible to investigate effects in adulthood in a timely manner.

Chapter 2 describes in detail the ovine model of moderately preterm birth that is utilised throughout this PhD project. Body growth, body dimensions, arterial pressure, heart rate and blood chemistry are also compared between preterm and term lambs (males and females) in the immediate period after birth. Our model of preterm birth is based on a previously established ovine model, whereby De Matteo et al. (2010) have optimised an ovine model of moderately preterm birth. Utilising this approach, lambs were born at 132 ± 1 days of gestation (0.9 of term) equivalent to 32 - 34 weeks gestation in humans. This is the earliest time point in which viable preterm lambs can be born without requiring significant respiratory support in the form of mechanical ventilation.

In the studies of De Matteo et al. (2010), low sub-clinical doses of betamethasone (3.7 mg) were administered to the ewes assigned to deliver preterm in order to facilitate survival of the preterm lambs after birth. Given that it has previously been shown that betamethasone is necessary for survival of the preterm lambs, in the studies described in this thesis, we chose to use a clinically relevant dose of betamethasone. The timing and dose of betamethasone administered to the ewes was the same as that routinely administered to women at risk of preterm delivery (2 injections; 11.4 mg each, 24 hours apart).

2.2 Animal model and methods

2.2.1 Animals

All experimental procedures were approved by the Monash University Animal Ethics Committee (approval MMCA-2011/01) in accordance with the National Health and Medical Research Council's Code of Practice (Australia) for the care and handling of animals for scientific purposes.

Lambs studied in this PhD project were born during 2011, 2012 and 2013. It is to be noted that I was directly involved in the prenatal and postnatal treatment and care of all ewes and lambs. In the initial animal studies, Prof. Richard Harding and Dr. Robert De Matteo provided expert advice in relation to the induction, delivery and care of the ewes and preterm lambs. Other people who were also involved in the animal studies and assisted me in the treatment and care of ewes and lambs were: Dr. Graeme Polglase (supervisor), Ms. Natasha Blasch (animal technician), Mr. Dalibor Stanojkovic (animal technician), Dr. Beth Allison (senior post-doctoral researcher), Dr. Shanti Diwakarla (post-doctoral researcher) and Ms. Alison Moxham (senior research assistant).

My PhD studies involved the delivery of two cohorts of preterm and term lambs; one cohort was used for short-term experiments, the other cohort was used for long-term experiments. Border Leicester ewes were mated with White Suffolk rams, of known mating date and ewes only carrying singletons were used. Ewes and lambs for the short-term studies were delivered and housed at Monash Medical Centre Animal Facility (MMCAF) in Clayton (see following), whilst ewes and lambs for the long-term studies were delivered and housed at the Monash University Animal Facilities in Churchill, Gippsland (see later – Chapter 3).

2.2.2 Care of ewes and induction of labour

Pregnant ewes in the short-term studies were transported to MMCAF from Monash University Animal Services (Gippsland) approximately 3 - 4 weeks before expected delivery of lambs. Whilst in Gippsland, ewes were housed in paddocks and fed on normal pasture. A week before transport to MMCAF, the ewes were housed indoors where they were acclimatised to inside housing and changed to a feed of lucerne hay and chaff, in preparation for the housing and feeding conditions at MMCAF.

Once at MMCAF, ewes were kept in individual single-sized lambing pens. They were fed 800 - 1000 g of lucerne hay/chaff twice daily (in the morning and afternoon) and given unlimited water in buckets to drink *ad libitum*. The ewes were monitored daily to make sure they were healthy and alert. After birthing, they were given as a once off, extra lucerne hay and also approximately 200 g of sheep pellets (Rumevite, Ridley AgriProducts, Victoria, Australia) for additional nutrition. Within the MMCAF, the sheep were housed indoors with a 12 hour day/night cycle; daytime between 8 am and 8 pm. MMCAF staff were responsible for monitoring the temperature and humidity daily (which ranged from 19.5 - 21.5^{o}C and 50 - 75%, respectively) as well as cleaning out the pens every morning. Prior to induction of labour, ewes were moved to double-sized lambing pens and after delivery the lambs remained in these pens with the ewes.

The ewes were randomly assigned to deliver at term (147 ± 1 days gestation; n=15) or preterm (132 ± 1 days gestation; n=21). All lambs were delivered vaginally. To induce birth, ewes were given epostane (Sanofi-Synthelabo, New South Wales, Australia); 50 mg in 2 ml 100% ethanol (EtOH) administered intravenously via the jugular vein, approximately 43 hours before expected preterm delivery or approximately 24 hours before expected term delivery. Epostane, a 3-beta-hydroxysteroid dehydrogenase inhibitor, is involved in preventing the production of progesterone from pregnenolone. Progesterone is imperative to help maintaining pregnancy in sheep and therefore, without it, labour is induced. If induction or the progress of labour was taking longer than expected, an extra dose of epostane was given on the morning the lamb should have been delivered if deemed necessary. Vaginal checks were also conducted on ewes to assess the position of the lambs in the birthing canal; this process also helped to stimulate dilation of the cervix and thus delivery.

For the preterm group, the corticosteroid, betamethasone (Celestone Soluspan, Schering-Plough, North Ryde, New South Wales, Australia) was also administered. Ewes were given two injections of betamethasone (11.4 mg in an aqueous vehicle in each dose) intramuscularly, into the hind leg. The first dose was administered approximately 48 hours before the expected delivery time (approximately 5 hours before the epostane injection) and the second dose administered 24 hours later. Ewes delivering term lambs were not administered betamethasone.

2.2.3 Delivery and care of lambs

2.2.3.1 Preterm lambs

Around the expected time of delivery, ewes were monitored from a separate room via video cameras set up above their pens. During this time, it was important to minimise movement and mitigate distractions whilst the ewe was in labour. Evidence that lambing had begun was indicated by the appearance of a thick whitish vaginal discharge and the onset of contractions. In addition, a fluid-filled sac (amniotic sac) appeared, protruding from the vagina which eventually ruptured to establish a lubricated passage through the vagina as uterine contractions became stronger and more frequent. From this point, the ewe was expected to deliver within 2 hours; any longer than this, it was likely that the lamb was in a breeched position and the ewe would then require assistance with delivery. In the normal delivery process, as labour progressed, the ewe would spend more time lying on her side, stretching legs out with her head raised up. As the ewe continued to push and contract, the tip of the nose and the front legs of the lamb delivered first and once the head and shoulders passed through the pelvis, the delivery of the whole lamb was rapid.

When the preterm lambs were delivered, they were closely monitored; if there were problems with breathing, intervention was immediately implemented (see later – Section 2.2.4). If there were no apparent problems, the lamb was left to bond with the ewe for a short while before removal to record body weight and physiological measurements. Bonding between the ewe and lamb was facilitated by allowing the ewe to lick and smell the lamb; this ensured that the ewe did not reject the lamb.

After recording of measurements (see later – Section 2.2.4), preterm lambs were placed back in the pen with the ewe in a tub lined with hay and warmed with hot water bottles wrapped in towels. An overhanging heating lamp was fixed to the side of the lambing pen and the tub was placed underneath to keep the lamb warm. The tub also prevented ewes from trampling the lambs as they could not yet stand to support themselves at this point. For the first 24 hours, lambs were bottle-fed expressed milk from the ewes. Feeding took place every 4 - 6 hours, at 80 ml/kg/day. As lambs got stronger, they were encouraged to stand and suckle from the ewe. This normally occurred within 24 hours.

2.2.3.2 Term lambs

The ewes that were to deliver at term were also monitored but usually no assistance in the birthing process was necessary. Assistance was only required when labour was longer than normal when the ewe appeared to be straining heavily and there was no sign of the lamb after a long period of time; it was then assumed the lamb may be too large for the ewe to push out by herself or it was breeched. At this stage, assistance in delivery was provided by manual extraction of the lamb. Then bonding between lamb and ewe was permitted before removing the lamb for measurements before its first feeding.

2.2.4 Monitoring at birth

After bonding, preterm and term lambs were taken from ewes to be cleaned and dried by towels and hairdryers and excess fluid suctioned from the mouth and nose. After this, the lambs were weighed, and temperature, blood oxygen saturation levels and heart rate were measured using a thermometer and pulse oximeter (Masimo, Irvine, California, USA), respectively. The pulse oximeter measurements were recorded continuously for approximately 30 minutes after birth, and then averaged. Arterial pressure was measured using an Advisor® Vital Signs Monitor (SurgiVet®; Smiths Medical PM, Massachusetts, USA), which involved the non-invasive measurement of blood pressure via an inflatable blood pressure cuff attached to the front limb of the lamb. These measurements were recorded every 5-10 minutes for approximately 30 minutes after birth and then averaged. Figure 2.1 shows a lamb after birth lying on a hot water bottle wrapped in a towel to maintain its temperature and having its oxygen saturation measured via a sensor on the front right limb and arterial pressure measured with an inflatable cuff attached to a sphygmomanometer on the front left limb.

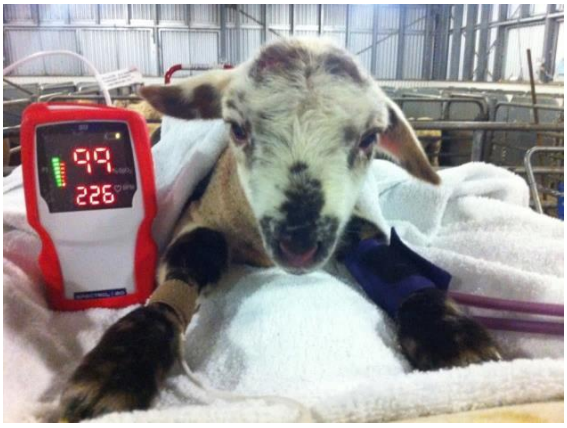

Figure 2.1. *Lamb having its oxygen saturation (sensor on front right limb) and arterial pressure (cuff on front left limb) measured after birth.*

After recording of measurements, term lambs were returned to ewes to stand and feed on their own as they did not require further assistance. However, if preterm lambs had low saturation levels, extra oxygen supplementation (Continuous Positive Airway Pressure) was provided via a nose cone connected to the Neopuff (Fisher & Paykel Healthcare, Panmure, Auckland, New Zealand) until saturation levels were stable at above 80% as seen in Figure 2.2. Once the preterm lamb was stable, they were placed back with the ewe.

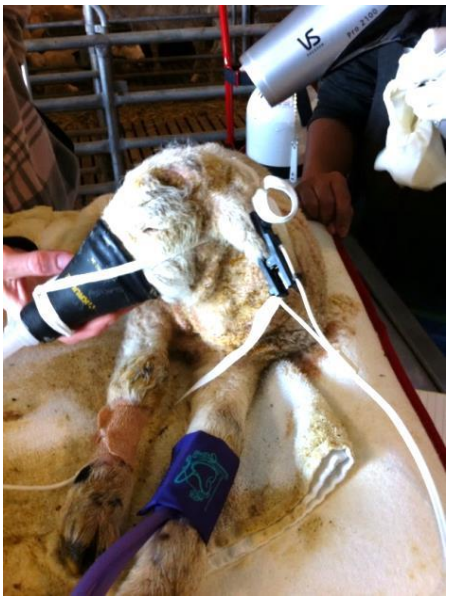

Figure 2.2. *Preterm lamb receiving oxygen supplementation via a nose cone, whilst having its oxygen saturation (sensors on front right limb and ear) and arterial pressure (cuff on front left limb) recorded. A hair dryer was also utilised to help dry and warm the lamb after birth.*

2.2.5 Postnatal measurements

After the initial recordings at birth, body weight, body temperature (rectal temperature), oxygen saturation, heart rate and arterial pressure were measured every 4-6 hours. The measurements recorded for the first 24 hours after birth were then averaged for day 1 data. On postnatal day 2, morphometric measurements were taken including: crown-rump length (CrL), thoracic girth (TG), front limb length (FLL) and hind limb length (HLL). Lambs were then anaesthetised with 1 ml/kg Sodium Thiopentone (50 mg/ml; Jurox, Rutherford, New South Wales, Australia), administered intravenously via the jugular vein, and maintained under general anaesthesia by inhalation of 2% Isofluorane in oxygen (Isoflo; Abbott, Sydney, New South Wales, Australia) and mechanically ventilated with 100% oxygen. A catheter was inserted into the femoral artery and mean arterial pressure, oxygen saturation, heart rate and temperature (via rectal probe) were continuously recorded (Advisor® Vital Signs Monitor; SurgiVet®, Smiths Medical PM, Massachusetts, USA) for approximately 60 minutes. Measurements at every 10 minutes were averaged for day 2 data. During recording, ultrasound analyses on the heart, kidneys and blood vessels (aorta, carotid arteries and renal arteries) were performed by Mr. Paul Lombardo, as part of his PhD project. At the termination of recording, the lamb was administered 1 ml of heparin (25000 IU in 5 ml; Pfizer Australia, West Ryde, New South Wales, Australia) via the femoral arterial catheter to prevent blood clotting. Twenty ml of blood was drawn from this same catheter for collection of plasma and another 1 ml was collected for blood chemistry analyses whilst the lamb was still under anaesthesia and mechanically ventilated with 100% oxygen. In regards to the blood chemistry analyses, not all animals were analysed (5 out of 28 lambs), as collection of the sample was overlooked in a few animals at the time of necropsy. Lambs were then euthanised with 0.5 - 1 ml of Lethabarb (325 mg/ml; Pentobarbitone, Verbac Animal Health, New South Wales, Australia) undiluted. This was administered via the femoral artery catheter or injected into the jugular vein.

2.2.6 At necropsy

At necropsy, the heart and lungs along with portions of the great vessels were dissected from the thoracic cavity and carefully separated. The heart was then weighed and placed in a solution of physiological saline with 1 ml of papaverine hydrochloride (1.2 mg/ml; Sigma-Aldrich, Missouri, USA) added for every 100 ml of saline. Small samples from the RV free wall

and LV apex were sampled and frozen in liquid nitrogen, and then the remainder of the heart was perfusion fixed. To do this, the heart was perfused with saline via a catheter in the aorta to clear the vasculature of blood; papaverine hydrochloride (0.12 mg) was also administered via the catheter to dilate the blood vessels and 0.5 M potassium chloride (0.1 ml) (Merck, Germany) administered to arrest the heart in diastole. After the blood was cleared from the coronary arteries, the heart was then perfused with 4% paraformaldehyde and subsequently stored in 10% formalin.

Also at the time of necropsy, several blood vessels were collected including the left and right renal arteries, left and right carotid arteries, lower thoracic aorta, abdominal aorta (below where the left renal artery is attached), third-order mesenteric arteries and ascending aortic arch (attached to the heart when removing the heart). All vessels were washed in saline with papaverine hydrochloride added (1 ml papaverine/100 ml saline). Portions of the blood vessels were divided for freezing in liquid nitrogen or immersion fixation in 10% formalin. Other organs collected at necropsy included the lungs, brain, left and right kidneys, liver, spleen, thymus and gut; these were weighed and immersion-fixed and/or frozen in liquid nitrogen for analysis by other researchers.

2.2.7 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics Version 21 (IBM SPSS, Illinois, USA) and graphed using GraphPad Prism Version 6.0 (GraphPad Software, California, USA). Kaplan-Meier survival curve analysis was used to analyse the survival between preterm and term female and male lambs. Data examining arterial pressure, heart rate, oxygen saturation and temperature at birth and day 1 were analysed using a three-way repeated measures analysis of variance (ANOVA), with time (P_T; birth or day1), gestational age at birth (P_G; preterm or term) and sex (P_S; male or female) as factors. All other data in this chapter were analysed using a two-way analysis of variance (ANOVA), with gestational age at birth (P_G; preterm or term) and sex (P_S; male or female) as factors. Significant interaction effects detected in the ANOVAs were further analysed by a Tukey's post-hoc test. Data are represented as means ± standard error of mean (SEM) and statistical significance was accepted at the level of $P<0.05$.

2.3 Results

2.3.1 Timing of deliveries (Figure 2.3)

Figure 2.3 shows the delivery times of all lambs after epostane administration in the ewes. Overall, the average delivery time for all lambs born preterm was 45 ± 1 hours after epostane administration and ranged from 36 to 55 hours. However, of the 21 lambs born preterm, 9 of them received an extra dosage of epostane on the day of expected delivery; the average time to delivery for the ewes receiving only one dose of epostane was 42 ± 1 hours, whereas it was 50 ± 1 hours in ewes that received a second dose.

For all term lambs, the average delivery time was 32 ± 1 hours after epostane administration, and ranged from 23 to 47 hours. Similar to the preterm group, 6 out of the 15 ewes in the term group received an extra dosage of epostane on the day of expected delivery. Ewes that received only one injection of epostane delivered 33 ± 2 hours after administration, whilst those that were given an extra injection on the day of delivery, ended up delivering more quickly at 32 ± 1 hours after the first epostane injection.

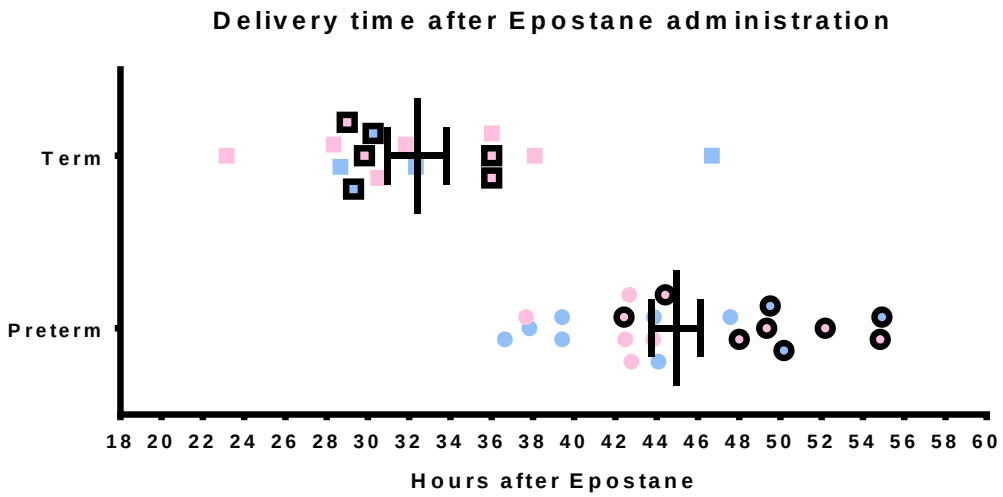

Figure 2.3. *Delivery times of all preterm and term male and female lambs after the initial administration of epostane. Deliveries highlighted in pink indicate female lambs and those in blue indicate male lambs. Those that received an extra dose of epostane on the day of expected delivery are indicated by the open squares or circles.*

2.3.2 Birth weights (Figure 2.4)

As expected, preterm lambs were significantly lighter (P_G<0.0001) at birth compared to those born at term; both female and male preterm lambs were lighter than their term counterparts as seen in Figure 2.4. In female preterm lambs, the average birth weight was 3.77 ± 0.16 kg compared with 5.57 ± 0.17 kg in female term lambs. The birth weights of male preterm lambs averaged 4.26 ± 0.19 kg compared with 5.29 ±0.24 kg in male term lambs. No sex differences were observed in birth weights (P_S=0.591).

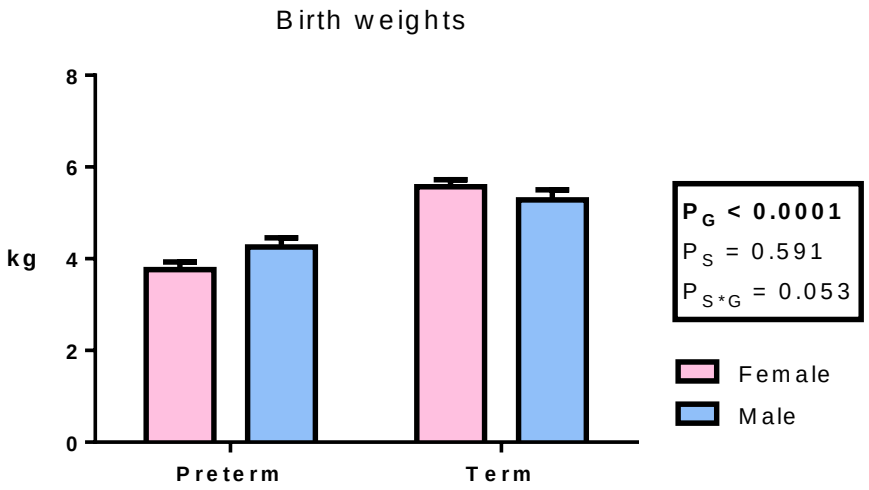

Figure 2.4. *Birth weights of preterm (n=12 female, n=8 male) and term (n=10 female, n=5 male) lambs. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

2.3.3 Survival after birth (Figure 2.5)

Figure 2.5 shows the percentage survival for all lambs over the first 48 hours of life. For the term group, all the lambs survived (10 females and 5 males) after birth. For preterm lambs, the percentage survival was 62%, with 13 out of 21 lambs surviving; 64% of female preterm lambs (7 out of 11) survived and 60% of male preterm lambs (6 out of 10) survived. Therefore, when comparing preterm lambs to term lambs, there was a significant difference (P=0.008) in the percentage survival. There was no significant difference (P=0.073) in survival rates between preterm males and preterm females.

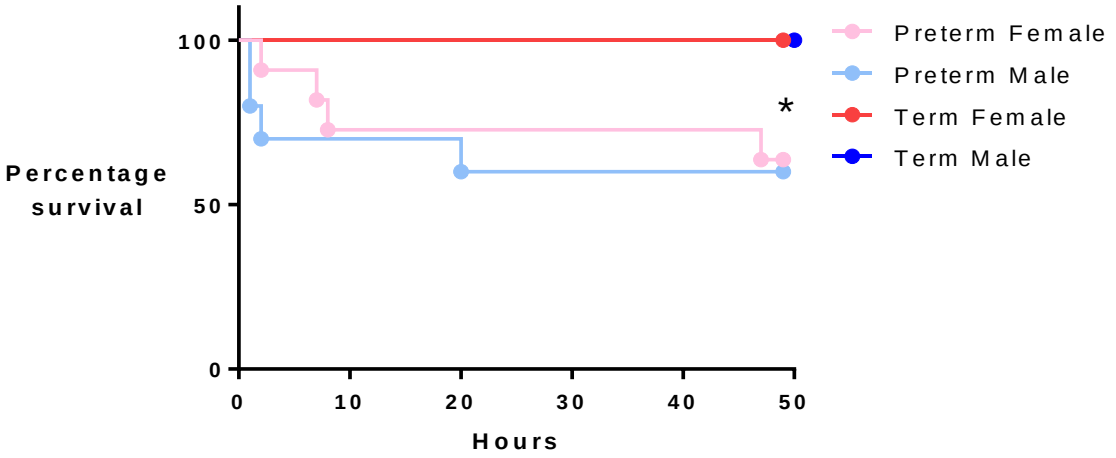

Figure 2.5. *Percentage survival of all preterm (male and female) and term (male and female) lambs in the first 50 hours after birth. Pink circles represent preterm females, pale blue circles represent preterm males, red circles represent term females and dark blue circles represent term males. Curve comparisons were performed by log-rank tests, with *P<0.05 observed between preterm vs. term lambs.*

2.3.4 Necropsy weights (Figure 2.6)

In the immediate period following birth, the term lambs and surviving preterm lambs had an average weight gain of 412 g/day and 71 g/day, respectively, until necropsy at 2 days of age. Even though all lambs increased in weight in the 2 days after birth, term lambs had almost 6 times greater weight gain compared to preterm lambs. Hence, at necropsy, body weights remained significantly reduced (P_G<0.0001) in the preterm group compared to term controls as seen in Figure 2.6. No sex differences were observed in body weights at necropsy (P_S=0.718).

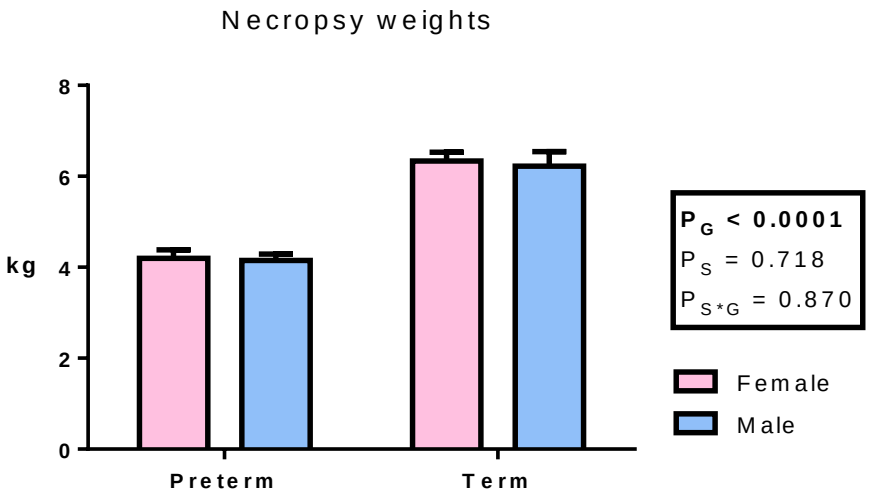

Figure 2.6. *Necropsy weights of preterm (n=7 female, n=6 male) and term (n=10 female, n=6 male) lambs. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

2.3.5 Organ weights (Table 2.1)

Table 2.1 reports the absolute and relative weights of organs collected at necropsy. Absolute organ weights of the heart, brain, left kidney, right kidney, liver and spleen were all significantly heavier in the lambs born at term when compared to preterm lambs. When adjusted for body weight, lungs, brain, right kidney and liver weights were all significantly higher in the preterm lambs compared to the term controls.

Males had significantly lighter absolute right kidneys than females (P_S=0.037), whereas there were no other differences in organ weights between sexes. When adjusted for body weight, there were no significant differences in relative organ weights between sexes.

Organs collected at necropsy	Preterm		Term		P-values
	Females n=7	Males n=6	Females n=10	Males n=5	
Absolute weights (g)					
Heart	39.43 ± 2.40	35.86 ± 2.59	54.70 ± 2.00	53.48 ± 2.83	**P_G<0.0001**
Lungs	128.70 ± 8.82	134.20 ± 9.53	131.03 ± 7.38	155.74 ± 10.44	NS
Brain	50.26 ± 1.33	48.65 ± 1.43	56.11 ± 1.17	59.00 ± 1.57	**P_G<0.0001**
Left Kidney	16.65 ± 1.24	15.18 ± 1.34	22.73 ± 1.04	19.22 ± 1.47	**P_G<0.001**
Right Kidney	17.04 ± 1.27	14.85 ± 1.27	22.19 ± 1.04	18.85 ± 1.39	**P_G=0.001** **P_S=0.037**
Liver	128.39 ± 6.84	119.76 ± 7.38	169.66 ± 5.72	157.48 ± 8.09	**P_G<0.0001**
Spleen	8.18 ± 0.61	8.50 ± 0.66	12.02 ± 0.51	11.33 ± 0.72	**P_G<0.0001**
Relative to body weight (g/kg)					
Heart	9.49 ± 0.42	8.62 ± 0.45	8.63 ± 0.35	8.68 ± 0.49	NS
Lungs	30.93 ± 1.69	32.43 ± 1.83	20.65 ± 1.42	25.24 ± 2.00	**P_G<0.0001**
Brain	12.16 ± 0.53	11.79 ± 0.57	8.86 ± 0.46	9.60 ± 0.62	**P_G<0.0001**
Left Kidney	4.01 ± 0.23	3.66 ± 0.25	3.60 ± 0.19	3.10 ± 0.27	P_G=0.054
Right Kidney	4.05 ± 0.23	3.58 ± 0.23	3.48 ± 0.19	3.03 ± 0.26	**P_G=0.025** P_S=0.058
Liver	30.62 ± 0.82	28.92 ± 0.88	26.78 ± 0.68	25.43 ± 0.97	**P_G<0.0001**
Spleen	1.96 ± 0.13	2.07 ± 0.14	1.90 ± 0.11	1.84 ± 0.16	NS

Table 2.1. *Absolute and relative weights of organs collected from preterm (female and male) and term (female and male) lambs at necropsy 2 days after birth. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM. Abbreviations: NS – not significant.*

2.3.6 Arterial pressure and heart rate (Figure 2.7 and Table 2.2)

Arterial pressure and heart rate were measured daily from birth as seen in Figure 2.7 and Table 2.2. From birth to day 1, there were no significant differences observed in systolic blood pressure, diastolic blood pressure, mean arterial pressure and heart rate in preterm lambs compared to term lambs. Additionally, there was no sex differences in either arterial pressure or heart rate observed between male and female lambs. Similarly, on postnatal day 2, there were no differences in arterial pressure or heart rate between any of the lambs whilst under anaesthesia.

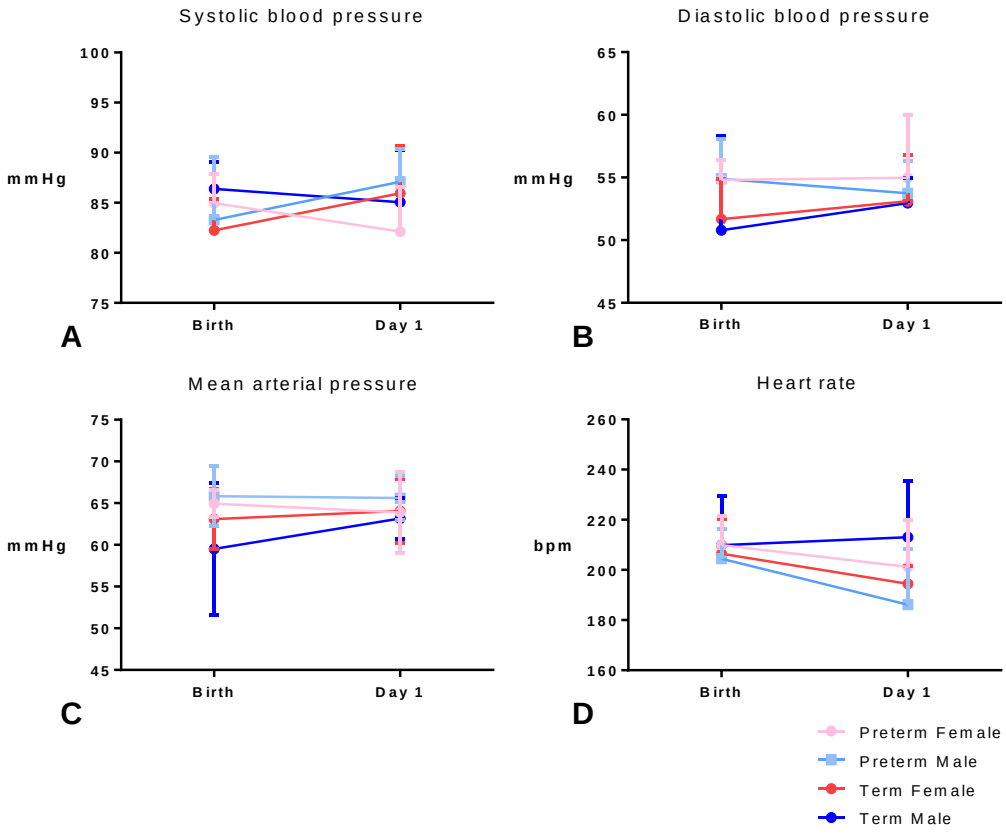

Figure 2.7. *Average systolic blood pressure (A), diastolic blood pressure (B), mean arterial pressure (C) and heart rate (D) in preterm (n=7 female, n=6 male) and term (n=10 female, n=5 male) conscious lambs at birth and 1 day after birth. Pink circles represent preterm females, pale blue circles represent preterm males, red circles represent term females and dark blue circles represent term males. Data were analysed using a 3-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and time (P_T; birth and day 1) as factors. Values are means ± SEM.*

	Preterm		Term		P-values
	Females n=7	Males n=6	Females n=10	Males n=5	
Systolic blood pressure	64.7 ± 8.2	67.5 ± 8.8	83.5 ± 6.9	79.8 ± 9.7	P_G=0.078
Diastolic blood pressure	42.3 ± 6.3	44.5 ±6.8	52.8 ±5.3	48.3 ± 7.5	NS
Mean arterial pressure	52.1 ± 7.0	54.1 ± 7.6	64.9 ± 5.9	60.3 ± 8.3	NS
Heart rate	181.5 ± 14.2	169.5 ± 14.2	164.0 ± 11.0	162.7 ± 15.5	NS

Table 2.2. *Average systolic blood pressure, diastolic blood pressure, mean arterial pressure and heart rate in preterm (n=7 female, n=6 male) and term (n=10 female, n=5 male) lambs under anaesthesia whilst being mechanically ventilated with 100% oxygen. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM. Abbreviations: NS – not significant.*

2.3.7 Oxygen saturation and temperature (Figure 2.8 and Table 2.3)

In addition to arterial pressure and heart rate, oxygen saturation and body temperature were also measured daily from birth (Figure 2.8 and Table 2.3). Preterm lambs had a significantly lower oxygen saturation percentage at birth compared to terms at birth (preterm: 77 ± 3% vs. term: 92 ± 3%; P_G=0.005). By one day after birth, oxygen saturation had significantly increased in preterm lambs (P_T=0.024) and was similar to term lambs at this time point. . At two days after birth, there was no significant difference in oxygen saturation between any of the lambs. There were also no significant differences in temperature at birth between preterm and term lambs. However, preterm lambs had significantly reduced body temperature one day after birth (preterm: 38.8 ± 0.1°C vs. term: 39.1 ± 0.1°C; P_G=0.043) and there was a trend for temperature to also be lower two days after birth, but this was not statistically significant (P_G=0.070).

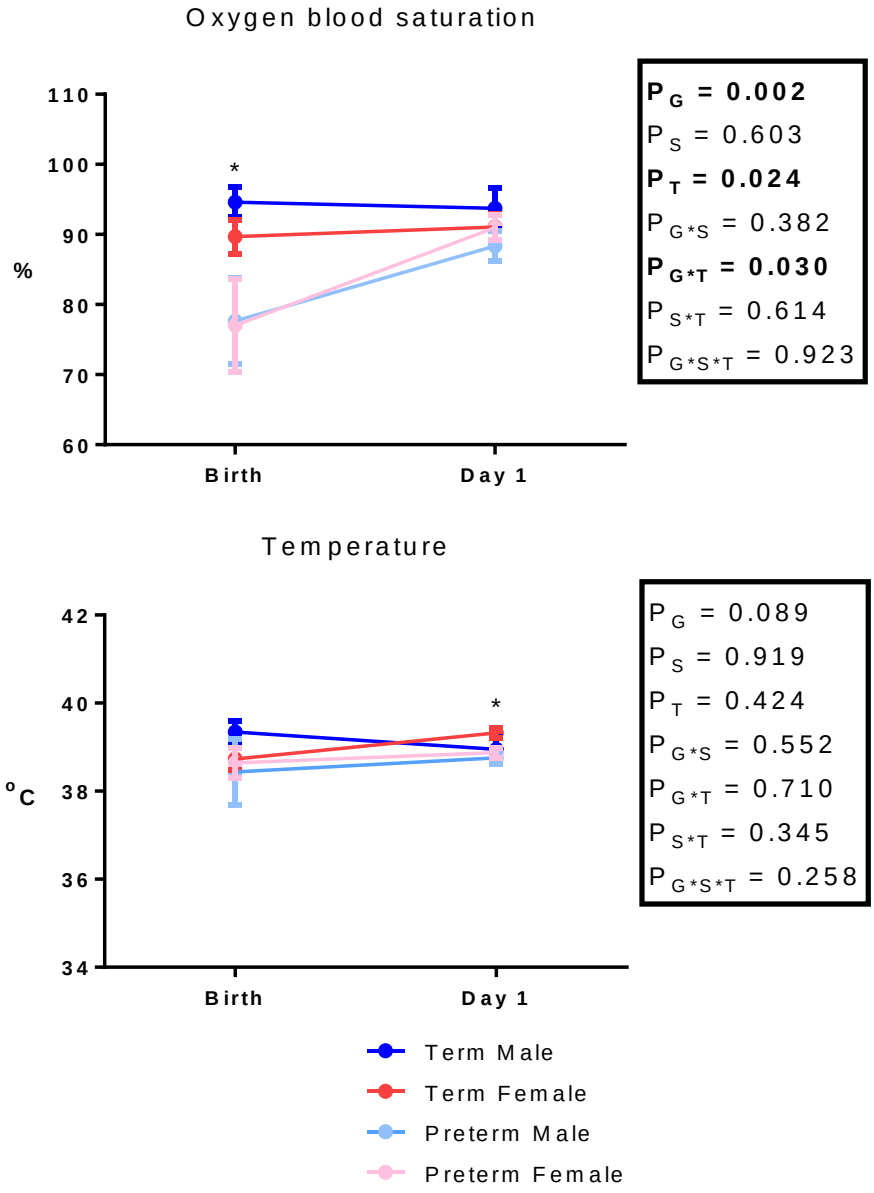

Figure 2.8. *Average oxygen saturation and body temperature in preterm (n=7 female, n=6 male) and term (n=10 female, n=5 male) lambs at birth and 1 day after birth. Pink circles represent preterm females, pale blue circles represent preterm males, red circles represent term females and dark blue circles represent term males. Data were analysed using a 3-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and time (P_T; birth and day 1) as factors. A 2-way ANOVA was also used to further analyse each time point with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. *P<0.05 between preterm vs. term lambs at that time point. Values are means ± SEM.*

	Preterm		Term		P-values
	Females n=7	Males n=6	Females n=10	Males n=5	
Oxygen saturation	93.5 ± 2.6	88.3 ± 2.8	94.5 ± 2.8	97.7 ± 4.0	NS
Temperature	39.2 ± 0.2	38.8 ± 0.2	39.5 ± 0.1	39.2 ± 0.3	P_G=0.070

Table 2.3. *Average oxygen saturation and rectal temperature in preterm (n=7 female, n=6 male) and term (n=10 female, n=5 male) lambs under anaesthesia whilst being mechanically ventilated with 100% oxygen. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM. Abbreviations: NS – not significant.*

2.3.8 Blood chemistry (Table 2.4)

Blood chemistry was analysed at postnatal day 2 from a 1 ml blood sample taken via the femoral artery catheter whilst lambs were still under anaesthesia and mechanically ventilated with 100% oxygen (Table 2.4). There were no significant differences observed in arterial oxygen saturation levels (sO_2%), pH levels, partial pressure of carbon dioxide (pCO_2) and glucose levels (cGlu) in blood samples of preterm and term lambs. However, preterm lambs had significantly higher haemoglobin levels (ctHb) (P_G=0.036) and lactate levels (cLac) (P_G=0.001) compared to lambs born at term. Between sexes, female lambs had higher $sO_{2\%}$ (P_S=0.014) and lower pCO_2 levels (P_S=0.016) compared to male lambs. Additionally, there was a significant interaction between sex and gestational age at birth in the ctHb levels (P_{S*G}=0.01) and also partial pressure of oxygen (pO_2) (P_{S*G}=0.043).

	Preterm		Term		P-values
	Females n=6	**Males n=5**	**Females n=7**	**Males n=5**	
Oximetry Values					
ctHb (g/dL)	12.3 ± 0.6	13.6 ± 0.6	12.7 ± 0.5	10.6 ± 0.6	**P_G=0.036** **P_{S*G}=0.010**
sO_2 (%)	99.3 ± 0.6	98.8 ± 0.6	99.5 ± 0.6	96.7 ± 0.6	**P_S=0.014**
Blood Gas Values					
pH	7.24 ± 0.07	7.20 ± 0.08	7.38 ± 0.07	7.17 ± 0.08	NS
pCO_2 (mmHg)	45.7 ± 15.9	96.1 ± 15.9	40.1 ± 13.5	71.5 ± 15.9	**P_S=0.016**
pO_2 (mmHg)	233.6± 44.2	287.9 ± 48.5	278.7 ± 41.0	135.2 ± 48.4	**P_{S*G}=0.043**
Metabolite Values					
cGlu (mmol/L)	6.6 ± 1.0	4.9 ± 1.0	5.3 ± 0.8	6.4 ± 1.0	NS
cLac (mmol/L)	4.1 ± 0.5	3.6 ± 0.6	1.9 ± 0.5	1.8 ± 0.6	**P_G=0.001**

Table 2.4. *Blood chemistry of the preterm (female and male) and term (female and male) lambs under anaesthesia whilst being mechanically ventilated with 100% oxygen on postnatal day 2 prior to necropsy. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. It should be noted that not all animals had their blood samples collected; the n values per group are shown. Values are means ± SEM. Abbreviations: NS – not significant.*

2.3.9 Morphometric measurements (Figure 2.9)

Figure 2.9 shows the morphometric measurements taken at postnatal day 2. In accordance with the body weight data, there was a significant reduction in CrL (P_G=0.003), TG (P_G<0.0001), FLL (P_G=0.013), HLL (P_G=0.001) and ponderal index (PI) (P_G=0.035) in preterm lambs compared to term controls. There were no significant differences in any of the morphometric parameters measured between male and female lambs.

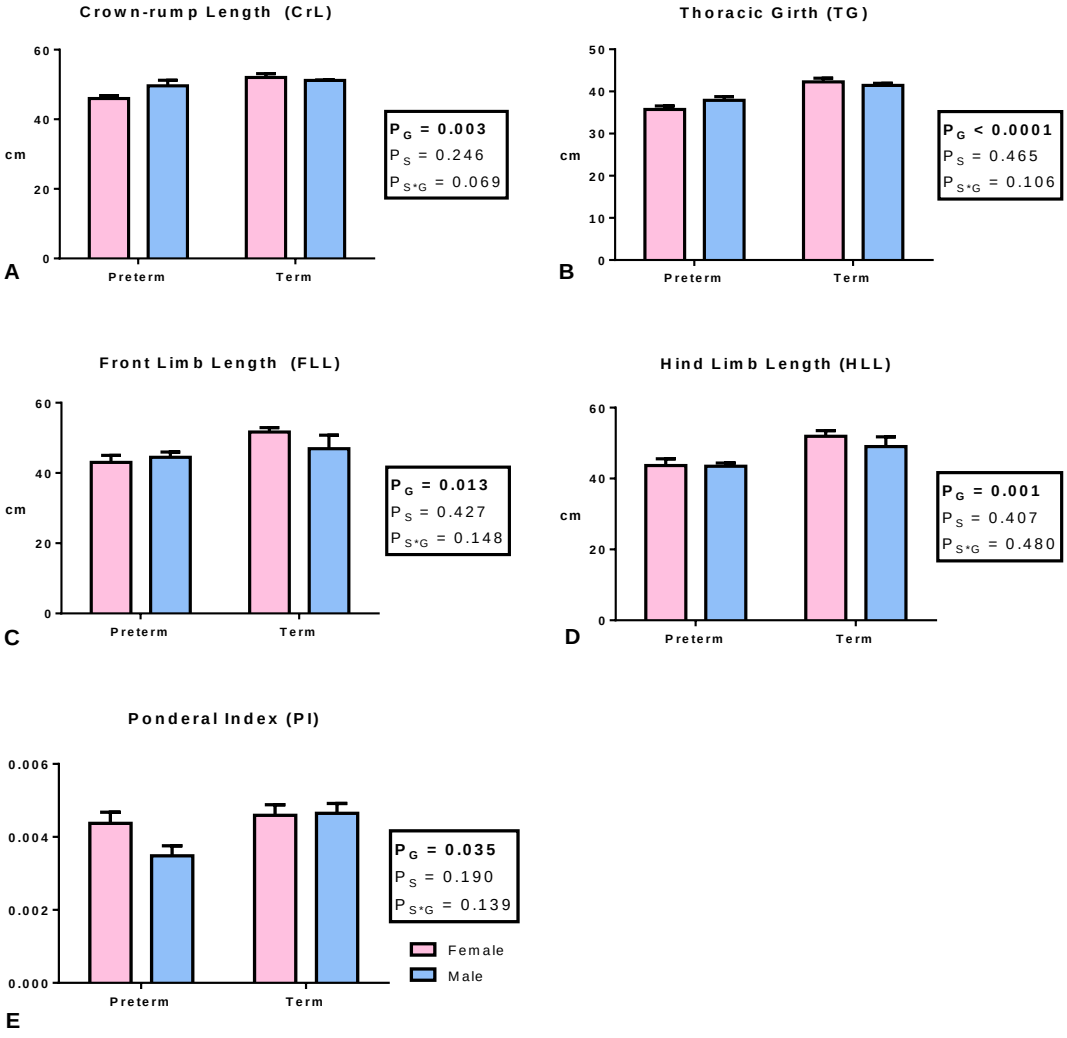

Figure 2.9. *Morphometric measurements of crown-rump length (A), thoracic girth (B), front limb length (C), hind limb length (D) and ponderal index (E) in preterm (n=7 female, n=6 male) and term (n=10 female, n=5 male) lambs at postnatal day 2. Pink shading represents females and blue shading represents males. Data were analysed at each time point using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

2.4 Discussion

2.4.1 Summary

In this chapter, I have successfully developed an ovine model of moderately preterm birth that has then been used in all subsequent chapters in this thesis. Overall, preterm lambs were lighter at birth and remained lighter in the 2 days after birth. In accordance with body weights, preterm lambs were smaller in all morphometric measures at 2 days after birth, including ponderal index which is a measure of 'leanness'. Arterial pressure and heart rate increased to a similar level immediately after birth in preterm and term lambs, and remained similar during the first two days after birth. Oxygen saturation was found to be significantly lower in preterm lambs compared to term lambs at birth. By two days after birth, oxygen saturation was normalised in preterm lambs, whereas haemoglobin and lactate levels were significantly elevated compared to term lambs.

2.4.2 Establishment of the model

In this thesis, I have developed a model of moderate preterm birth in sheep, adapted from the previously published study of De Matteo et al. (2010). At the commencement of my studies, valuable advice was obtained from Dr. Robert De Matteo and Prof. Richard Harding. In the previous studies by De Matteo et al. (2010), preterm labour was induced in Merino x Border Leicester ewes by administration of epostane, the progesterone inhibitor, at the same dose (50 mg in 2 ml of 100% EtOH) as used in my studies; however, the dose of betamethasone administered to the ewes was much less. In their studies, they explored 3 different protocols relating to the timing of administration of epostane and the timing and dose of betamethasone. These included: 1) epostane (50 mg) given 6 hours after betamethasone (3 mg), 2) epostane (50 mg) given 6 hours after a higher dosage of betamethasone (5.7 mg) and 3) epostane (50 mg) given 42 hours after betamethasone (5.7 mg). It was concluded in their study that neither changing the dose of betamethasone nor the length of time between epostane and betamethasone injections improved the survival rates of preterm lambs any further (De Matteo et al., 2010). In this regard, it is important to note that both doses of betamethasone used in their studies were well below the dose routinely administered to women at risk of preterm delivery.

In my studies, given that betamethasone is essential for postnatal survival in the preterm lambs, we decided to use a clinical dose of betamethasone in order to mimic the current treatment of pregnant women at risk of preterm delivery. Border Leicester ewes mated with White Suffolk rams were given an initial dose of betamethasone (11.4 mg, intramuscularly) 45 - 52 hours before expected delivery time and another dose 24 hours later. This is the same regimen currently used clinically; where pregnant women who are at risk of delivering preterm are also given 11.4 mg of betamethasone, 24 hours apart.

Overall, my experimental protocol led to the successful delivery of preterm lambs at the target gestational age of 132 ± 1 days, which is equivalent to birth at 32 - 34 weeks gestation. The preterm lambs at this gestation did not require ventilatory support, thus excluding the potential confounding factor of postnatal ventilation. This experimental approach therefore provides an excellent model in future chapters to investigate the effect of moderate preterm birth, after exposure to clinically relevant doses of betamethasone, on the cardiovascular system in the offspring. However, it is important to note, that because the preterm lambs in this study have been exposed to antenatal corticosteroids, there is the potential for any significant differences observed in the preterm lambs to be the result of exposure to corticosteroids *in utero* rather than preterm birth *per se*, and this must be kept in mind when interpreting the findings in this thesis.

2.4.3 Survival of preterm lambs

As mentioned previously, De Matteo et al. (2010) found that administration of 5 mg of betamethasone to pregnant ewes did not significantly improve survival rates of preterm lambs compared to lambs of ewes that received only 3.7 mg of betamethasone. Interestingly, this remained the case when the dosage was markedly increased in my studies to two doses of 11.4 mg 24 hours apart. De Matteo et al. (2010) reported an overall preterm survival rate of 60%, which was similar as the overall survival rate of 62% in my study. This suggests that the higher dose of corticosteroids administered to women at risk of preterm birth may not confer any extra survival advantage compared to a much lower dose. Indeed, based on my findings, this should be further explored. Certainly, it is well known that the administration of antenatal corticosteroids has the potential to lead to adverse side effects in the mother and their offspring. Hence, if the dose of betamethasone could be lowered, the benefits on survival may still remain but the potential maternal adverse side effects of anaemia and leucocytosis

(Romejko-Wolniewicz et al., 2014) may be lessened, and also the elevated arterial pressure observed in adult offspring following exposure to excess corticosteroids may be prevented (Singh et al., 2012).

Importantly, in the studies of De Matteo et al. (2010), the survival rates in the preterm lambs differed between the sexes; with the survival of the male preterm lambs significantly less than preterm females (44% of preterm males survived compared to 76% of preterm females). These findings are similar to that observed in the neonatal intensive care unit where there appears to be a 'male disadvantage' amongst preterm infants with prematurity in males associated with higher rates of both neonatal mortality and morbidity (Ingemarsson, 2003). In contrast, female infants born premature appear to be less vulnerable to preterm birth and have better long-term outcomes after birth. For example, a higher proportion of preterm males develop neonatal respiratory distress syndrome as well as chronic lung disease (Elsmén et al., 2004a). In addition, there are a number of studies linking preterm males with delayed lung development, with females exhibiting advanced lung maturation relative to males during late gestation (Fleisher et al., 1985) and also advanced cardiomyocyte maturation compared to males (Lumbers et al., 2009). Importantly, in our study, we did not observe any significant differences in the survival rates between sexes in the preterm lambs; overall there was a 64% survival in preterm female lambs and 60% survival in preterm male lambs. Therefore, it is likely that the higher dose of antenatal corticosteroids may have led to improved survival in male lambs.

In regards to the survival of the preterm female lambs, the percentage survival was less in my study compared to that of De Matteo et al. (2010) (64% survival 2 days after birth vs. 76% survival 2 weeks after birth, respectively). Hence, there is no evidence in my study that the higher dose of antenatal betamethasone is of benefit to the survival of the preterm female lambs; in fact, it may be detrimental. Further studies exploring the doses of antenatal betamethasone are necessary to verify this. An alternative explanation relating to differences in survival between my studies and that of De Matteo et al. (2010) may relate to the different cross breeds of sheep studied; in my study Border Leicester ewes were crossed with White Suffolk rams, whereas in the studies by De Matteo et al. (2010) Border Leicester ewes were crossed with Merino rams.

2.4.4 Body growth is affected in the immediate period after birth

As expected, birth weights in moderately preterm lambs were significantly lower when compared to term lambs. Body weight in the preterm lambs remained lower until the time of necropsy at postnatal day 2. During the immediate 2 day period after birth, both preterm and term lambs gained weight with an average weight gain of 71 g/day and 412 g/day, respectively. Hence, the term lambs had a 6 times greater growth rate in the immediate period after preterm delivery. The slower growth in the preterm lambs may, in part, relate to differences in feeding regimens. Preterm lambs were bottle-fed at set intervals for the first 24 hours, until independent suckling from mothers was observed, whereas terms were strong enough to feed *ad libitum* from birth. Furthermore, it is well known that infants born moderately preterm are delivered during the period of the highest growth increase *in utero* (Cooke et al., 2004); hence, being born before or during this period can subsequently lead to body growth restraints. This may be a result of feeding problems and/or immaturity of the gastrointestinal system in preterm infants (Sangild, 2006, Puntis, 2006). Indeed, appropriate nutrition and growth in early life are important factors in determining long-term developmental outcomes for preterm subjects.

As expected, the severity of effects on the postnatal growth of premature babies depends on their degree of prematurity. The earlier and more immature the infant is at birth, the greater the initial weight loss and the longer time it takes to regain initial birth weight (Blackwell et al., 2005). This may in turn impact on long-term adult health (Osmond and Barker, 2000, Barker et al., 2005, Thureen, 2007). Studies have reported that infants born extremely and very preterm suffer significant weight loss (14%) during the first weeks of life whilst in the neonatal intensive care unit and after discharge (Gill et al., 1986). This leaves them growth retarded for the first years of life, only able to maintain a low weight gain thereafter and subsequently can take 4 - 7 years to sustain stable weight gain within normal ranges (Niklasson et al., 2003). For moderately preterm infants, Bocca-Tjeertes et al. (2011) have reported an average decrease of 8% in birth weight at approximately 5 days after birth but then after this initial decrease, slow weight gain was observed up to 6 months of age.

In contrast to the findings in human infants, our moderately preterm lambs did not experience weight loss after birth; however, a low weight gain was evident during the first two days of life

when compared to term lambs. It is likely that if the lambs were born earlier, more severe effects on growth would have been observed.

2.4.5 Arterial pressure and heart rate are unchanged after birth

Hypotension (low blood pressure) and depressed heart rate are often observed in preterm infants in the neonatal intensive care unit. To date, there has been a paucity of data specifically relating to the effects of moderate preterm birth on arterial pressure and heart rate in the immediate period after birth and in the long-term; hence, this is specifically addressed in this thesis. In my studies, there were no significant differences in systolic blood pressure, diastolic blood pressure and mean arterial pressure between moderately preterm lambs compared to term lambs at birth, or in the two days after birth. There was no evidence of hypotension in the moderately preterm lambs. However, it must be noted that the sphygmomanometrical measurement of arterial pressure using a cuff can be stressful to unconditioned animals and hence this method may in fact lead to an elevation of arterial pressure due to stress. Therefore, arterial pressure cuff measurements should be interpreted with caution due to the fact that this method may be measuring a stress response in the lambs. If this was the case, there were still no significant differences in arterial pressure between preterm and term lambs.

In addition, heart rate was normal in the preterm lambs. This is in contrast to clinical studies which have reported that heart rate is lower in very and moderately preterm infants (median gestational age of 33 weeks) immediately after birth but then is higher once haemodynamic stability is achieved compared to those born at term (Dawson et al., 2010).

Hypotension is a common complication in preterm infants whilst in the neonatal intensive care unit. However, this is usually observed in preterm infants born very or extremely preterm (Watkins et al., 1989, Georgieff et al., 1996, Subhedar, 2003, Witcombe et al., 2008). These babies often require inotropic support to normalise their arterial pressure and this is thought to be due to immaturity of their cardiovascular system (Kluckow and Evans, 2000, Harper, 2000). Interestingly, in such infants, arterial pressure often increases more rapidly within the first month of life when compared to term controls (Pejovic et al., 2007).

Over recent years there have been a multitude of studies reporting an elevation in blood pressure in children, adolescents and adults born preterm compared to those born at term (Bonamy et al., 2005, Johansson et al., 2005, Doyle, 2008, Keijzer-Veen et al., 2010), which is

in direct contrast to the hypotension often observed in preterm infants in the neonatal period. Given that, hypertension is a major risk factor for cardiovascular disease, it is imperative to gain an understanding of the mechanisms leading to hypertension in adulthood in subjects that were born preterm. In the following chapter, the long-term effects of moderate preterm birth on arterial pressure are comprehensively addressed.

2.4.6 Blood chemistry following preterm birth

Oxygen saturation was found to be significantly lower in preterm lambs compared to term lambs at birth and was normalised within 24 hours after birth. By two days after birth, all blood chemistry measurements were similar between preterm and term lambs, except for haemoglobin and lactate levels, which were significantly elevated in preterm lambs.

It is important to note that there was some unexpected findings in the blood chemistry results at 2 days of age. For example, high haemoglobin levels in preterm male lambs compared to term male lambs, low pH in term male lambs (pH=7.17), low pO_2 in term male lambs (135.2 mmHg) and high pCO_2 in preterm male and term male lambs (96.1 mmHg and 71.5 mmHg, respectively). This may be due to a number of factors, including different operators monitoring the ventilation equipment and in particular, the use of an adult ventilator to ventilate the lambs as we did not have access to a paediatric ventilator. Since the ventilator was designed for adults, it is likely to have affected the accuracy of the measurements. In addition, there may have been some technical issues that were not detected at the time, such as the carbon dioxide absorber may not have been switched on for some of the animals and/or there may have been mixing of room air rather than 100% oxygen.

Notably, even though there is variation in the blood gas findings, the results in all groups were within the normal range, except for abnormally high pO_2 values in the preterm males and females and term female lambs. This can be accounted for by the fact that these lambs were mechanically ventilated with 100% oxygen.

2.4.7 Conclusion

In conclusion, in this chapter an ovine model of moderate preterm birth has been established which is then utilised in subsequent chapters to examine the effects of moderate preterm birth on the immature cardiovascular system. Given that 80% of all preterm infants are born moderately preterm, it is essential to gain an understanding of the effects of moderate

preterm birth on the heart and blood vessels. Our preterm model, with concomitant antenatal corticosteroid administration, at a clinically relevant dose, replicates the common clinical scenario where all women 'at risk' of preterm delivery are administered antenatal corticosteroids. In Chapter 3, body growth and composition and physiological measurements of arterial pressure and heart rate are investigated in sheep maintained to young adulthood (14.5 months of age). In Chapter 4, heart structure and cardiomyocyte growth are investigated and compared in preterm and term sheep at 2 days after birth and 14.5 months of age. Lastly, Chapter 5 examines the structure and composition of the thoracic aorta and left carotid artery in preterm and term sheep in the short-term and long-term cohorts.

Chapter 3:

The Effect of Moderate Preterm Birth on Survival, Body Growth, Arterial Pressure and Heart Rate from Birth until Early Adulthood

3.1 Introduction

Over the last few decades, with the steady increase in the survival of preterm infants, it has become clearly apparent that preterm birth can lead to vulnerability to disease later in life, including a strong susceptibility for the development of hypertension (Saigal and Doyle, 2008, Keijzer-Veen et al., 2010, Crump et al., 2011, Sutherland et al., 2014). This is of particular concern given that hypertension (which is a sustained elevation in arterial pressure) is one of the major risk factors for the development of cardiovascular disease (Kannel, 1996, Berry et al., 2012, Kahan, 2014). Hence, with the marked improvement in survival of preterm infants (Goldenberg et al., 2008), it is imperative to gain an understanding of the aetiology of their hypertension later in life. This can potentially lead to therapeutic strategies to reduce arterial pressure in adults born preterm, which in turn would alleviate their vulnerability to cardiovascular disease.

With preterm infants, there is usually poor postnatal growth in the early neonatal period, and they often require interventions to promote better growth (Clark et al., 2003, Dusick et al., 2003, Roggero et al., 2011). Compared to babies born at term, preterm infants have slower growth rates and there is often growth failure in the immediate period after birth (Gairdner and Pearson, 1971). This growth impairment during early infancy can lead to permanent deleterious effects, as body and organ growth abnormalities that develop during this time can persist into adulthood. Notably, the negative impact on postnatal growth in relation to body weight, length and head circumference in preterm infants is inversely proportional to gestational age at birth (Clark et al., 2003).

Although neonatal growth is impaired, the majority of preterm infants do experience some catch up growth during childhood and adolescence, resulting in faster growth than normal to match term growth trajectories (Altigani et al., 1989, Euser et al., 2008, Gong et al., 2013). Rapid weight gain is beneficial for neurodevelopmental outcomes as the early postnatal period is critical for brain growth (Latal-Hajnal et al., 2003, Leppänen et al., 2013), but this has also been linked to adverse metabolic effects in later life (Ong et al., 2000, Hales and Ozanne, 2003, Gianni et al., 2012). In general, catch up growth in preterm infants is more pronounced in regards to head circumference, body weight and body mass index rather than height (Saigal et al., 2006); head circumference increases due to brain growth being prioritised, after which then there is catch up growth in weight and lastly height (Ghods et al., 2011). Notably, the

gain in weight during catch up growth for preterm infants has been reported to be mainly due to increases of fat mass rather than lean mass (Ahmad et al., 2010, Gianni et al., 2012).

Indeed, the development of hypertension in subjects born preterm may relate to abnormal changes in postnatal body growth. Epidemiological studies demonstrate that it is when there is catch up in body growth in low birth weight infants (which can result from intrauterine growth restriction and/or preterm birth) that leads to the highest risk of cardiovascular and metabolic disease in adulthood (Eriksson et al., 1999, Ong et al., 2000, Ibanez et al., 2006).

Hence, the aims of the studies in this chapter were to investigate, in our sheep model, the effects of moderate preterm birth on: body growth (weight and body dimensions), arterial pressure and heart rate from birth until adulthood and body composition in adulthood.

3.2 Methods

3.2.1 Care and management of animals

The animal studies in this chapter utilise the same ovine model of moderately preterm birth as described in Chapter 2. In this chapter, the preterm and term lambs were left to develop until 14.5 months of age (early adulthood). Body weight, body dimensions, arterial pressure and heart rate were measured weekly for the first 12 weeks after birth then monthly up until necropsy, whilst body composition and indwelling recordings of arterial pressure were measured only at 13.5 months of age.

Lambs from only singleton pregnancies were delivered over a period of two and a half months in 2012 and housed for approximately 13 months following birth at Monash University Animal Services in Churchill, Gippsland. During this time, the animal services staff fed and monitored the sheep daily.

Overall, care of ewes and induction of labour was the same as that described in Chapter 2. In this long-term cohort, the ewes (n=28 assigned to deliver preterm and n=18 assigned to deliver at term) were housed in the main farm shed at Monash University Animal Services in Gippsland a week before expected delivery, then moved to individual pens (still within the shed) lined with hay when the first injections of epostane (20 mg in 2 ml 100% EtOH, i.v.; Sanofi-Synthelabo, New South Wales, Australia) and Betamethasone (only to the preterm group; 22.8 mg, i.m.; Celestone Soluspan, Schering-Plough, North Ryde, New South Wales) were administered (as previously described in Chapter 2, Section 2.2.2, page 43). Overall, conditions of the shed were slightly different from the housing of the ewes described in Chapter 2 (Section 2.2.2, page 43) at the Animal Facilities at Clayton; the shed was not fully enclosed, therefore temperature could not be regulated and as a consequence, was lower in comparison.

3.2.2 Immediate postnatal care

After birth, care of preterm lambs was the same as described in Chapter 2 (Section 2.2.3, page 44). Lambs were separated from the ewes (after initial bonding time) for weighing and recording of physiological parameters, using the same monitoring techniques and associated equipment as described in Chapter 2 (Section 2.2.4, page 45-47). In addition, all lambs were

administered 0.5 ml of Alamycin intramuscularly (Alamycin 10, 2 - 4.5 ml/50 kg, Victoria, Australia), which is a broad spectrum antibiotic to prevent any potential infections and facilitate survival. Furthermore, surfactant was administered immediately after delivery to a few of the preterm lambs that appeared to be experiencing significant respiratory distress. A single bolus dose of CUROSURF® (1.25 ml/kg; Chiesi USA, Inc., North Carolina, USA) was administered via a feeding line through the intratracheal tube in intubated lambs. Following removal of the intratracheal tube, Continuous Positive Airway Pressure was applied to ensure the surfactant reached the lungs and spread evenly and quickly. After the administration of CUROSURF®, it was usual for there to be a relatively quick improvement of gas exchange and subsequent increase in oxygen saturation levels. Lambs experiencing respiratory difficulties were kept warm in tubs lined with towels and hot water bottles. Heart rate and arterial oxygen saturation were continuously monitored with a pulse oximeter (sensor wrapped around a shaved front limb or tail). Once oxygen saturation was stable, at about 80%, lambs were placed back with their mothers.

The feeding regimes of the preterm and term lambs were the same as in Chapter 2 (Section 2.2.3, page 44). Usual practices of ear marking, tail docking, vaccinations (see later) and weaning were all performed by Monash University animal house staff in Gippsland. All lambs were ear tagged for identification purposes a day after birth. Tail docking was performed on all lambs by banding at approximately 4 weeks of age. When applying the band, local anaesthetic (1 ml Bupivicaine, Marcain, AstraZeneca, New South Wales, Australia) was administered to the tail to reduce the pain of banding. At the time of tail banding, lambs were also vaccinated against Ovine Johne's Disease (Gudair®, 1 ml, Zoetis, New South Wales, Australia), which is an infectious fatal wasting disease and Cheesy Gland and other clostridial diseases such as black disease, pulpy kidney and tetanus, using Glanva® 6S (1 ml, Zoetis, New South Wales, Australia).

3.2.3 Long-term care and monitoring

Lambs were housed in the sheep shed with ewes for approximately 5 weeks, until deemed strong enough to graze in the paddocks with the ewes. Once in the paddocks, lambs and ewes jointly grazed with alpacas (as a preventative measure to avoid foxes from attacking the lambs). All lambs were weaned (separated from ewes) at 12 weeks of age. The males were not

castrated, and therefore males and females were maintained in separate paddocks from weaning.

To assess body growth, sheep from both preterm and term groups were weighed and measured weekly for the first 12 weeks after birth then every 4 weeks up until 12 months of age; this included morphometric measurements of CrL, TG, FLL and HLL. Temperature, oxygen saturation, heart rate and arterial pressure were also measured at these same times using the same monitoring techniques as described in Chapter 2 (Section 2.2.4, page 45-46). I had assistance in recording these measurements from Ms. Caitlin Youde and Ms. Tamara Black, at the time, both undergraduate students completing a Bachelor of Science (Veterinary Bioscience) at Monash University Gippsland.

Lambs were housed in paddocks at Gippsland Monash University Animal Services until approximately 13 months of age; by this stage they were sexually mature sheep. The sheep were then transported to Monash Animal Services (MAS) at Monash University in Clayton, where they were housed over a period of four to six weeks.

3.2.4 Body composition assessed using Dual-energy X-ray absorptiometry (DEXA)

Within one to two weeks after being transported to MAS at Clayton, sheep were taken to the Large Animal Facility at the Department of Physiology, Monash University where Dual-energy X-ray absorptiometry (DEXA) analyses were performed. DEXA is used as a method to measure body composition including: bone mineral content, fat mass and lean mass as well as total mass. In order to do this, sheep were placed in individual metabolic cages on wheels. Each sheep was moved into the room with the DEXA machine one at a time. Sheep were administered Domitor® (Medetomidine, 0.1 ml/10 kg, i.v., Zoetis, New South Wales, Australia) intravenously on the basis of body weight, which is a sedative and analgesic. This enabled the sheep to be laid onto the DEXA machine bed for scanning, which took approximately two minutes and the scanned image can be seen in Figure 3.1. Large bolsters were placed under the sheep's head and around the lower rump area to hold it in place. Heart rate and oxygen saturation were measured before and after scanning using a pulse oximeter. After the scan, the sheep was moved back into the metabolic cage and a reversal drug, Antisedan (Atipamezole, 0.05 mg/kg, i.v., Zoetis, New South Wales, Australia), was immediately administered intravenously. Sheep were standing and showing no effects, usually within a

minute after administration. At completion of scanning, the sheep were transported back to MAS housing facilities.

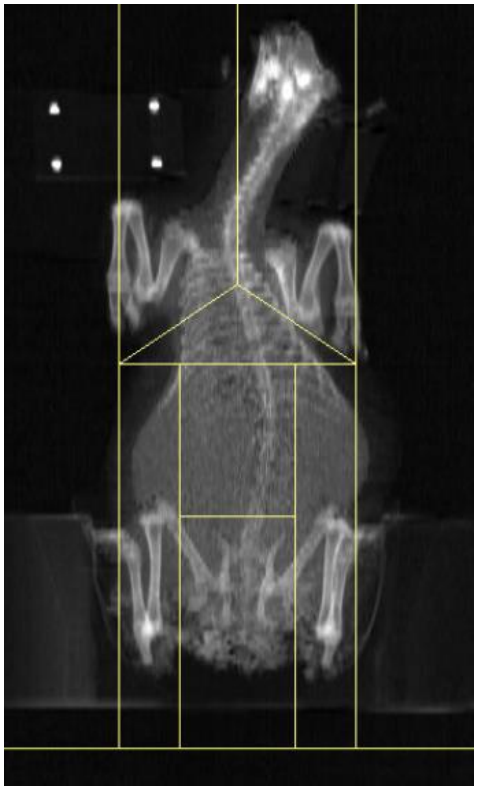

Figure 3.1. *Scanned image of a preterm sheep on the DEXA machine.*

Sheep were transported to MMCAF approximately three weeks before necropsy and housed in individual cages. During this time, staff continued to monitor and feed them twice daily with lucerne hay.

3.2.5 Surgery

Two weeks before necropsy, sheep underwent aseptic surgery to insert catheters into the right femoral artery and vein and to place a flow probe around the left femoral artery. Prior to surgery, sheep were fasted for approximately 15 to 20 hours, however, drinking of water was still allowed. On the day of surgery, the wool along the neck was clipped and the sheep were sedated by injection of 20 ml of Sodium Thiopentone (50 mg/ml; Jurox, Rutherford, New South Wales, Australia) into the jugular vein. Once sedated, the sheep was placed on its back and with the help of a laryngoscope intubated with a cuffed endotracheal tube. General

anaesthesia was initially induced by spontaneous inhalation of 5% Isofluorane in O_2 (Bomac, Animal Health, New South Wales, Australia), whilst the hind limbs and abdomen area of the lamb was shaved and cleaned thoroughly with alcohol and concentrated iodine solution. Also during this time, antibiotics were administered intravenously: 5 ml of engemycin (100 mg/ml; Coopers Animal Health, Bendigo East, Victoria, Australia) and 1 g of ampicillin (1 g/5 ml, Aspen Pharmacare Australia Pty Ltd, New South Wales, Australia). After prepping the sheep, the concentration of isofluorane was reduced to 2% in O_2 and administered via the endotracheal tube by use of a positive pressure ventilator; this was maintained for the remainder of the surgery.

Strict aseptic conditions were followed during these surgeries; facemasks, surgical hats and sterile surgery gowns were worn by all participating in the surgery. Once the sheep were prepped for surgery, they were placed on the surgery table then covered with a sterile plastic drape and surgical linen drapes, leaving the incisions sites uncovered. Incisions were made on the medial surface of each of the hind limbs to expose the femoral artery and vein. An arterial catheter was inserted through a small hole in the femoral artery wall of the right hind limb and secured with a silk tie. Likewise, the same was done with the venous catheter inserted into the right femoral vein. The inserted catheters were then flushed with heparinised saline. In the opposite limb, a vascular flow probe (size: 4 mm, Transonic Systems, Ithaca, New York, USA) was placed around the left femoral artery for measurement of blood flow. Catheters and the flow probe were tunnelled subcutaneously along the side of the sheep's body and allowed to exit the body via keyhole incisions in the middle of the side of the body. All incisions were then sutured. Netting was wrapped around the middle of the sheep's body to keep catheters in place and to avoid the sheep pulling out their sutures. After surgery, sheep were taken off anaesthesia, but kept on the ventilator, until spontaneous breathing occurred; they were then extubated. Sheep were taken back to their individual cages for recovery, where they were continuously monitored and given free access to food. Water was given once the sheep were standing again.

After surgery, sheep also received analgesia for 72 hours using a transdermal fentanyl patch (Janssen Cilag, North Ryde, New South Wales, Australia). Antibiotics of 5 ml engemycin (100 mg/ml; Coopers Animal Health, Bendigo East, Victoria, Australia) and 500 mg of ampicillin (1g/5ml, Aspen Pharmacare Australia Pty Ltd, New South Wales, Australia) were also administered intravenously daily for three days following surgery. Catheters were regularly

flushed with heparinised saline and daily blood samples were taken from the arterial catheter to monitor blood gases (ABL30; Radiometer, Copenhagen, Denmark).

It should be noted that Dr. Ilias Nitsos and Dr. Beth Allison performed the catheterisation surgeries. I assisted in the surgeries for all the sheep; assistance was also provided by Mr. Dalibor Stanojkovic (animal technician).

3.2.6 Cardiovascular and metabolic studies

The arterial and venous catheters in the right femoral artery and vein were used for assessment of glucose metabolism. These metabolic studies were conducted a week after surgery by Dr. Robert De Matteo from the Department of Anatomy and Development Biology at Monash University in collaboration with Dr. Kathy Gatford from the University of Adelaide.

The flow probe surrounding the left femoral artery was used for cardiovascular studies on blood flow, arterial pressure and heart. These studies were performed a week after the metabolic studies by Dr. Beth Allison from the Hudson Institute, Clayton (formerly known as Monash Institute of Medical Research). Basal femoral arterial blood pressure and blood flow was measured in conscious lambs five days post-surgery. Femoral artery blood pressure (DTX Plus Transducer, Becton Dickinson, Singapore) and flow were continuously recorded (Powerlab; ADinstruments, Castle Hill, NSW, Australia). Transducers were fixed to the back of the lambs and calibrated using a two point calibration with a manometer. Distance from transducer position to tip of the femoral artery catheter was measured at post mortem; this measurement was used in post analysis to obtain corrected blood pressure. These basal measurements were recorded continuously for one hour in non-stressed lambs standing normally. It should be noted that even though I was not responsible for directly carrying out these measurements, I was involved in the preparation and organisation of these procedures and I assisted the main researchers as much as my time would allow.

3.2.7 Ultrasounds

On the day of necropsy, similar to that in Chapter 2 (Section 2.2.5, page 47-48), sheep were anaesthetised (using the same method as described previously in Section 3.2.5, page 71). Once unconscious, sheep were monitored carefully whilst ultrasound analyses of the heart, kidneys and blood vessels (aorta, carotid arteries and renal arteries) were performed. These

ultrasound assessments were performed by Mr. Paul Lombardo as part of his PhD project. His ultrasound measurements provide valuable information on cardiovascular structure and function, which complement the morphological analyses conducted in my PhD thesis. Collectively, our findings will lead to comprehensive joint first author publications of the effects of moderate preterm birth on the cardiovascular system.

After completion of the ultrasounds, 20 ml of blood was drawn from the femoral arterial catheter for collection of plasma and also a 1 ml blood sample for blood chemistry analyses. In regards to the blood chemistry analyses, not all animals were analysed, as collection of the sample was overlooked in a few animals at this time. The sheep was then administered 1 ml of heparin (25000 IU in 5 ml; Pfizer Australia, West Ryde, New South Wales, Australia) via the femoral arterial catheter to prevent blood clotting during necropsy. Sheep were then euthanised with a 20 ml injection of Lethabarb (325 mg/mL; Pentobarbitone, Verbac Animal Health, New South Wales, Australia) via the femoral arterial catheter.

3.2.8 At necropsy

Sheep were aged 14.5 months ± 2 weeks at necropsy. Similar to Chapter 2 (Section 2.2.6, page 47-48 the heart, lungs, brain, kidneys, liver, spleen, thymus, gut, adrenal glands and blood vessels were collected in the same manner as described previously. Some additional tissues (not collected in the short-term studies) were also collected for analysis by Dr. Kathy Gatford at the University of Adelaide. This included dissection of the soleus, vastus and semitendinosus muscles, and the pancreas; each were weighed and samples of each tissue were immersion-fixed, snap frozen in liquid nitrogen and embedded frozen in an optimal cutting temperate compound (OCT). Subcutaneous, abdominal and perirenal fat were also collected and immersion-fixed, snap frozen in liquid nitrogen and embedded frozen in OCT.

3.2.9 Statistical analysis

For all measurements conducted throughout the duration of the long-term study (such as body weight, morphometric measurements, arterial pressure and heart rate), the data were analysed using a three-way repeated measures ANOVA, with time after birth (P_T), gestational age at birth (P_G; preterm or term), and sex (P_S; female or male) as factors. A two-way ANOVA was also used to analyse differences at each individual time point, with a Tukey's post-hoc test performed if significant interactions were detected. For the DEXA analysis and all data

collected at post-mortem, such as blood chemistry and organ weights, a two-way ANOVA was utilised with gestational age at birth (P_G; preterm or term) and sex (P_S; female or male) as factors. Similarly, significant interaction effects detected in the 2-way ANOVA were further analysed by a Tukey's post-hoc test. All data are represented as means ± SEM and statistical significance was accepted at the level $P<0.05$. Statistical analysis was performed using IBM SPSS Statistics Version 21 (IBM SPSS, Illinois, USA) and graphed using GraphPad Prism Version 6.0 (GraphPad Software, California, USA).

3.3 Results

3.3.1 Survival rates (Figure 3.2)

3.3.1.1 Preterm cohort

Over the study period, 57% of all preterm lambs survived (16 out of 28 preterm lambs); 56% of male preterm lambs (9 out of 16) survived and 58% of female preterm lambs (7 out of 12) survived. There was no significant difference (P=0.919) between survival rates of preterm males and preterm females.

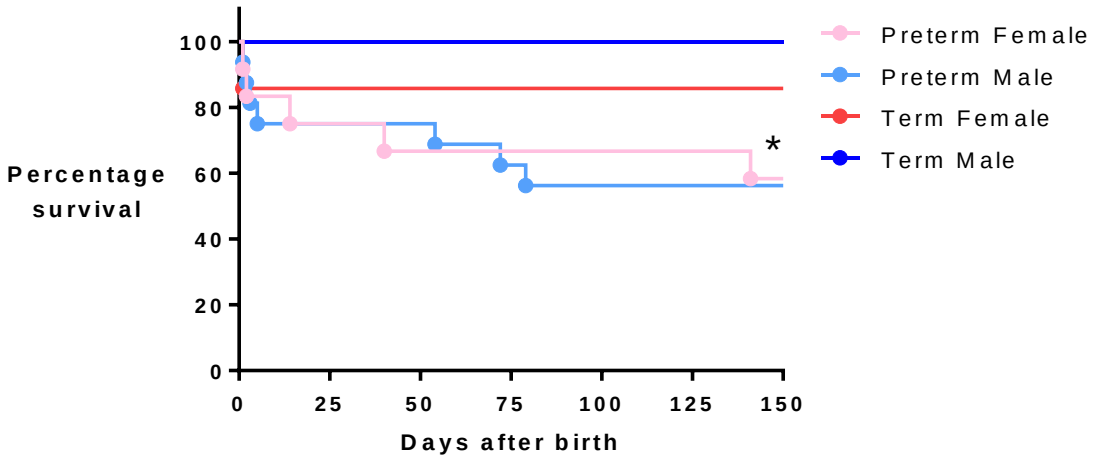

Figure 3.2. *Percentage survival of all preterm (male and female) and term (male and female) lambs until 5 months of age. After 5 months of age, all lambs survived until the end of the study period. Pink circles represent preterm females, pale blue circles represent preterm males, red circles represent term females and dark blue circles represent term males. Curve comparisons were performed by log-rank tests, with *P<0.05 observed between preterm lambs versus term lambs.*

Of the 43% of preterm lambs that did not survive, the majority of them (7 out of 12; 4 preterm males and 3 preterm females) died within the two-week period following birth, due to respiratory failure. Therefore, the survival rate of lambs after the first two weeks of life was 75% for both preterm males and preterm females, with 21 out of 28 preterm lambs surviving.

Unexpectedly, another five preterm lambs (3 preterm males and 2 preterm females) died between 1 and 5 months after birth. Pathology tests identified that the deaths were likely due to white muscle disease which can be caused by a deficiency in Vitamin E and/or selenium. Symptoms of stiffness in limbs, obvious pain in walking and the inability to stand were observed in some of the preterm lambs, whilst term lambs did not appear to be affected. After a couple of lambs died, autopsy reports and blood tests of the remaining surviving preterm lambs showed severe muscle wasting and low levels of Vitamin E. However, the muscle wasting appeared to be so severe that it was considered unlikely that a low level of Vitamin E was the only contributing factor and it was suggested that there may have also been ionophore toxicity; this could have occurred if the lambs had eaten ewe-specific pellets. Once alerted to these potential causes, all lambs were treated with Vitamin E and given lamb-specific pellets in their diet.

3.3.1.2 Term cohort

For the term group, 94% of lambs survived (11 out of 11 males and 6 out of 7 females). One female lamb died during the birthing process as it was abnormally positioned in the birthing canal (breeched) and we were unable to save the lamb during its delivery.

When comparing preterm lambs to term lambs, there was a significant difference (P=0.009) in the percentage survival. There was no significant difference in the percentage survival between males and females and the percentage survival of preterm males was similar to the percentage survival of preterm females. Figure 3.2 shows the survival curve up from birth to 5 months of age; from this point onwards, all the lambs survived until the end of the study period.

3.3.2 At birth

3.3.2.1 Birth weight and morphometric measurements (Figure 3.3 and Table 3.1)

At birth, preterm lambs were significantly lighter (P_G<0.0001) than term controls (Figure 3.3). There was no significant difference in birth weight between males and females.

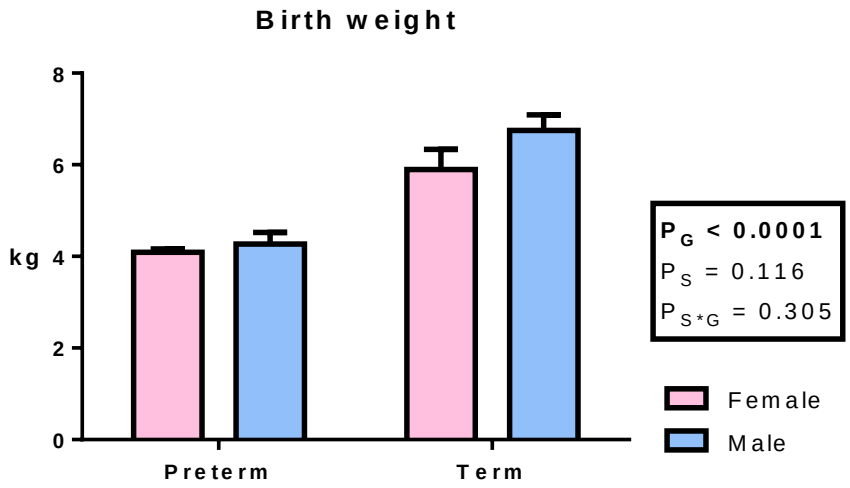

Figure 3.3. *Birth weights of preterm (n=7 female, n=9 male) and term (n=6 female, n=11 male) lambs. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

The first morphometric measurements were recorded 2 days after birth, once lambs were stable. In accordance to body weight, all body dimensions of CrL, TG, FLL and HLL were significantly reduced (P_G<0.0001) in the preterm group compared to the term group. Only PI (a measure of "leanness") was found to be similar between all groups. There were no significant differences in any of the morphometric measurements between male and female lambs (Table 3.1).

	Preterm		Term		P-values
	Females n=7	Males n=9	Females n=6	Males n=11	
Crown-rump length (cm)	47.04 ± 1.45	45.93 ± 1.27	51.70 ± 1.56	52.31 ± 1.15	**P_G<0.0001**
Thoracic girth (cm)	39.43 ± 1.24	37.09 ± 1.09	43.53 ± 1.34	43.36 ± 0.99	**P_G<0.0001**
Front limb length (cm)	43.97 ± 1.24	44.53 ± 1.09	50.43 ± 1.34	52.28 ± 0.99	**P_G<0.0001**
Hind limb length (cm)	42.91 ± 1.11	43.47 ± 0.98	50.17 ± 1.19	50.78 ± 0.88	**P_G<0.0001**
Ponderal index (kg/cm^3)	40.12 ± 4.52	43.86 ± 3.99	46.43 ± 4.88	51.80 ± 3.61	NS

Table 3.1. *Morphometric measurements of preterm (female and male) and term (female and male) lambs at 2 days of age. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM. Abbreviations: NS – not significant.*

3.3.2.2 Arterial pressure and heart rate (Figure 3.4)

At birth, there were no significant differences in systolic blood pressure or mean arterial pressure between preterm lambs and term lambs (Figure 3.4A and Figure 3.4C). However, diastolic blood pressure was found to be significantly higher in the preterm lambs compared to the term lambs (Figure 3.4B; preterm: 60.2 ± 2.1 mmHg vs. term: 53.4 ± 2.1 mmHg; P_G=0.030) and heart rate was significantly lower in the preterm lambs (Figure 3.4D; preterm: 210.6 ± 11.2 bpm vs. term: 245.1 ± 11.2 bpm; P_G=0.038). There were no significant differences between males and females in any of the parameters measured except for mean arterial pressure, which was significantly higher in female lambs compared to male lambs (female: 70.9 ± 2.1 mmHg vs. male: 65.0 ± 1.7 mmHg; P_S=0.033).

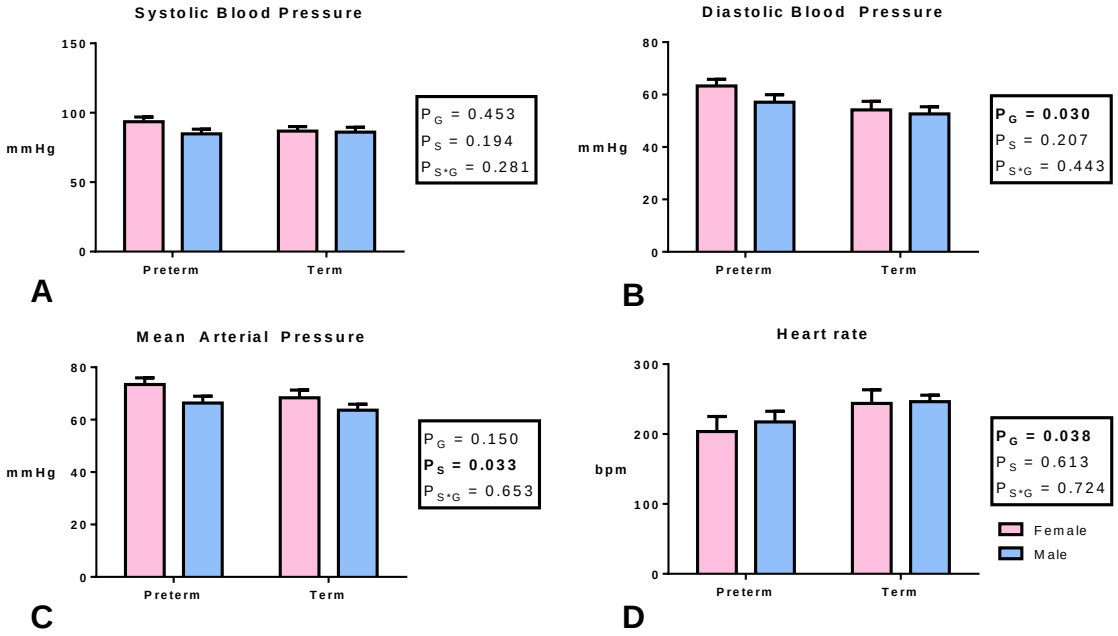

Figure 3.4. *Average systolic blood pressure (A), diastolic blood pressure (B), mean arterial pressure (C) and heart rate (D) in preterm (n=7 female, n=9 male) and term (n=6 female, n=11 male) lambs at 2 days of age. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

3.3.3 Postnatal growth, arterial pressure and heart rate

3.3.3.1 Body weights (Figure 3.5)

Body weights were recorded weekly for the first 12 weeks, then every four weeks until 14.5 months of age (60 weeks) (Figure 3.5). During the first 12 weeks after birth there was a steady rise in body weight in both preterm and term lambs (Figure 3.5). Around 12 weeks of age, there was a slowing of growth in all lambs and there was there was a marked reduction in body weight of preterm sheep, compared to controls. From 12 weeks until about 30 weeks of age, although the rate of growth was similar to that of term animals there were persistent significant reductions in body weights in the preterm sheep compared to term sheep, with preterm males most affected. From approximately 30 weeks of age until necropsy, there was a gradual catch-up in body weight in the preterm female sheep, with no differences in body weight at most time points from 30 weeks of age to necropsy (Figure 3.5B). In contrast, the

male preterm sheep remained significantly lighter than male term sheep throughout the experimental period (Figure 3.5A). At necropsy body weight was significantly lower in male preterm sheep compared to term sheep but there was no difference in body weight in preterm and term female sheep.

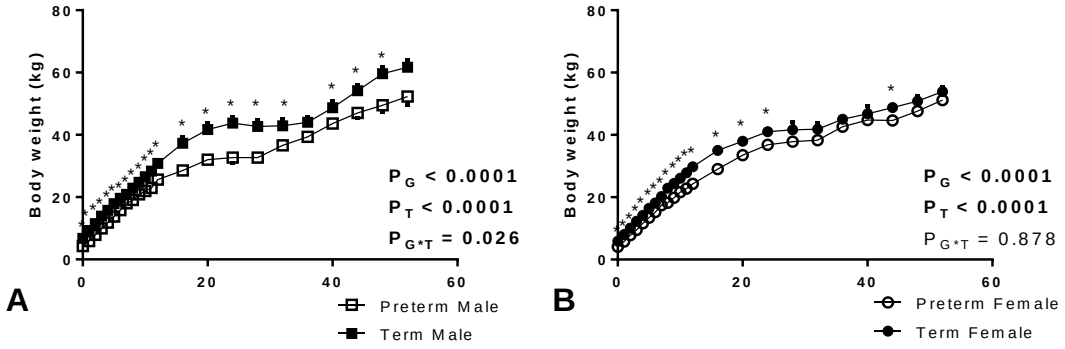

Figure 3.5. *Body weights of all surviving male (A: preterm and term) and female (B: preterm and term) sheep until 52 weeks of age. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G) and time after birth (P_T: repeated measures) as factors. *P<0.05 between preterm vs. term within the same sex, following a Tukey's post-hoc analysis.*

3.3.3.2 Morphometric measurements (Figure 3.6)

Throughout the experimental period, measurements of CrL, TG, FLL and HLL were taken at the same time as body weight as seen in Figure 3.6. In the first 12 weeks after birth, there was a marked increase in CrL, TG, FLL and HLL. After this time, there was little change in these measurements; with only a small increase observed between 12 to 52 weeks of age in these growth parameters. Overall, there were no significant differences in the patterns of growth for CrL, TG, FLL and HLL between any of the groups over time; only PI showed a significant difference (P_{G*T}<0.001) between preterm and term sheep over time.

Hence, given that the preterm lambs started off significantly smaller at birth, they have remained smaller during the experimental period. Overall, all body dimensions (CrL, TG, FLL and HLL) of the preterm sheep were significantly less (P_G<0.0001) than in the term sheep over the study period, except for PI which was significantly higher (P_G<0.0001) in preterm sheep.

Furthermore, females had significantly larger TG (P_S<0.0001), shorter FLL (P_S<0.0001) and lower PI (P_S<0.0001) compared to male sheep.

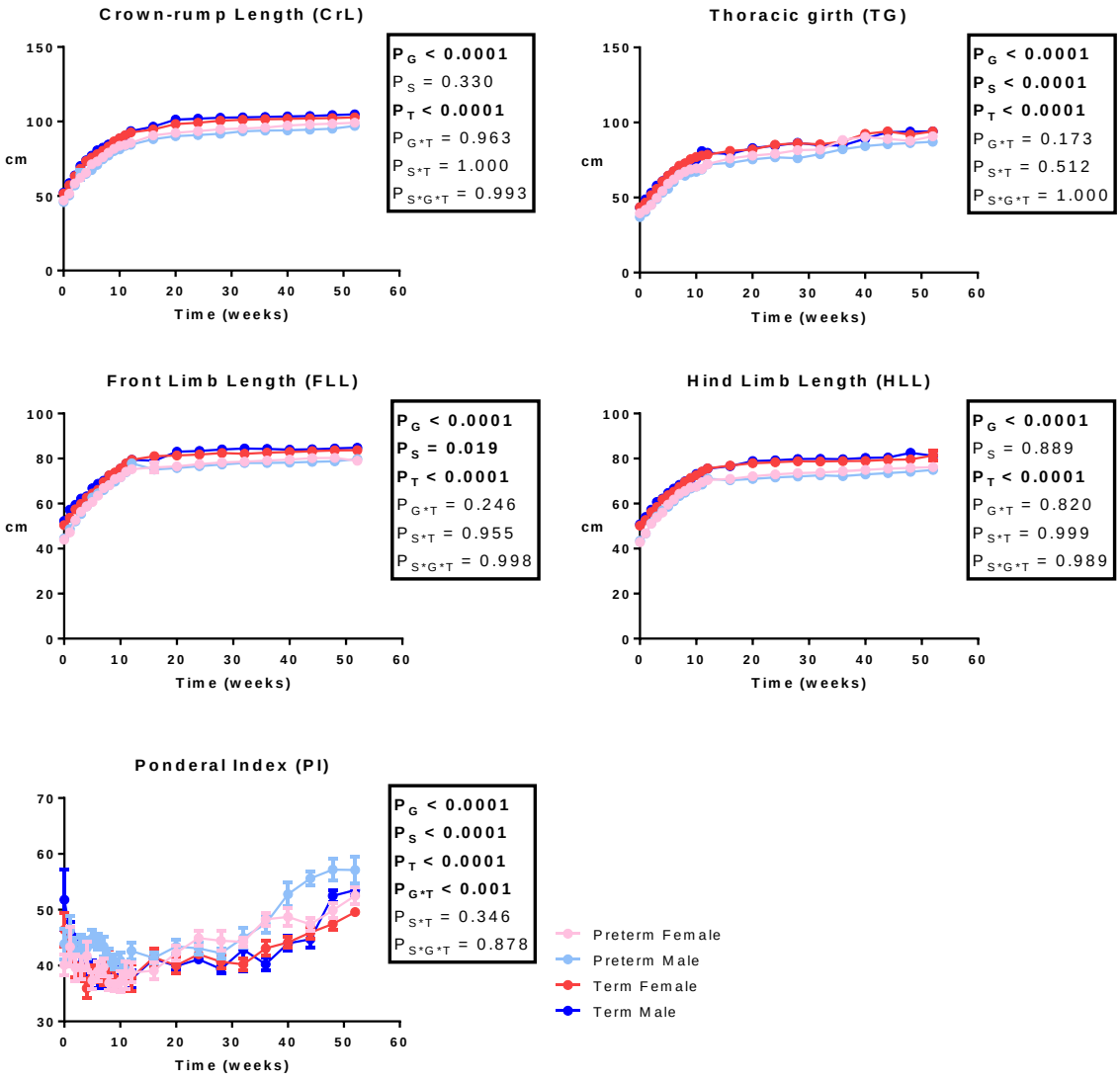

Figure 3.6. *Morphometric measurements of preterm (n=7 female, n=9 male) and term (n=6 female, n=11 male) lambs over the study period. Pink circles represent preterm females, pale blue circles represent preterm males, red circles represent term females and dark blue circles represent term males. Data were analysed using a 3-way repeated measures ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and time (P_T) after birth as factors. Values are means ± SEM.*

3.3.3.3 Arterial pressure and heart rate (Figure 3.7)

During the first 12 weeks after birth, systolic blood pressure, diastolic blood pressure and mean arterial pressure increased for both preterm and term lambs (Figure 3.7). However, during this time, systolic blood pressure and mean arterial pressure was significantly lower in preterm lambs compared to term lambs, whilst diastolic blood pressure was only significantly reduced in preterm male lambs compared to term male lambs. After weaning at 12 weeks of age until 30 weeks of age, there were no longer any significant differences between any of the groups. From approximately 30 weeks of age until 52 weeks, preterm female sheep had significantly lower diastolic blood pressure compared to term female sheep, but there were no differences in systolic blood pressure or mean arterial pressure. In contrast, preterm male sheep had significantly higher systolic blood pressure compared to term male sheep; however, there were no differences in diastolic blood pressure or mean arterial pressure. It is important to note there was high variability in all measurements of blood pressure; hence, when individual time points were examined, there were very few significant differences in blood pressure observed.

Heart rate decreased significantly from birth until weaning for both preterm and term lambs. During this time, preterm male lambs had a significantly higher heart rate compared to term male lambs. However after this period, from 12 weeks until 30 weeks of age, male preterm sheep had significantly lower heart rate compared to term male sheep. From approximately 30 weeks of age until 52 weeks, the heart rate of all males decreased over time but there were no differences between male preterm and term sheep. In contrast in female preterm sheep, there were no differences in heart rate compared to term female sheep throughout the study period.

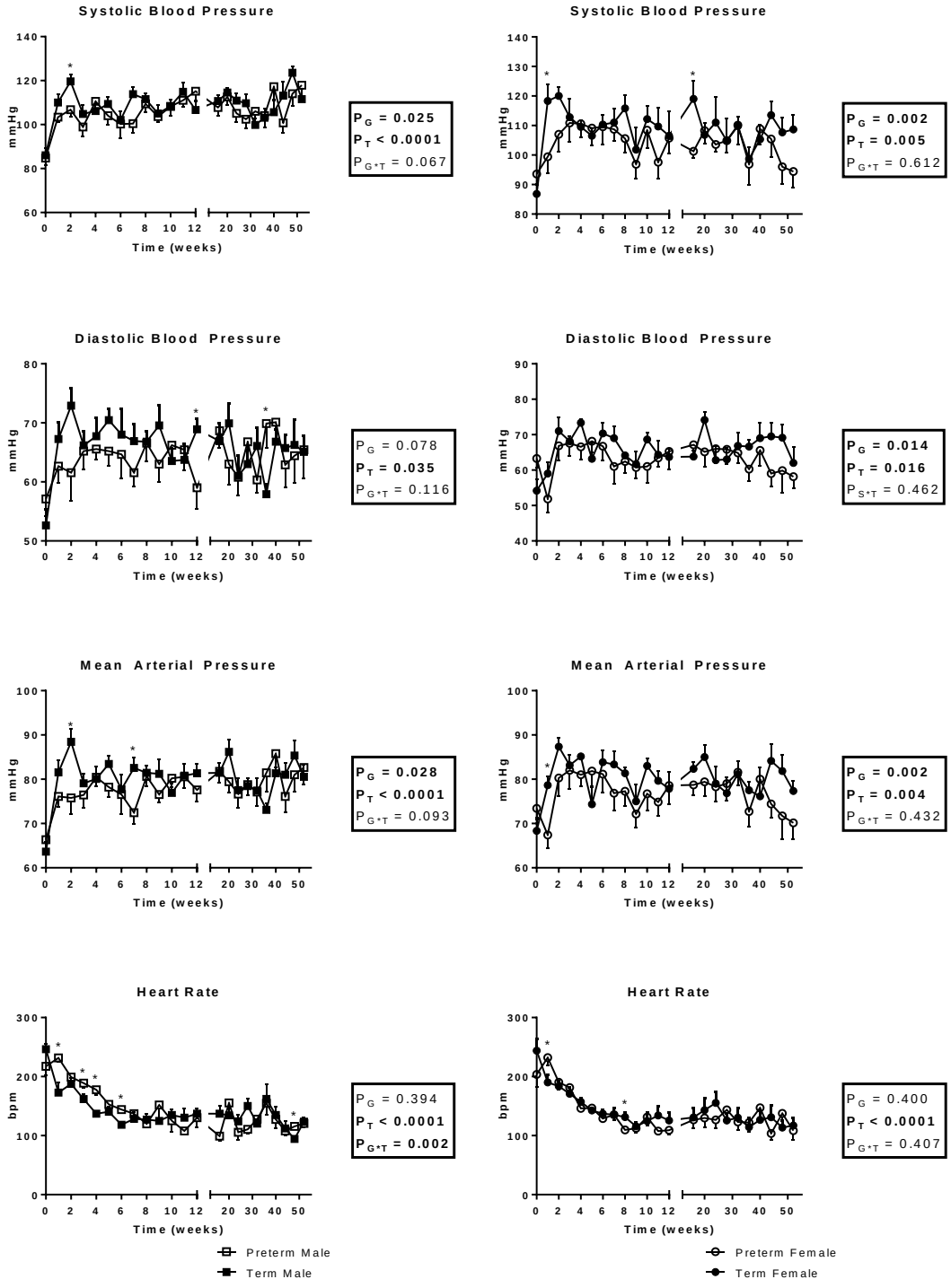

Figure 3.7. *Systolic blood pressure, diastolic blood pressure, mean arterial pressure and heart rate in male (left panel: preterm and term) and female (right panel: preterm and term) lambs over the study period. Data was analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term) and time (P_T: repeated measures) after birth as factors. *P<0.05 between preterm vs. term within the same sex, following a Tukey's post-hoc analysis. Values are means ± SEM.*

3.3.3.4 Post Term Equivalent Age (PTEA)

Given that body weights and body measurements of growth were recorded weekly, this enabled me to look at body weight and size at term equivalent age (TEA) in the preterm lambs and to compare body growth relative to post term equivalent age (PTEA) during the first 12 weeks of life.

3.3.3.4.1 Body weights (Figure 3.8)

In the initial 12 week period after birth, there were no significant differences in the growth trajectory of body weights between preterm lambs at PTEA and term lambs, however female lambs were found to be significantly lighter (P_S=0.001) compared to male lambs.

Interestingly at TEA, preterm lambs were significantly heavier (P_G<0.001) than term lambs. The average body weight for preterm lambs at TEA was 7.86 ± 0.44 kg for preterm females and 7.91 ± 0.39 kg for preterm males whereas in the term lambs at birth, the average birth weight for term females was 5.89 ± 0.47 kg and 6.75 ± 0.35 kg for term males. A significant elevation in body weight was also observed in the preterm lambs at 1 week PTEA compared to term lambs. However, from 2 weeks PTEA, all lambs (from the preterm and term groups) were of similar weight up until the time of weaning as shown in Figure 3.8.

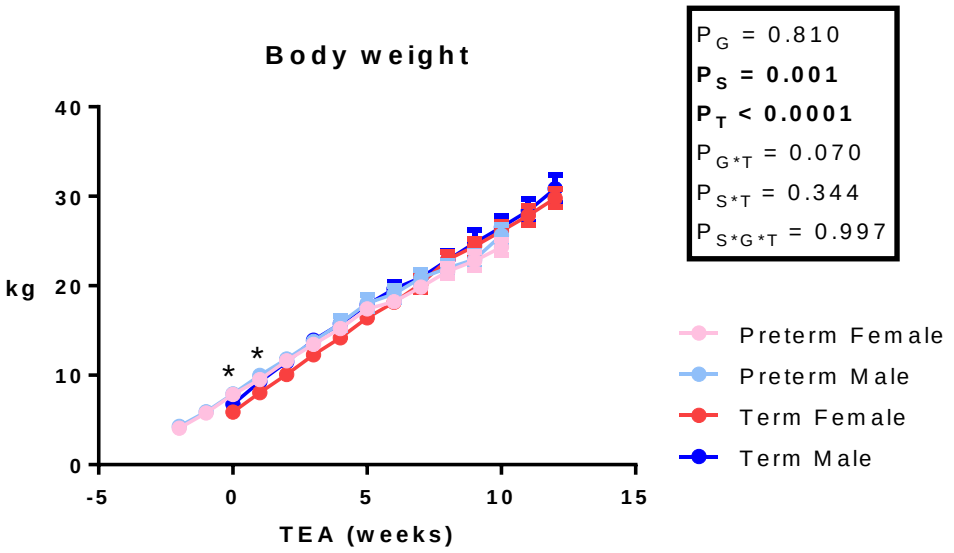

Figure 3.8. *Body weight increases of preterm (n=7 female, n=9 male) lambs when adjusted for TEA and term (n=6 female, n=11 male) lambs for 12 weeks after birth. Pink circles represent preterm females, pale blue circles represent preterm males, red circles represent term females and dark blue circles represent term males. Data was analysed using a 3-way repeated measures ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and time (P_T) after birth as factors. A 2-way ANOVA was also utilised (with gestational age at birth and sex as factors) to analyse each individual time point. *P<0.05 between preterm vs. term lambs at the individual time point. Values are means ± SEM.*

3.3.3.4.2 Morphometric measurements (Figure 3.9)

Throughout the 12 weeks, the patterns of growth were found to be significantly different between preterm lambs at PTEA and term lambs for CrL (P_{G*T}<0.0001), TG (P_{G*T}<0.0001) and PI (P_{G*T}=0.008) as seen in Figure 3.9. Overall, preterm lambs at PTEA had significantly reduced TG (P_G=0.003) and HLL (P_G=0.011) compared to term lambs.

At TEA, the CrL of preterm lambs was significantly longer (P_G<0.001) than in term lambs; there were no significant differences in TG, FLL and HLL. Interestingly by around 10 weeks after TEA, term lambs had significantly greater CrL (P_G=0.004), TG (P_G=0.012), and HLL (P_G=0.018) compared to preterm lambs.

Additionally, female lambs exhibited significantly reduced FLL (P_S=0.023) and PI (P_S<0.0001) compared to male lambs.

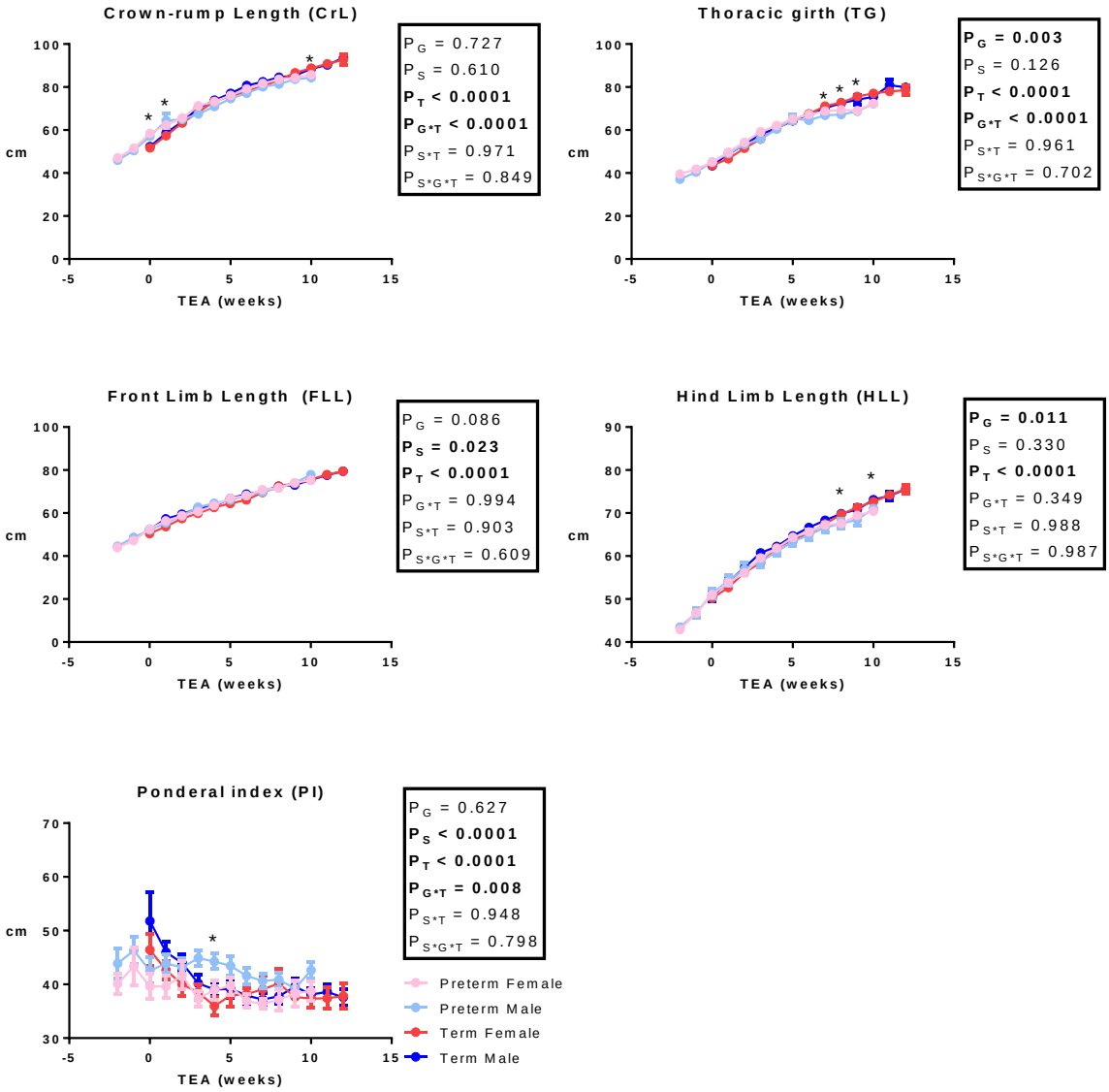

Figure 3.9. *Morphometric data of preterm (n=7 female, n=9 male) lambs when adjusted for TEA and term (n=6 female, n=11 male) lambs for 12 weeks after birth. Pink circles represent preterm females, pale blue circles represent preterm males, red circles represent term females and dark blue circles represent term males. Data was analysed using a 3-way repeated measures ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and time (P_T) after birth as factors. A 2-way ANOVA was also utilised (with gestational age at birth and sex as factors) to analyse each individual time point. *P<0.05 between preterm vs. term lambs at the individual time point. Values are means ± SEM.*

3.3.4 At 14.5 months (adulthood)

3.3.4.1 Body growth and composition (Table 3.2 and Figure 3.10)

At the end of the experimental period, morphometric measurements of CrL, TG and FLL were all found to be significantly lower in the preterm sheep compared to term sheep, with the differences predominantly observed between preterm male and term male sheep, as seen in Table 3.2. There was a trend for preterm sheep to be lighter than term sheep and have reduced HLL, but these did not quite reach statistical significance (body weight, P_G=0.076; HLL, P_G=0.074). The trend of reduced body weight in preterm sheep is mostly attributed to preterm male sheep being lighter (P=0.065; however this did not quite reach statistical significance) compared to term male sheep, whereas females had caught up in body weight (P=0.464)

Overall, there were no significant differences in body growth between males and females.

	Preterm		Term		P-values
	Females n=7	**Males n=9**	**Females n=6**	**Males n=11**	
Body weight (kg)	54.36 ± 2.45	53.11 ± 2.17#	55.75 ± 2.65	60.27 ± 1.96	P_G=0.076
Crown-rump length (cm)	109.70 ± 2.57	108.64 ± 2.27*	114.45 ± 2.78	116.00 ± 2.05	**P_G=0.019**
Thoracic girth (cm)	105.64 ± 2.80	99.72 ± 2.47#	108.58 ± 3.02	108.42 ± 2.23	**P_G=0.036**
Front limb length (cm)	89.06 ± 2.01	85.82 ± 1.77*	93.15 ± 2.17	93.88 ± 1.60	**P_G=0.003**
Hind limb length (cm)	82.87 ± 1.75	84.46 ± 1.55	84.87 ± 1.90	88.61 ± 1.40	P_G=0.074
Ponderal index (kg/cm^3)	41.52 ± 2.42	41.41 ± 2.14	37.95 ± 2.61	40.05 ± 1.93	NS

Table 3.2. *Body weights and morphometric measurements of preterm (female and male) and term (female and male) lambs at 14.5 months (60 weeks) of age. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors, followed by a Tukey's post-hoc test. *P<0.05 between preterm vs. term within the same sex. #P<0.07 between preterm vs. term within the same sex. Abbreviations: NS – not significant. Values are means ± SEM.*

Body composition was analysed at approximately 13 months ± 2 weeks of age with DEXA scans in sheep. Figure 3.10 shows there were no significant differences in bone mineral content, fat mass and lean muscle mass between preterm and term sheep or the percentage of fat and lean muscle mass.

Overall, females had higher fat mass (11.43 kg vs. 9.84 kg; P_S=0.021) and percentage of fat (20.4% vs. 16.7%; P_S<0.0001), but lower lean muscle mass (43.47 kg vs. 48.02 kg; P_S=0.038) and percentage of lean muscle mass (77.9% vs. 82.5%; P_S<0.001) when compared to males.

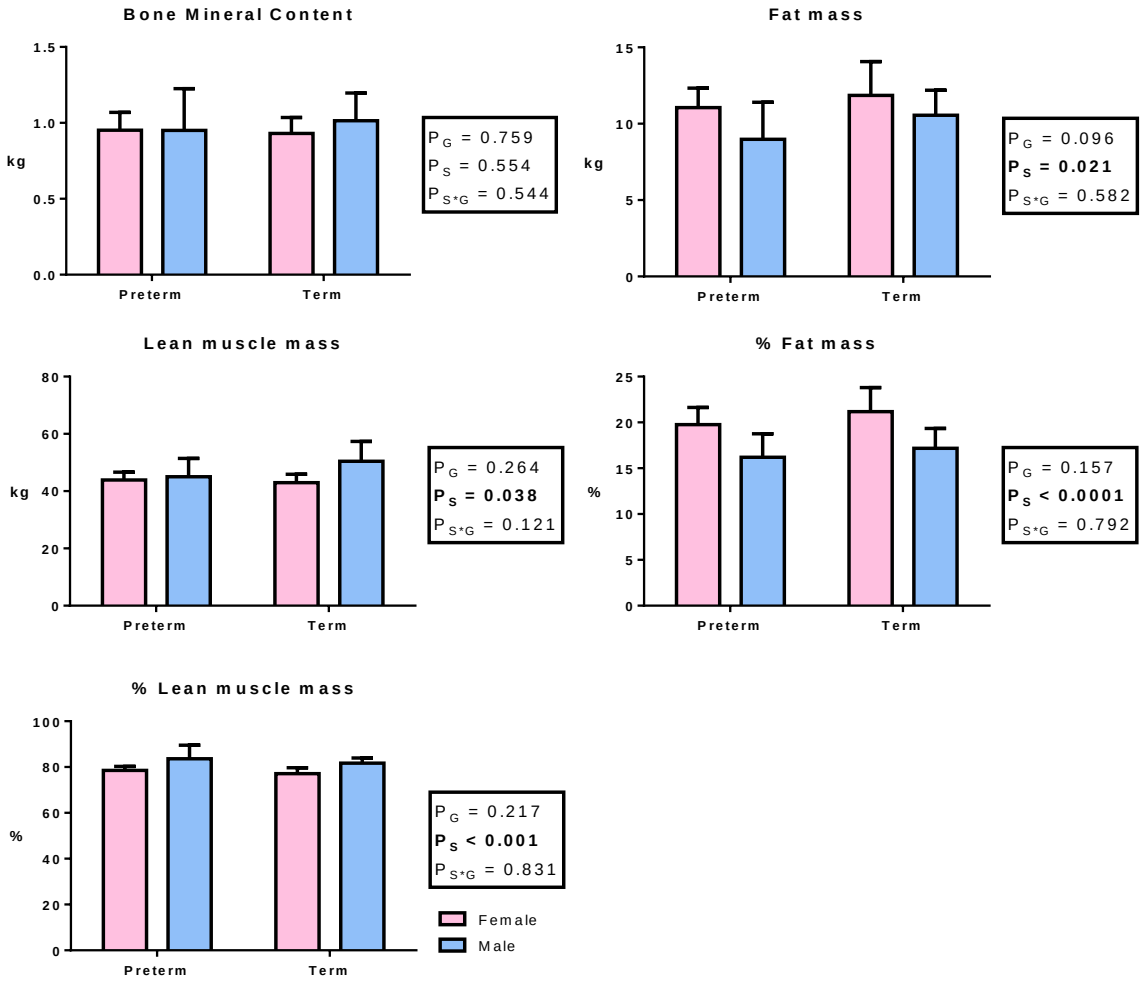

Figure 3.10. *Bone mineral content, fat mass, lean muscle mass, percentage of fat and percentage of lean mass in preterm (n=7 female, n=9 male) and term (n=6 female, n=11 male) sheep at approximately 13 months ± 2 weeks of age. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

3.3.4.2 Arterial pressure and heart rate (Figure 3.11)

At the end of the experimental period, arterial pressure and heart rate were measured in conscious sheep via an indwelling catheter in the femoral artery. At this final recording session, there were no differences in arterial pressure or heart rate between preterm and term sheep (Figure 3.11). In addition, differences between the sexes were observed in systolic blood pressure; with female sheep exhibiting significantly elevated systolic blood pressure compared to male sheep (female: 127.19 ± 2.07 mmHg vs. male: 117.54 ± 3.17 mmHg; P_S=0.019).

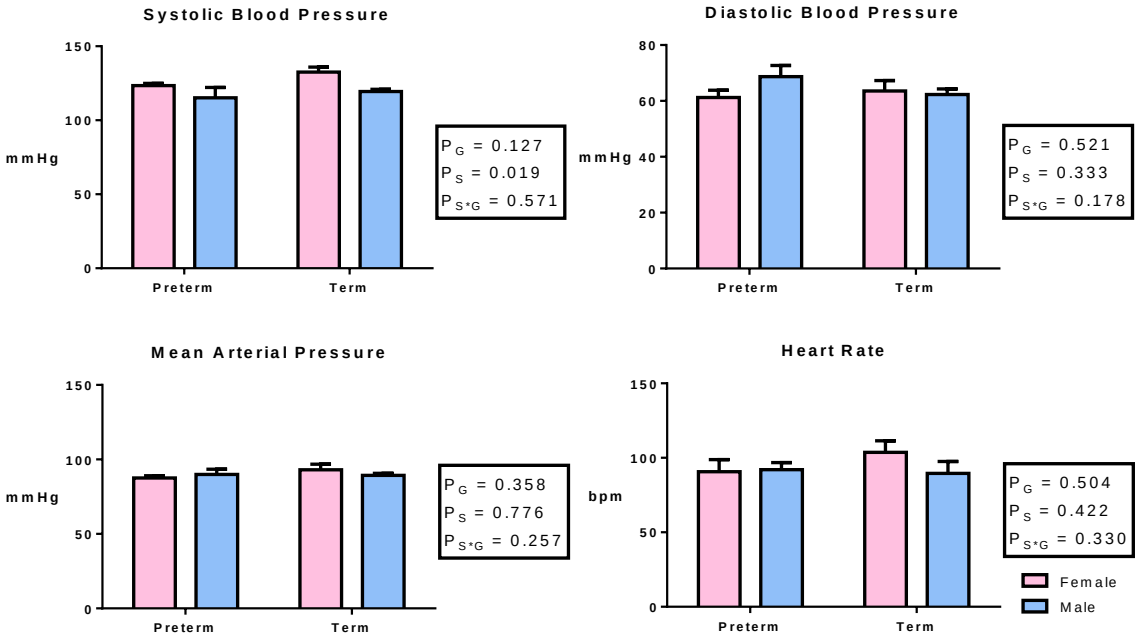

Figure 3.11. *Average systolic blood pressure, diastolic blood pressure, mean arterial pressure and heart rate in preterm (n=7 female, n=9 male) and term (n=6 female, n=11 male) sheep at approximately 14 months ± 2 weeks of age. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

3.3.4.3 Blood chemistry (Table 3.3)

Blood chemistry was analysed just prior to necropsy from a 1 ml blood sample taken via the femoral arterial catheter whilst sheep were under anaesthesia and mechanically ventilated with 100% oxygen (Table 3.3). There were no significant differences observed in haemoglobin levels (ctHb), oxygen saturation levels (sO_2), pH, pCO_2, glucose levels (cGlu) and Lactate levels (cLac) between preterm and term sheep or male and female sheep.

	Preterm		Term		P-values
	Females n=7	**Males n=7**	**Females n=5**	**Males n=10**	
Oximetry Values					
ctHb (g/dL)	10.3 ± 0.4	10.4 ± 0.6	10.7 ± 0.5	9.3 ± 0.3	NS
sO_2 (%)	98.0 ± 0.2	98.2 ± 0.3	97.8 ± 0.2	97.6 ± 0.3	NS
Blood Gas Values					
pH	7.42 ± 0.02	7.48 ± 0.01	7.47 ± 0.03	7.39 ± 0.05	NS
pCO_2 (mmHg)	34.9 ± 1.5	35.8 ± 1.0	33.8 ± 1.0	37.2 ± 1.0	NS
pO_2 (mmHg)	227.0 ± 15.8	222.7 ± 15.8	197.6 ± 18.6	200.8 ± 13.2	NS
Metabolite Values					
cGlu (mmol/L)	4.0 ± 0.2	4.3 ± 0.4	4.4 ± 0.4	3.7 ± 0.2	NS
cLac (mmol/L)	0.8 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	NS

Table 3.3. *Blood chemistry of the preterm (female and male) and term (female and male) sheep collected before necropsy whilst under anaesthesia and mechanically ventilated with 100% oxygen at approximately 14.5 months ± 2 weeks. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM. Abbreviations: NS – not significant.*

3.3.4.4 Organ weights (Table 3.4)

Table 3.4 reports the absolute and relative weights of all organs collected at necropsy. Absolute organ weights of the heart, lungs and right adrenal were all significantly lighter in

sheep born preterm when compared to term sheep. When adjusted for body weight, only the right adrenal remained significantly lighter in preterm lambs compared to term lambs.

The absolute weights of the pancreas and soleus muscle were found to be significantly heavier in male sheep when compared to female sheep and there was a trend for the heart to be heavier in males (this was most evident between male and female term sheep). Relative to body weight only the soleus muscle was still significantly lighter in the female lambs compared to term lambs.

There were significant interactions between sex and gestational age at birth in the absolute weights of the lungs, left kidney, right kidney, spleen and semitendinosus muscle; and also in the relative weight of the spleen. In the preterm sheep, preterm females had heavier organs (of those mentioned above) compared to preterm males, whereas within the term sheep, males had heavier organs compared to female sheep. Additionally, there was a trend for an interaction effect on the absolute heart weight, with a big difference in heart size between preterm and term male sheep, whereas the difference was much less pronounced between preterm and term females.

Organs	Preterm		Term		P-values
	Females n=7	Males n=9	Females n=6	Males n=11	
Absolute weights (g)					
Heart	265.75 ± 12.51	265.42 ± 11.04	273.96 ± 13.52	318.95 ± 9.98	**P_G=0.014** P_S=0.069 P_{S*G}=0.066
Lungs	559.29 ± 23.35	538.89 ± 20.59	592.50 ± 25.22	671.82 ± 18.63	**P_G<0.001** **P_{S*G}=0.032**
Brain	107.38 ± 2.46	105.82 ± 2.17	108.62 ± 2.66	113.41 ± 1.96	P_G=0.068
Left Kidney	85.66 ± 4.00	82.00 ± 3.53	82.48 ± 4.32	94.45 ± 3.35	**P_{S*G}=0.050**
Right Kidney	89.61 ± 3.93	87.26 ± 3.47	84.64 ± 4.25	100.05 ± 3.14	**P_{S*G}=0.024**
Liver	883.57 ± 28.99	892.22 ± 25.57	842.50 ± 31.32	926.36 ± 23.13	NS
Spleen	246.62 ± 29.85	181.59 ± 26.32	192.83 ± 32.24	246.27 ± 23.81	**P_{S*G}=0.045**
Pancreas	56.11 ± 4.18	63.24 ± 3.69	49.66 ± 4.52	62.25 ± 3.34	**P_S=0.019**
Left Adrenal	1.61 ± 0.16	1.68 ± 0.14	1.84 ± 0.19	2.05 ± 0.14	NS
Right Adrenal	1.44 ± 0.14	1.50 ± 0.12	1.89 ± 0.14	1.76 ± 0.11	**P_G=0.010**
Soleus muscle	50.62 ± 2.93	54.19 ± 2.59	47.43 ± 3.17	59.51 ± 2.34	**P_S=0.009**
Vastus muscle	386.43 ± 34.07	362.78 ± 30.04	387.50 ± 36.79	367.31 ± 27.17	NS
Semitendinosus muscle	155.37 ± 8.95	136.28 ± 7.89	137.79 ± 9.67	153.93 ± 7.14	**P_{S*G}=0.046**
Relative to body weight (g/kg)					
Heart	4.89 ± 0.20	5.02 ± 0.18	4.90 ± 0.22	5.35 ± 0.16	NS
Lungs	10.28 ± 0.44	10.27 ± 0.39	10.65 ± 0.47	11.25 ± 0.35	NS
Brain	1.98 ± 0.08	2.03 ± 0.07	1.96 ± 0.09	1.90 ± 0.07	NS
Left Kidney	1.58 ± 0.09	1.58 ± 0.08	1.48 ± 0.10	1.66 ± 0.08	NS
Right Kidney	1.65 ± 0.09	1.67 ± 0.08	1.52 ± 0.09	1.68 ± 0.07	NS
Liver	16.26 ± 0.72	17.15 ± 0.64	15.15 ± 0.78	15.48 ± 0.58	P_G=0.051
Spleen	4.56 ± 0.64	3.67 ± 0.56	3.52 ± 0.69	4.13 ± 0.51	**P_{S*G}=0.045**
Pancreas	1.03 ± 0.08	1.21 ± 0.07	0.90 ± 0.09	1.04 ± 0.07	P_G=0.064 P_S=0.052
Left Adrenal	0.030 ± 0.003	0.032 ± 0.003	0.034 ± 0.004	0.035 ± 0.003	NS
Right Adrenal	0.026 ± 0.002	0.028 ± 0.002	0.034 ± 0.002	0.030 ± 0.002	**P_G=0.044**
Soleus muscle	0.93 ± 0.05	1.02 ± 0.04	0.85 ± 0.05	1.00 ± 0.04	**P_S=0.010**
Vastus muscle	7.10 ± 0.53	6.87 ± 0.47	6.96 ± 0.57	6.02 ± 0.42	NS
Semitendinosus muscle	2.86 ± 0.13	2.58 ± 0.12	2.47 ± 0.14	2.57 ± 0.11	NS

Table 3.4. *Absolute and relative weights of organs collected from preterm (female and male) and term (female and male) sheep at necropsy 14.5 months ± 2 weeks after birth. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM. Abbreviations: NS – not significant.*

3.4 Discussion

3.4.1 Summary

In this chapter, I have delivered lambs moderately preterm or at term and followed them from birth to young adulthood at 14.5 months of age. During this time, body growth, arterial pressure and heart rate were closely monitored. Overall, preterm lambs were lighter and smaller throughout the experimental period compared to term controls; however, by 14.5 months of age preterm females had caught up in body weight, whereas body weight remained attenuated in preterm males. At necropsy, the sheep born preterm were physically smaller with reduced CrL, TG and FLL; however, body composition was not different compared to terms. Prior to weaning, arterial pressure was significantly reduced in preterm lambs compared to term lambs. As the sheep grew to adulthood, there was no evidence of an elevation of arterial pressure in preterm sheep, with no detectable differences in arterial pressure or heart rate at 14.5 months of age when assessed in conscious animals using an indwelling catheter.

3.4.2 Preterm birth adversely impacts survival in the first 5 months of life

In this long-term cohort, the survival rate of preterm lambs after the first two days of life was not significantly different between sexes; 83% for preterm female lambs and 88% for preterm male lambs. Similarly, there were no sex differences in the survival of preterm lambs in the short-term cohort (previously described in Chapter 2, Section 2.3.3, page 51-52); 64% for preterm females and 60% for preterm males. However, the overall preterm survival rate in the first two days after birth was significantly higher (P=0.049, 86% vs. 62%) in the cohort studied in this chapter when compared to the survival of preterm lambs of the short-term cohort (Chapter 2, Section 2.3.3, page 50-51). This is likely due to the extra care given to the preterm lambs of the long-term cohort (in this chapter) in the immediate period following birth; all preterm lambs were administered a 0.5 - 0.7 ml intramuscular injection of Alamycin (which is a broad spectrum antibiotic) to prevent potential infections and also four of the preterm lambs received a single bolus dose of surfactant to further improve respiratory function. Furthermore, the level of experience in preterm deliveries and confidence of the team working with the animals was enhanced in this second series of experiments compared

to the initial studies with the short-term cohort; by this stage our experimental protocols during immediate postnatal care were well-established.

After the first two weeks of life, the survival rate dropped to 75% for both preterm male and female lambs. Deaths within this time were due to ongoing respiratory challenges even though a clinically relevant dose of betamethasone was administered to ewes delivering premature and an extra single bolus dose of surfactant administered postnatally to preterm lambs experiencing respiratory distress. At autopsies, these preterm lambs showed extremely unaerated lungs. Overall, the survival of preterm female lambs was similar to that reported by De Matteo et al. (2010) (76% after two weeks); hence, the higher dose of antenatal betamethasone administered to ewes that were to deliver preterm in the present studies (this chapter and Chapter 2) did not improve survival of preterm females compared to the earlier studies of De Matteo et al. (2010). In contrast, the survival of preterm male lambs in my study in the first two weeks of life was much higher than that reported by De Matteo et al. (2010) for moderately preterm male lambs delivered at a similar gestational age (75% compared to 44%). Hence, it is likely that the higher dose of antenatal corticosteroids used in my studies has led to improved survival of male preterm lambs. Based on the differences in survival between the sexes between these studies, further studies are required to explore different dosages of antenatal corticosteroids. Indeed, my findings suggest that the current doses of antenatal corticosteroids administered to women at risk of preterm delivery may potentially be lowered if the woman is carrying a female fetus.

Unexpectedly, in the first 5 months of life there were additional deaths in the sheep that were born preterm; during this time, the overall survival rate dropped further to 56% for preterm male sheep and 58% for preterm female sheep. As mentioned previously in Section 3.3.1 (page 75-76), this was due to severe muscle wasting as a result of white muscle disease with pathology tests reporting low levels of Vitamin E and ionophore toxicity. White muscle disease is a degenerative muscle disease which is usually caused by deficiencies in Vitamin E and/or selenium (Oldfield et al., 1960, Hogue et al., 1962). Additionally, ionophore toxicity can contribute to muscle weakness by inhibiting sodium and potassium ion transport across cell membranes resulting in mitochondrial failure and decreased ATP activity; and this was thought to be attributed to the lambs eating ewe-specific pellets given to their mothers. The problem was first identified when lambs were observed walking stiffly and in obvious pain when walking; eventually they were not able to stand. After symptoms appeared, all lambs were treated with Vitamin E and given lamb-specific pellets to provide nutrients in their diet

and avoid toxicity from ewe-specific pellets. Interestingly, the development of white muscle disease was only observed in lambs that were born preterm thus suggesting that preterm lambs were more vulnerable to developing this disease; likely due to the immaturity of muscle tissues and the gastrointestinal tract. Further studies would be required to elucidate this. At the termination of the experiment, we have collected and fixed pieces of muscle (semitendinosus, vastus and soleus) from the hind limbs of all surviving lambs so this can be tested in a future student project.

My findings of an increased vulnerability to white muscle disease in preterm lambs supports the many studies in preterm human subjects with prematurity linked to the aetiology of many adverse health problems throughout the lives of those born preterm (Institute of Medicine (US) Committee on Understanding Premature Birth and Assuring Healthy Outcomes, 2007); the earlier an infant is born, the increased risk of developing adverse complications. Indeed, some of these problems may not even emerge for several years until childhood or even adulthood. For example, preterm birth has been linked to gastrointestinal problems such as necrotising enterocolitis (Neu, 2007, Ng, 2009, Sangild et al., 2013), infections such as pneumonia, sepsis and meningitis (Stoll et al., 2004), vision complications (Repka, 2002, Madan et al., 2005), hearing loss (Hintz et al., 2005, Vohr et al., 2005) and dental problems (Seow, 1997, Seow et al., 2005). Notably, the risk of developing these complications decreases with increasing gestational age and sometimes may be exacerbated by the treatments to the conditions following preterm birth (Committee on Understanding Premature Birth Assuring Healthy Outcomes, 2007).

3.4.3 Lifelong growth attenuated following preterm birth

Our findings show that preterm lambs were generally lighter and smaller in stature throughout life compared to term lambs, but body composition was not affected. At the time of necropsy, however, the difference in body weight between term and preterm sheep did not quite reach statistical significance (P_G=0.076) when the body weight data was only compared at that time point. This indicates that the difference in body weight between preterm and term lambs was much less at 14.5 months of age compared to birth (7.65% compared to 40.75%, respectively). Hence by adulthood, there was clearly evidence of catch up in body weight but not for body dimensions. However, it is important to note that preterm males did not fully catch up in body weight as they remained attenuated at 14.5 months of age

(P=0.065); whereas there was no difference in the body weight of preterm females compared to term females at 14.5 months of age (P=0.464).

As previously observed in Chapter 2, body growth of the preterm lambs in the immediate period following birth was different to that in human infants. Preterm lambs gained weight from the time of birth, albeit at a slower growth rate than the term lambs from birth until weaning. Overall, however, the long-term postnatal growth trajectory of our preterm sheep was comparable to that of humans born preterm. In this regard, it is well described in the literature that there is poor postnatal growth and slower growth rates in individuals that were born preterm (Dusick et al., 2003, Roggero et al., 2011, Leppänen et al., 2013). Preterm infants, including those born moderately preterm, generally have a marked reduction in weight during the first weeks of life compared to term infants (Niklasson et al., 2003); low weight gain is apparent from the start of life and becomes more prominent with higher degrees of prematurity (Clark et al., 2003). Additionally, preterm babies are significantly shorter at birth and there is attenuated growth during the first few years of life in relation to both weight and height. Throughout childhood and into late adolescence, preterm subjects remain lighter and shorter, however, differences in weight are reported to decrease over time with some catch up growth in body weight but height differences persist (Batista et al., 2012, Roberts et al., 2013).

Interestingly in my studies, the preterm sheep showed no differences in body composition compared to term sheep in young adulthood. These findings are in contrast to human data of preterm subjects. Preterm babies are born with relatively low fat stores (Rigo et al., 1998), but as they grow they develop more visceral fat than peripheral fat (Uthaya et al., 2005). By one year corrected gestational age, preterm infants are reported to have increased fat mass and lower lean mass (Cooke et al., 1999), with fat mass in female preterm infants greater than preterm male infants (Pieltain et al., 2001). In adulthood, increases in fat mass and altered fat distribution remain in preterm subjects as indicated by DEXA scan analyses (Mathai et al., 2013). It is interesting that in my sheep studies, the preterm sheep did not have an increase in fat distribution compared to term sheep in early adulthood. Given that an increase in fat deposition predisposes to metabolic syndrome, the apparent difference in fat deposition following preterm birth between species is an important finding which should be investigated in future studies.

The only significant difference observed in body composition was between sexes, with female sheep exhibiting a higher fat mass but lower lean mass compared to male sheep. The differences in body composition between the sexes are also a common finding in human studies. It is well established in the literature that women generally have a higher amount of body fat mass but lower amount of lean body mass compared to men, with females having a high proportion of body fat in the upper leg region but males having more abdominal fat in adulthood (Lemieux et al., 1993, Blaak, 2001, Nindl et al., 2002, Wells, 2007, Kirchengast, 2010). Interestingly, it has been reported that newborn girls have significantly higher relative fat mass and lower lean body mass compared to newborn boys, but by 6 months of age, these differences appeared to diminish (Fields et al., 2009). By puberty and adolescence, sexual dimorphism in body composition is apparent again, and ultimately most evident in adulthood (Wells, 2007, Kirchengast, 2010). Hormonal factors have been implicated in the difference in body composition; however, the exact mechanisms behind these sex differences in body fat distribution patterns are largely unknown.

3.4.4 Growth relative to post-term equivalent age (PTEA)

Given that body weight and the morphometric parameters were measured weekly in the first 12 weeks of life, this provided the opportunity to assess growth relative to TEA. We asked the question: is growth of preterm lambs at TEA the same as for term lambs? Our findings have shown that preterm lambs at TEA were significantly heavier and had significantly longer CrL than lambs born at term. This demonstrates that preterm lambs had greater growth *ex utero* compared to that of term lambs *in utero* during the period equivalent to the last two weeks of gestation. This is likely due to the differences in nutrition *in utero* versus *ex utero*. During the two weeks *ex utero*, preterm lambs were bottle-fed milk expressed from the ewes at set intervals for the first two days of life; once strong enough the lambs suckled from the ewes *ad libitum* until weaning at 12 weeks of age. Indeed, given the difference in body growth compared to that *in utero*, it is conceivable that the preterm lambs may have been overfed during the bottle-feeding period. This situation is similar to that of human infants where carers may unintentionally feed preterm babies more than necessary. Alternatively, it may be due to the preterm lambs developing greater muscle mass simply by walking/running around *ex utero* as opposed to the minimal movement during the extra 2 weeks *in utero*.

In contrast, it is important to note that in very preterm infants, body weight gain *ex utero* is actually less than *in utero* during the same gestational age period (Hay, 2008). Furthermore, preterm infants (born between 30 and 33 weeks of gestation) have been found to be significantly lighter compared to term counterparts at term-equivalent age, even though catch up growth was evident. This is likely to be due to the immaturity of the gastrointestinal tract which limits the absorption of necessary nutrients in the very preterm infant (Sangild, 2006). Interestingly, our moderately preterm lambs appear to have a sufficiently mature gastrointestinal system allowing for the greater rates of growth after birth.

By 10 weeks post term-equivalent age, preterm lambs had similar body weights to term lambs, however, CrL, TG and HLL remained significantly reduced from TEA until 10 weeks post term-equivalent age compared to term lambs. This indicates that the *ex utero* growth rates of term lambs relating to body stature are greater than the *ex utero* growth rates of preterm lambs.

3.4.5 Arterial pressure was not elevated in adulthood following preterm birth

In my studies, there was an overall reduction in arterial pressure in preterm sheep compared to term sheep over the experimental period; however, by early adulthood, arterial pressure was similar between groups. There were no apparent differences in arterial pressure at 14 months of age when measured via an indwelling catheter.

These findings are in contrast to those reported in humans where there are now many studies reporting a significant elevation in arterial pressure in children, adolescents and adults born preterm (Johansson et al., 2005, Doyle, 2008, Keijzer-Veen et al., 2010, Kerkhof et al., 2012, Sutherland et al., 2014). The discrepancy in findings between our studies and those in humans may relate to the degree of prematurity. In my studies, the preterm lambs were delivered at 132 ± 1 days of gestation, which is 0.9 of term and equivalent to 32 - 34 weeks of gestation in humans. Given the findings in humans, it is conceivable that if the preterm lambs were born earlier (that is, greater severity of prematurity) that an elevation in arterial pressure may have developed. Indeed, the observed increases in arterial pressure in human subjects born preterm is dependent on the age of delivery at birth; with increased severity of preterm birth associated with increasing levels of arterial pressure (Siewert-Delle and Ljungman, 1998, Lawlor et al., 2007, Cooper et al., 2009). For example, Johansson et al. (2005) showed in young adults that there was a 0.31 mmHg increase in systolic blood pressure for every week born

less than 37 completed weeks in of gestation. Hence, given that the lambs were born moderately preterm, it is conceivable that hypertension may never develop or alternatively may not manifest until much later in life. In future studies, it would be interesting to monitor arterial pressure in moderately preterm sheep through to old age.

Interestingly and in contrast to the findings in my studies, many studies have demonstrated an association between infants exposed to antenatal corticosteroids and the development of higher arterial pressures after birth (Demarini et al., 1999, Doyle et al., 2000, Smith et al., 2000, Seckl and Holmes, 2007, Gwathmey et al., 2011). In my study, only ewes delivering preterm were administered betamethasone, but we did not find any evidence of significantly elevated arterial pressure in the preterm group after birth. To further investigate the effects of antenatal corticosteroids, it would be beneficial to study another cohort of term lambs that were also exposed to the same dose of betamethasone and another cohort of preterm lambs that were not exposed to betamethasone (however these preterm lambs may not be viable).

3.4.6 Heart rate initially lower following preterm birth but normalises postnatally

In my studies, heart rate was significantly lower in the preterm lambs compared to term lambs at birth, but after birth through to early adulthood, heart rate was similar between preterm and term sheep. Fluctuations in heart rate over the study period, however, were significantly different between preterm and term sheep.

In general, heart rate is considered to be a significant clinical indicator of the health of an infant after birth (Apgar, 1953); with increases after birth believed to be a valuable guide of effective resuscitation and indicative of the newborn's ability to transition from *in utero* to *ex utero* life (Wyllie, 2006). It has been reported that heart rate is lower in preterm babies immediately after birth and that it rises more slowly in preterm neonates, taking longer to reach a heart rate of 100 beats per minute (considered normal), than babies born at term (Dawson et al., 2010). However, once preterm neonates are haemodynamically stabilised, it is reported that heart rate becomes elevated compared to term babies and this is linked to immaturity of the autonomic nervous system (Eiselt et al., 1993, Patural et al., 2008, Sweeney and Blackburn, 2013). Indeed, the autonomic nervous system plays a key role in the regulation of both heart rate and arterial pressure and it is made up of two components: the parasympathetic nervous system and the sympathetic nervous system. The parasympathetic

component is responsible for reducing heart rate and arterial pressure, whilst the sympathetic component is responsible for increasing heart rate and arterial pressure (Saladin, 2012). It is known that as a fetus advances to birth, the autonomic nervous system increases its activity and complexity. The sympathetic nervous system develops quickly in the first trimester but slowly thereafter and often matures within the first year of life, however, the parasympathetic nervous system becomes more dominant around 25 to 30 weeks of gestation (Walker et al., 1978, Gagnon et al., 1987, Wakai, 2004). Overall, significant maturation of the autonomic nervous system occurs between 31 and 38 weeks of gestation, when heart rate decreases and heart rate variability (fluctuation in time between heart beats) increases due to increasing parasympathetic influence (Sahni et al., 2000). Therefore, being born early, when the maturation of the parasympathetic nervous system is still ongoing, can directly impact heart rate in the neonatal period. For example, Patural et al. (2004) has reported that infants born preterm (born between 25 and 37 weeks of gestation) have lower parasympathetic activity at TEA compared to those born at full term, and the greater the prematurity the lower the parasympathetic activity. Similarly, Verklan and Padhye (2004) observed that preterm infants had less heart rate variability compared to term infants. This indicated that preterm birth may prevent maturation of the parasympathetic component therefore suggesting immaturity of the autonomic nervous system in preterm infants.

Importantly, there have been a number of studies suggesting that the increases in heart rate that develop postnatally in preterm infants are the result of their immature autonomic nervous system at the time of birth (Fyfe et al., 2014). For example, it has been reported that preterm infants at TEA have higher resting heart rates which persist until 7 months of age compared to those born at term (Katona et al., 1980, Eiselt et al., 1993). It has been proposed that this impaired autonomic control in preterm infants might contribute to cardiovascular complications in later life.

3.4.7 Conclusion

In conclusion, this study shows sexual dimorphism in body growth in preterm sheep; in females there was a catch up in body weight by adulthood, whereas there was persistent attenuation of body weight in males. All preterm sheep remained smaller in stature throughout the study period. Overall, there was no evidence of hypertension in adulthood as a result of moderate preterm birth.

Chapter 4:

The Effect of Moderate Preterm Birth on Heart and Cardiomyocyte Growth in the Immediate Period after Birth and in Early Adulthood

4.1 Introduction

Over recent decades, there has been substantial improvement to the survival rates of infants born preterm. With the advancements in medical technology and knowledge, there is increased survival of babies born as early as 22 weeks (Rysavy et al., 2015) and current statistics show that neonates born at 25 weeks (extremely preterm) have an 80% survival rate (Kutz et al., 2009, Lorenz, 2011).

Even so, infants born preterm continue to exhibit a higher risk of death and disability in the neonatal period (Christie, 2000, Lumley, 2003) and autopsies in neonates indicate that the majority of deaths after preterm birth are due to challenges in the respiratory, immune and cardiovascular systems (Barton et al., 1999, Elder and Zuccollo, 2005). In addition, survival past infancy does not guarantee that there will be no long-term health problems in subjects born preterm; it is common for neurological deficits and also complications in other organ systems to develop over time (Stoll et al., 2004, Vohr et al., 2005).

To date, the majority of research has focused on the effect of preterm birth on an immature respiratory system, and the findings have been crucial for the development of strategies to facilitate survival in the neonatal period. However, to date the effects of preterm birth on other organ systems is not as well studied. The focus of this study is to investigate the effect of moderate preterm birth on the immature cardiovascular system and in particularly, this chapter focusses on the structure of the myocardium and cardiomyocyte growth.

The heart is one of the first organs in vertebrates to form, thus allowing circulation of blood to the developing organs of the embryo/fetus in order to provide oxygen and nutrients for growth. Cardiomyocytes are the functional units of the heart. During early gestation, heart growth is achieved by hyperplasia (proliferation of cardiomyocytes), whereas in late gestation, cardiomyocytes undergo a process of maturation whereby they become terminally differentiated (Huttenbach et al., 2001, Burrell et al., 2003, Bubb et al., 2007). After birth, there is a reduced capacity for cardiomyocytes to proliferative (Mollova et al., 2013), with postnatal cardiac growth mainly due to hypertrophy of cardiomyocytes and extracellular matrix deposition. Sheep make an excellent model to study heart growth, as the timeline of cardiomyocyte maturation in the developing heart is very similar to the human (Huttenbach et al., 2001). In sheep, mature cardiomyocytes are easily identified as they are binucleated, whereas, immature cardiomyocytes are mononucleated (Burrell et al., 2003).

It is now well established that adaptations within organ systems, in response to perturbations in growth, during early life can lead to the programming of diseases in adulthood (Barker, 1995, Barker, 2007). Perturbations and interruptions in growth during critical times of development *in utero* have clearly been shown to lead to adverse effects on the growth and maturation of cardiomyocytes, which may in turn, be linked to long-term vulnerability to cardiovascular disease (Corstius et al., 2005, Bensley et al., 2010). In addition to this, recent experimental studies show maladaptive modifications of the myocardium in response to preterm birth, which are likely to have long-term consequences. We content that the heart is likely to be particularly vulnerable given the major haemodynamic transition at birth and the relative immaturity of the myocardium at the time; and decreasing gestational age at birth is likely to exacerbate the vulnerability.

The haemodynamic transition at birth is the change from fetal to postnatal circulatory configuration, when a baby takes its first breath (Cuneo, 2013, Johnson et al., 2014). It involves the loss of the placenta, closure of shunts (foramen ovale, ductus arteriosus and ductus venosus) and alterations in cardiac output and blood flow (Blackburn, 2006). Before birth, the right ventricle is the dominant ventricle and is responsible for 66% of cardiac output, whereas after birth the left ventricle now pumps against a high pressure and high resistance while the RV pumps against a low resistance, low pressure (Rudolph, 1979). With removal of the placenta, the combination of these circulatory changes at birth and the steep changes to circulating hormone levels such as angiotensin II and catecholamines ultimately leads to an increase in heart rate and abrupt rise in systemic arterial pressure, thus increasing the load on the left ventricle (Louey et al., 2000, Hillman et al., 2012).

As the first survivors of very and extremely preterm birth are now reaching adulthood, studies are emerging demonstrating long-term alterations in the structure and function of the heart in young adults born preterm. In a study by Lewandowski et al. (2013a), significant increases in left ventricular mass were observed in adults (aged between 20-39) born preterm; with greater prematurity at birth associated with greater left ventricular mass. In addition, the overall geometry of the left ventricle was found to be different in individuals born preterm, such that they exhibited shorter ventricles, displaced apexes and smaller chamber diameters. Furthermore, left ventricular function appeared to be altered with significant reductions in systolic function (peak strain, strain rate and velocity) and diastolic function (peak strain rate and velocity) and rotational movement. In a follow up study by these investigators, it was found that the effects were more pronounced in the right ventricle, with preterm adults

exhibiting smaller right ventricles (reduced right ventricular end-diastolic volume, end-diastolic length and chamber diameter) but greater right ventricular mass compared to term born controls; again the severity of the cardiac changes were associated with decreasing gestational age at birth (Lewandowski et al., 2013b). Furthermore, the ejection fraction of the right ventricle was significantly lower in subjects born preterm illustrating impairment of right ventricular function. Hence, these clinical studies clearly demonstrate that preterm birth alters the way the heart grows after birth (Lewandowski and Leeson, 2014).

The studies in this chapter aim to further characterise the structural differences in the heart in the immediate period following moderate preterm birth (2 days of age) and in adulthood (14.5 months of age), with particular emphasis on cardiomyocyte growth. To address the aims, I have used a sheep model which avoids the many confounding variables associated with studies in humans. The specific aims were to examine RV and LV+S wall and chamber volume, RV and LV wall thickness, cardiomyocyte number and nuclearity within the LV+S and also RV and LV collagen content between preterm (female and male) and term (female and male) sheep at 2 days after birth and 14.5 months of age.

Due to time constraints, in this thesis I have only examined cardiomyocyte growth in the LV+S, as the LV is the dominant ventricle in postnatal life. The right ventricle was stored in 10% formalin for subsequent analyses; not a part of this thesis.

4.2 Methods

4.2.1 Heart collection and perfusion fixation

Hearts excised at necropsy, were weighed and placed in a container filled with physiological saline and papaverine hydrochloride (1.2 mg/ml; Sigma-Aldrich, Missouri, USA). Small cubes of tissue (approximately the area of 1 - 2 cm^2) from the free walls of the RV and LV were carefully dissected from the heart; these samples were excised from the same anatomical site in each animal. These pieces of ventricular wall were cut into smaller pieces and placed in labelled cryovials, which were immediately snap frozen in liquid nitrogen and later stored in a -80^oC freezer. The heart was then perfusion fixed via a catheter inserted into the aorta. Firstly, the heart was perfused with saline to clear the coronary vasculature. Papaverine hydrochloride (1.2 mg/ml; Sigma-Aldrich, Missouri, USA) was then administered via the catheter to dilate blood vessels and lastly 0.5 M potassium chloride (0.1 ml) (Merck, Germany) was administered to arrest the heart in diastole; saline was administered after both to ensure that they were flushed throughout the coronary vasculature. After blood was cleared from the coronary arteries, the heart was perfused with 4% paraformaldehyde at a perfusion pressure of approximately 60 mmHg in the 2 day old lambs (average mean arterial pressure recorded prior to necropsy) and 90 mmHg in the adult sheep (average mean arterial pressure measured during cardiovascular experiments) until the tissue appeared stiffer and lighter in colour. Once fixation was complete, hearts were stored in a container with 10% formalin until sampling (see later – Section 4.2.2).

4.2.2 Ventricular sampling (Figure 4.1)

Prior to sampling, the great vessels (aorta, pulmonary arteries and veins and vena cava) and connective tissues were dissected from the heart. The atria were also excised (Figure 4.1A-4.1C). The great vessels and atria were stored in 10% formalin for possible future examination by researchers in our laboratory group. Using a large knife, transverse slices approximately 10 mm thick for lambs hearts (short-term studies) and 17 mm thick for the adult hearts (long-term studies), were cut across the ventricles commencing at the plane of the atrioventricular valves (Figure 4.1D-4.1G). Using these slices, the volume of the ventricular walls and chambers were estimated using the Cavalieri Principle (Gundersen et al., 1999) and the ventricular wall thickness was measured (see later – Section 4.2.3).

In the transverse slices of the ventricles, the RV free wall was dissected away from the septum (keeping the septum intact with the LV) and the RV tissue was stored in 10% formalin (Figure 4.1H). The slices of the left ventricle plus septum (LV+S) were cut into smaller pieces (usually 3 - 4 pieces per slice in the lamb hearts and 12 - 14 pieces per slice for the adult hearts) and laid out in order of size from largest to smallest; 8 - 10 pieces were then sampled using a smooth fractionator approach (Figure 4.1I and 4.1J). This process involves choosing the first piece at a random point (between 1 and n) and then selecting every n^{th} piece from there to give a total number of 8 - 10 pieces per LV+S. Every sampling fraction was carefully recorded as the n number was sometimes different between animals.

If these selected tissue pieces were too large for direct embedding into glycolmethacrylate or paraffin (particularly for the larger animals of the long-term cohort), the selected pieces from the first sample were then further sliced at 2 mm thickness each using a custom made razor blade slicing device and again, using a smooth fractionator approach (as described previously), 8 - 10 pieces were sampled. These samples of the LV+S were subsequently processed and embedded in glycolmethacrylate. Every adjacent piece was processed and embedded in paraffin wax (also 8 - 10 pieces per LV+S).

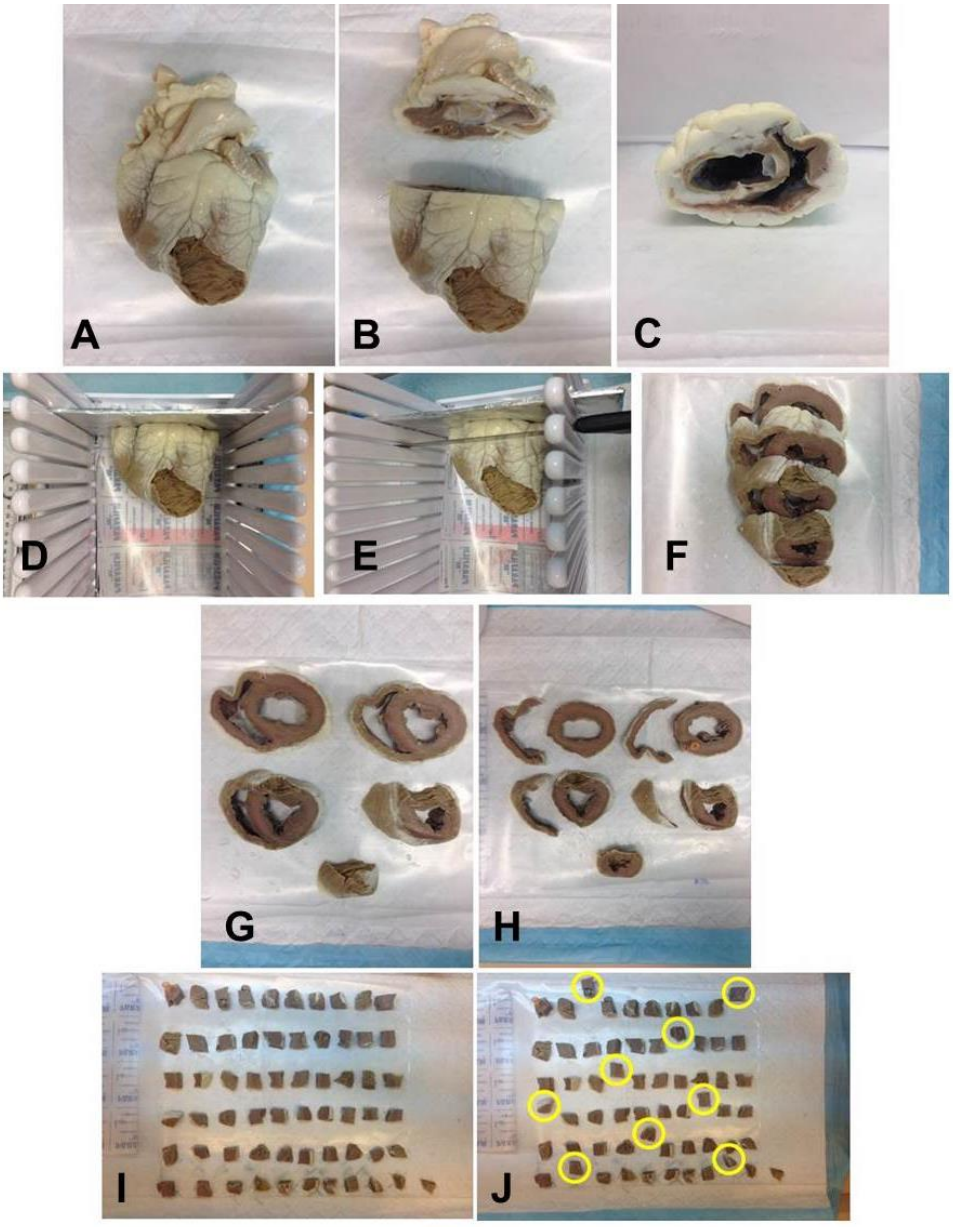

Figure 4.1. *Sampling method of hearts showing whole heart with great vessels attached (A), removal of the great vessels and atria (B), top of the ventricles (C), ventricles in preparation of being sliced (D), long knife used to cut transverse slices of the ventricles (E), transverse slices of the ventricles (F), ventricular slices laid out from top to bottom (apex) (G), right ventricle free wall separated from left ventricle plus septum (H), cut pieces of left ventricle plus septum ordered from largest to smallest (I) and 8-10 pieces of left ventricle plus septum sampled (circled in yellow) using the smooth fractionator approach (J).*

4.2.3 Measurement of wall volume, chamber volume and wall thickness

As mentioned previously, with the initial transverse slices (when both ventricles are still attached), measurements of ventricular wall volume, chamber volume and wall thickness was conducted.

For estimation of ventricular wall and chamber volume, the Cavalieri principle (Gundersen et al., 1999) was utilised. This involved superimposing an orthogonal grid onto every slice and counting the number of intersecting points which crossed the tissue or the open chamber area as seen in Figure 4.2. The total number of intersecting points for the RV free wall, LV+S and their respective chambers were added up from every slice. Total volume was estimated using this formula:

$$V = P \times T \times a$$

where V is the volume (in mm^3); P is the total number of grid points overlying heart tissue or chamber space; T is the thickness of tissue (10 mm for lamb hearts and 17 mm for adult hearts) and a is the area of the grid used (6.25 mm^2 for lamb hearts and 25 mm^2 for adult hearts).

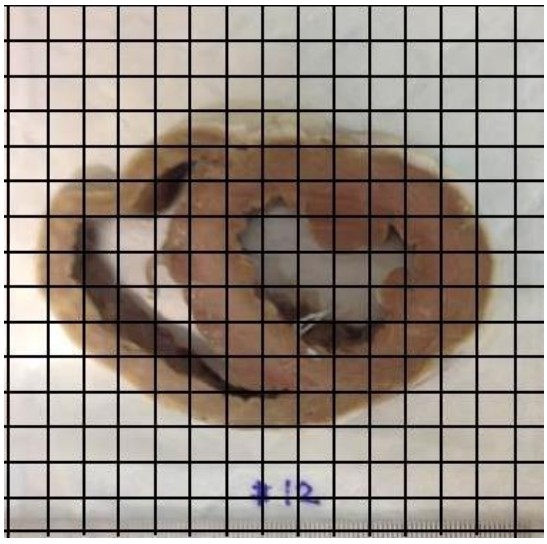

Figure 4.2. *A transverse ventricular slice with an orthogonal grid superimposed to determine ventricular wall and chamber volume.*

For measuring the ventricular and septum wall thickness, a digital photograph was taken of the base of the second transverse slice with a ruler placed below and another adjacent to the slice as shown in Figure 4.3. This photograph was digitally uploaded and examined using Image Pro-Plus Version 6.2 (Media Cybernetics, Bethesda, Maryland, United States). Calibration was performed for each image of the two rulers and the average calibration value was utilised. Using the image analysis software, ventricular and septum walls were traced manually and the average wall thickness measured.

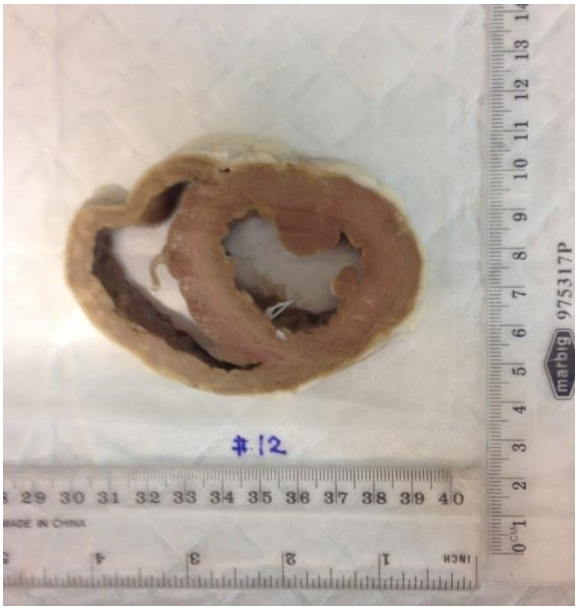

Figure 4.3. *The base of the second transverse ventricular slice with a ruler placed below and adjacent was photographed and ventricular wall thickness measured in the digital image.*

4.2.4 Processing, embedding, sectioning and staining

4.2.4.1 Glycolmethacrylate

The processing and embedding of tissues in glycolmethacrylate were performed with the help of Monash Histology staff. To process tissues in glycolmethacrylate, sampled pieces of the LV+S were initially placed in 70% EtOH overnight. The following day they were dehydrated through fresh 70% EtOH (1 x 1 hour), 100% EtOH (3 x 1 hour), 100% butanol (1 x 1 hour) then overnight in 100% butanol whilst gently shaken. The tissues were then infiltrated in the 'infiltration solution' overnight.

This 'infiltration solution' was prepared using a Technovit 7100 kit (Heraeus Kulzer, Wehrheim, Germany), which involves adding 1 g of Hardener I in 100 ml of Technovit 7100 solution and stirring with a magnetic stirrer for 30 minutes until dissolved fully. After infiltration, the LV+S pieces were placed in plastic embedding moulds with the face to be cut facing the bottom of the mould. The moulds were then filled with the 'embedding solution'.

The embedding medium was prepared by adding 1 ml of Hardener II to 15 ml of the above 'infiltration solution' and mixing it thoroughly with a wooden stick. Labels were added to the moulds approximately 20 minutes after filling as the embedding medium began to harden. The embedding solution was left to set at room temperature overnight or until set. Once the glycolmethacrylate embedding medium had hardened, the glycolmethacrylate blocks had backing blocks adhered to them.

These backing blocks were made from Technovit 3040 resin using a 2:1 ratio of powder to liquid. Backing blocks were attached by placing a couple of drops of this solution onto the back of the glycolmethacrylate block and quickly placing the backing block and allowing it to set.

Using a Leica 2165 microtome (Leica Systems, Nusloch, Germany), the glycolmethacrylate blocks were sectioned at 20 µm thickness, with every 40^{th} section collected, beginning with a random number between 1 and 40. This type of microtome uses a glass knife and an automated machine attached to count the number of sections taken. Once cut, sections were placed in a water bath for ease of picking up the sections and mounted carefully onto glass slides. The slides were then placed onto a hot plate to dry the section and ensure adherence to the slide.

All sections were stained with Harris's Haematoxylin (Amber Scientific, Queensland, Australia). This protocol involved placing the slides into a slide holder and submerging in filtered haematoxylin in a plastic microwaveable container. After covering with a lid, the container was put in the microwave (Samsung M945 (1000W output), South Korea) and heated for 4 minutes at 50% power. If the solution started bubbling, the microwave was paused for 30 seconds and then heating was resumed. The slides were allowed to stand in the heated haematoxylin for 2 - 3 minutes before rinsing the slides in a container of lukewarm tap water to wash away excess stain for 5 minutes. Each slide was carefully inspected to ensure no bubbles of haematoxylin were trapped under the sections. If so, these were gently removed. The slides were left to air dry overnight or alternatively in a 40^{o}C oven for at least 2 hours. The

haematoxylin was reused in the same staining session for subsequent slides. For staining subsequent slides, as the haematoxylin was already heated, the time in the microwave was decreased by 30 - 60 seconds; for example, the second slide holder to be stained was heated in the microwave for 3 minutes and 30 seconds and the subsequent slide holder 3 minutes. Sections were continuously monitored to see how the staining was progressing. After staining 4 - 5 slide holders, a fresh solution of filtered haematoxylin was prepared. Once slides dried completely, they were coverslipped with glass coverslips using DPX (Di-n-butyl phthalate in Xylene) as the mounting media.

4.2.4.2 Paraffin

Processing of the tissue for paraffin embedding was performed by an automatic processing machine (Leica Peloris II, Leica, New South Wales, Australia) located at Monash Histology. An automated processing protocol was chosen for the heart tissue samples, as described in the Table 4.1.

Step	Reagent	Step time (minutes)	Temperature (^{O}C)
1	10% buffered formalin	10	45
2	80% Ethanol	1	Ambient
3	Ethanol	1	Ambient
4	Ethanol	1	Ambient
5	Ethanol	20	45
6	Ethanol	20	45
7	Ethanol	45	45
8	Xylene	1	Ambient
9	Xylene	10	45
10	Xylene	45	45
11	Wax	10	65
12	Wax	10	65
13	Wax	40	65

Table 4.1. *Automated processing protocol performed on the samples of the left ventricle plus septum.*

Once processed, tissues were embedded in paraffin wax. To do this, processed tissues were placed flat on the bottom of the mould with the face to cut facing downwards. The mould was filled with paraffin wax, with a processing cassette placed onto the mould as the backing block and topped with wax to keep it in place. This mould was then moved to a cold plate so that the paraffin could solidify and when hardened the moulds were removed.

Sectioning was performed on a microtome. Initially, several sections at 4 µm were cut from each block and stained with Haematoxylin and Eosin. This was conducted in order to check

that the cardiomyocytes within the tissue sections were in the required orientation for measurement of nuclearity. Cardiomyocytes orientated in the long axis allowed for analysis of cardiomyocyte nuclearity. If necessary, the tissues were re-embedded at a 90 degree angle from the original plane to get the correct tissue orientation.

Once orientation was appropriate, blocks were sectioned at 3 µm thickness, with 2-3 sections collected (to ensure 1 good section), and mounted onto SuperFrost Plus slides. Sections were dewaxed with 3 changes of xylene at 10 minutes, 3 changes of EtOH at 10 minutes each and then washed in running water for 20 minutes. Slides were dipped in 70% EtOH with 1% ammonia for 10 minutes and then re-washed for a further 20 minutes with running water. Antigen retrieval was performed on the slides at 98^{o}C, for 1 hour, in Dako Target Retrieval solution (Dako, Denmark) then afterwards allowed to cool to room temperature. Dako Peroxidase Blocking Reagent (Dako, Denmark) was applied onto the slides for 10 minutes at room temperature. For the staining of the cell boundaries, anti-Laminin antibody at 1:40 (Raised in Rabbit, ab11575, Abcam, USA) was applied for 1 hour, then the secondary antibody, Dako anti-Rabbit EnVision+ FLEX (Dako, Denmark) was applied for 30 minutes. Following, wheat germ agglutinin-horseradish peroxidase (WGA-HRP) was applied at 1:20, diluted in Hank's Balanced Salt Solution (HBSS) for 45 minutes. Afterwards, diaminobenzidine (DAB, Dako DAB system, Denmark) was applied for 10 minutes. Lastly, for the staining of the nuclei, Harris' Haematoxylin (Amber Scientific, Queensland, Australia) was applied for 10 minutes, blued in Scott's tap water, dehydrated, cleared in xylene and coverslipped with DPX. This immunostaining protocol was performed on the Dako Autostainer Plus (DakoCytomation, Fort Collins, Colorado, USA) and Dako Automation Wash Buffer was the wash buffer used in between stains.

4.2.5 Estimating cardiomyocyte number - optical disector/smooth fractionator approach

Total cardiomyocyte number within the LV+S was estimated using an optical disector/fractionator approach (Bruel and Nyengaard, 2005) in haematoxylin-stained glycolmethacrylate sections. This involved the use of an Olympus BX51 microscope (Olympus, Tokyo, Japan), fitted with a 1.25x Olympus ApoE lens and a 100x Olympus ApoE oil immersion lens. The microscope was combined with a motorised stage which was controlled by a joystick

for movement in the *x* and *y* directions. The stage was also fitted with a *z* axis sensor which enabled the depth of focus to be measured.

C.A.S.T (Computer Aided Stereological Toolbox) software (Olympus, Denmark) was used to count the number of nuclei. Every time this software was launched, a calibration of the stage was performed automatically. Using the lowest magnification (1.25x), the outline of the tissue was traced using the C.A.S.T define feature tool to ensure only that area was to be scanned by the microscope. Then at 100x magnification, an unbiased counting frame (area of 544.5 μm^2) was superimposed over the field of view. Tissue sections were systematically sampled (uniform along the *x* and *y* axis, beginning from a random starting point with a step length of 2000 µm for lamb hearts and 1000 µm for adult hearts in both the *x* and *y* directions).

Cardiomyocyte nuclei could be easily identified as lightly stained and oval-shaped, with the presence of a dense nucleoli and chromatin. Nuclei were only counted if they came into focus within a 10 µm range in the middle of the 20 µm section (in the *z* plane). Only the middle 10 µm was counted to ensure that uneven cutting surfaces of the section were excluded from counting. An unbiased counting frame was used; as seen in Figure 4.4, cardiomyocyte nuclei were counted only if they came into focus within the counting frame but not crossing the forbidden lines (two lines in red of the counting frame were forbidden, the other two green lines were inclusion lines).

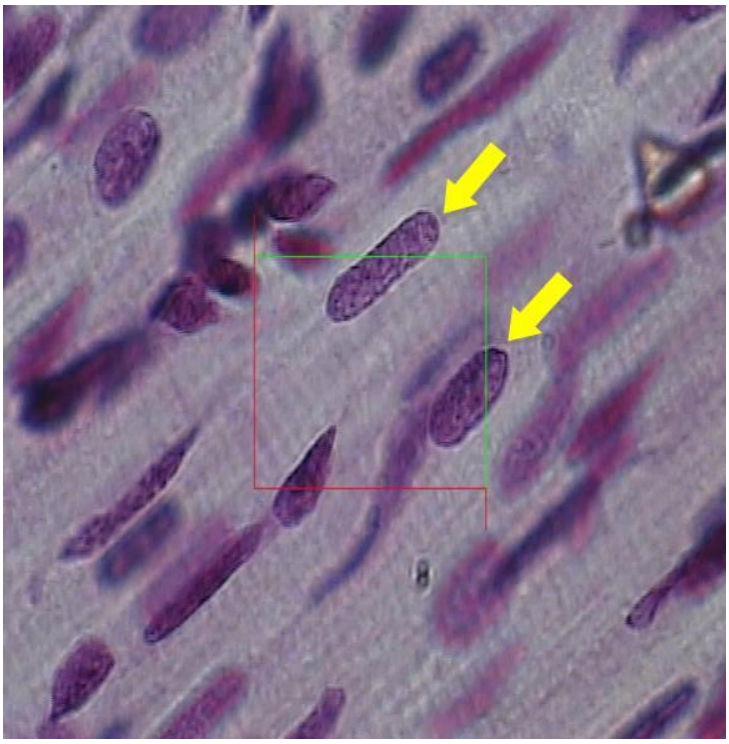

Figure 4.4. *Counting frame is superimposed over the field of view. The red lines are forbidden lines, whereas the green lines are the inclusion lines. Therefore, cardiomyocyte were only counted if whole or part of the nucleus was in the counting frame and not touching the red forbidden lines. In this image, both the nuclei marked with yellow arrows would be counted.*

The number of cardiomyocytes in the LV+S was subsequently calculated according to the formula:

$$\text{Cardiomyocyte number} = Q^{-} \times \frac{1}{F1} \times \frac{1}{F2} \times \frac{1}{F3} \times \frac{1}{F4} \times \frac{1}{F5}$$

where, Q^{-} is the number of nuclei counted using the optical disector/fractionator approach; $F1$ is the first sampling fraction using the smooth fractionator; $F2$ is the second sampling fraction also using the smooth fractionator (if further cut with the custom made razor blade device); $F3$ is the sampling fraction selected from the glycolmethacrylate sections (40, since every 40th slide was selected for counting analysis); $F4$ is the field of view on the section that is examined, calculated by multiplying the step length (1000 µm or 2000 µm) in the *x* and *y* axis by itself and dividing it by the counting frame (which was found on the C.A.S.T program;

area of 544.5 μm^2) and lastly $F5$ accounts for the fraction within the section where nuclei were counted (only the middle 10 μm of the section was used out of 20 μm therefore $F5$ equals 2).

In the haematoxylin-stained sections, only nuclei were visible and cell boundaries were not visible. Hence, in order to determine the total number of cardiomyocytes in the LV+S, adjustments were required based on the proportion of mono-, bi-, tri- and tetra- nucleated cardiomyocytes (see next section – Section 4.2.6).

4.2.6 Estimating cardiomyocyte nuclearity – image analysis

To determine cardiomyocyte nuclearity (proportion of mono-, bi-, tri- and tetra-nucleated cells) in the LV+S, image analysis was utilised.

In tissue sections stained with anti-Laminin antibody for cell boundaries and Harris' Haematoxylin for cell nuclei (see previous section – Section 4.2.4.2, page 114), cardiomyocytes were visualised using Image Pro-Plus Version 6.2 software (Media Cybernetics, Bethesda, Maryland, USA). Images of cardiomyocytes were captured in the long axis for analysis.

The number of nuclei per cell was counted within approximately 150 to 200 cardiomyocytes per animal. Nuclei were only counted within cardiomyocytes which had a fully intact and visible cell membrane and the nuclei were centrally located. Figure 4.5 illustrates the representations of mononucleated (Figure 4.5A), binucleated (Figure 4.5B), trinucleated (Figure 4.5C) and tetranucleated (Figure 4.5D) cardiomyocytes.

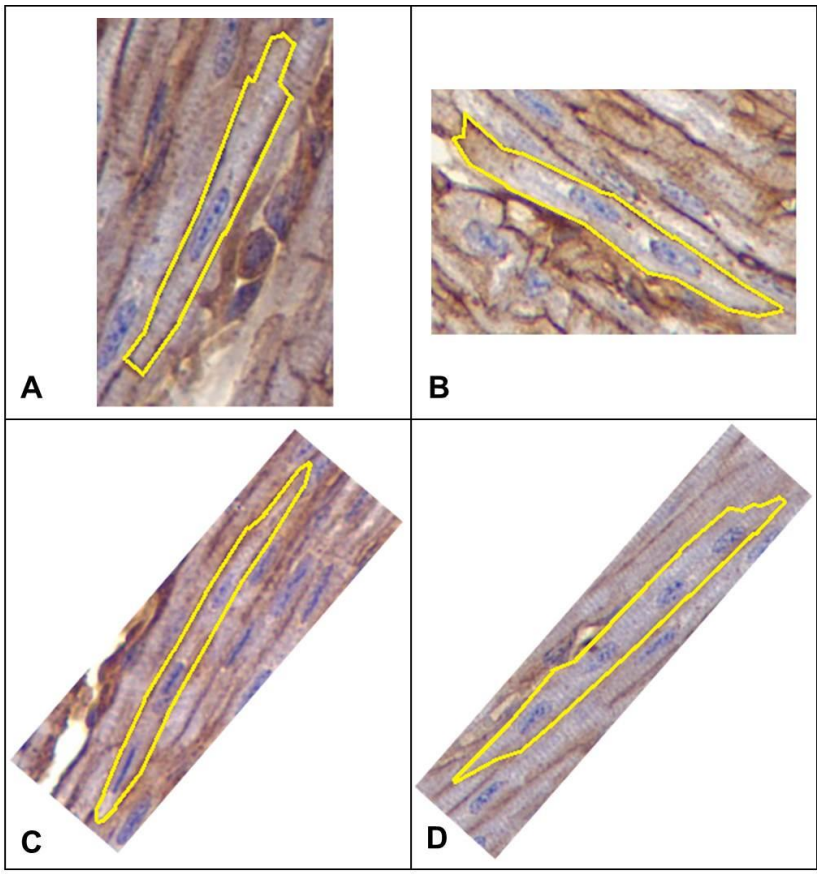

Figure 4.5. *Representative images of mononucleated (A), binucleated (B), trinucleated (C) and tetranucleated (D) cardiomyocytes in a LV+S section stained with anti-Laminin antibody (staining cell boundaries brown) and Harris' Haematoxylin (staining nuclei dark blue/purple). Cell boundaries are outlined in yellow.*

After assessing the percentage of mono-, bi-, tri- and tetra-nucleated cardiomyocytes present within the LV+S, the total number of cardiomyocytes was calculated by correcting for nucleation. Total number of nuclei was divided by the average number of nuclei for each cardiomyocyte per animal to give the total number of cardiomyocytes within the LV+S. This was only performed on hearts of the 2 day old lambs, as it is expected that by 14.5 months of age, all cardiomyocytes are mature and therefore binucleated.

4.2.7 Myocardial collagen content - hydroxyproline assay

In collaboration with A/Prof. Chrishan Samuel from the Department of Pharmacology at Monash University, the total collagen content (and therefore collagen concentration) was estimated in the RV and LV of the hearts of both short-term lambs and long-term sheep using a colorimetric-based hydroxyproline assay as previously described (Samuel, 2009). This assay determines the total amount of collagen by measuring the hydroxyproline content in the tissues, since collagen is one of the few proteins that contain the amino acid hydroxyproline.

Snap frozen tissues (collected at necropsy) were transferred into individual labelled Eppendorf tubes with holes pierced through the lid. The tissue was freeze-dried overnight in a lyophiliser down to dry weight and the dry weights recorded. These dried tissues were rehydrated by adding citrate buffer for approximately four hours in a cool room (4^{o}C). The rehydrated tissue was transferred to labelled Kimax^{TM} screw-capped glass tubes (Kimble/Kontes, Vineland, New Jersey, USA), making sure that the tissue was lying at the bottom of the tube. Approximately 0.5 - 1 ml of 6 M hydrochloric acid (HCl) was added to the tubes ensuring that the tissue was completely submerged. Tubes were tightly capped and placed in a heating block to hydrolyse tissue at 110^{o}C overnight. Once hydrolysed, the tubes were cooled at 4^{o}C before adding distilled water (or a strong base to neutralize the acid) and freeze-dried again in the lyophiliser overnight to evaporate all liquid. The following day, the dried tissues were dissolved in 0.1 M HCl according to the original dry weights, such that any weight less than 6 mg received 0.25 ml, between 6.001 - 12.500 mg received 0.5 ml and any higher received 1 ml. Samples were now ready for the hydroxyproline assay.

Each sample was assayed in duplicate by adding 10 µL of heart tissue hydrolyzate (in 0.1 M HCl) to 90 µL of distilled water into new glass tubes (separate glass tube per sample and replicate). In four separate tubes, labelled 0, 1, 2 and 3, aliquots of 0, 2, 4 and 6 µL of the 1 mg/ml hydroxyproline standard (in 0.1 M HCl) were added respectively. Distilled water was subsequently added to bring the volume to 100 µL. To all tubes (samples and standards), 200 µL of isopropanol was added, followed by 100 µL of oxidation buffer resulting in a total volume of 400 µL. Immediately all samples were vortexed thoroughly and left to stand at room temperature for 4 minutes. A further 1.3 ml of isopropanol was added to each tube and vortexed roughly. The tubes in the rack were covered with a large piece of foil and placed in a shaking water bath set at 60^{o}C for 25 minutes. After this, the samples were cooled at 4^{o}C for approximately 5 minutes. A further 3.3 ml of isopropanol was added to the cool tubes to give

a total volume of 5 ml per tube and again vortexed thoroughly. Once this was done, sample mixtures were transferred into disposable curvettes (Lake Charles Manufacturing, Lake Charles, Los Angeles, USA) and the absorbance of each sample was measured at 558 nm in a spectrophotometer, using the "blank" (tube labelled "0") to calibrate each assay.

From the absorbance readings, determination of hydroxyproline content and therefore collagen concentration was conducted. Using Microsoft Excel software, a standard curve was generated by plotting the absorbance levels (*y*-axis) vs. the amount of hydroxyproline standard added per tube (*x*-axis). A line of best fit, equation and R squared value was obtained. This generated equation from the standard curve in each assay was used to determine the amount of hydroxyproline in each sample. The duplicate hydroxyproline values were averaged out then multiplied by the total amount of 0.1 M HCl added to the hydrolyzates divided by 10 µL. For example if 0.5 ml was added, then multiplication is by 50 to calculate the total amount of hydroxyproline for each sample. The total amount of collagen content is then extrapolated by further multiplying the amount of hydroxyproline by 6.94 which is derived from the knowledge that hydroxyproline makes up 14.4% of the amino acid composition in most mammalian tissues (Gallop and Paz, 1975). Collagen concentration (expressed as a percentage) within the ventricular samples was then calculated by dividing the total collagen content by the dry weight of the tissues.

It should also be noted that some tissue samples were accidentally thawed after being left out of the freezer; therefore for the short-term cohort, analysis of preterm males and term males was unable to be completed. Therefore, only preterm females and term females were compared (student's t test) in the hearts of 2 day old lambs.

4.2.8 Statistical analysis

For statistical analysis, data was analysed using IBM SPSS Statistics Version 21 (IBM SPSS, Illinois, USA) and graphed using GraphPad Prism Version 6.0 (GraphPad Software, California, USA).

A student's t-test was used to analyse the hydroxyproline assay data; where by a comparison of the total collagen content within the LV and RV was compared between preterm and term female lambs at 2 days of age. Males were not analysed (see Section 4.2.7). All remaining data for the short-term cohort and long-term cohort were analysed using a two-way ANOVA, with gestational age at birth (P_G; preterm or term) and sex (P_S; female or male) as factors.

Significant interaction effects detected in the 2-way ANOVA were further analysed by a Tukey's post-hoc test. Similarly, to compare the differences between the short-term cohort and long-term cohort, a two-way ANOVA was used for each preterm group and term group separately, with time (P_T; short-term or long-term) and sex (P_G; female or male) as factors.

Data are represented as means ± SEM and statistical significance was accepted at the level of $P<0.05$.

4.3 Results

4.3.1 Immediate period after birth (2 days of age)

4.3.1.1 Body weights and absolute and relative heart weights (Table 4.2)

The body weights of preterm lambs 2 days after birth were significantly reduced (P_G<0.0001) compared to term lambs (Table 4.2 and previously shown in Figure 2.6, Chapter 2, Section 2.3.4, page 51-52). Heart weights in preterm lambs were significantly lighter (P_G<0.0001) than term lambs (Table 4.2 and previously shown in Table 2.1, Chapter 2, Section 2.3.5, page 52-53), however, when adjusted for body weight, there was no longer a difference between the groups.

There were no significant differences between the sexes in body weight, absolute heart weight or relative heart weight.

In examining the hearts after necropsy, there were no gross congenital abnormalities observed in the hearts of the preterm and term lambs at 2 days of age. Furthermore, during the slicing of the hearts and separation of ventricles, no irregularities were seen in the valves, or in the septation of the atria and ventricles.

	Preterm		**Term**		**P-values**
	Females n=7	**Males n=6**	**Females n=10**	**Males n=5**	
Body weight (kg)	4.19 ± 0.21	4.15 ± 0.23	6.34 ± 0.18	6.22 ± 0.25	**P_G<0.0001**
Absolute heart weight (g)	39.43 ± 2.40	35.86 ± 2.59	54.70 ± 2.00	53.48 ± 2.83	**P_G<0.0001**
Relative heart weight (g/kg)	9.49 ± 0.42	8.62 ± 0.45	8.63 ± 0.35	8.68 ± 0.49	NS

Table 4.2. *Body weights and absolute and relative heart weights of preterm (female and male) and term (female and male) lambs at necropsy 2 days after birth. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM. Abbreviations: NS – not significant.*

4.3.1.2 Ventricular wall volume (Figure 4.6)

Right ventricle

The RV wall volume was significantly reduced (P_G<0.0001) in preterm lambs compared to term lambs (Figure 4.6A); however, RV wall volume relative to body weight and heart weight was not significantly different between preterm and term lambs (Figure 4.6B and 4.6C).

There was no difference between females and males in absolute RV wall volume, or when adjusted for body weight or heart weight in lambs 2 days after birth.

Left ventricle plus septum

Similarly, preterm lambs also exhibited significantly reduced (P_G<0.0001) LV+S wall volume when compared to term lambs (Figure 4.6D); however when adjusted to body weight and heart weight, there was no longer a significant difference between preterm and term lambs (Figure 4.6E and 4.6F).

There was no significant difference between female and male lambs in absolute LV+S wall volume, or when adjusted for body weight or heart weight.

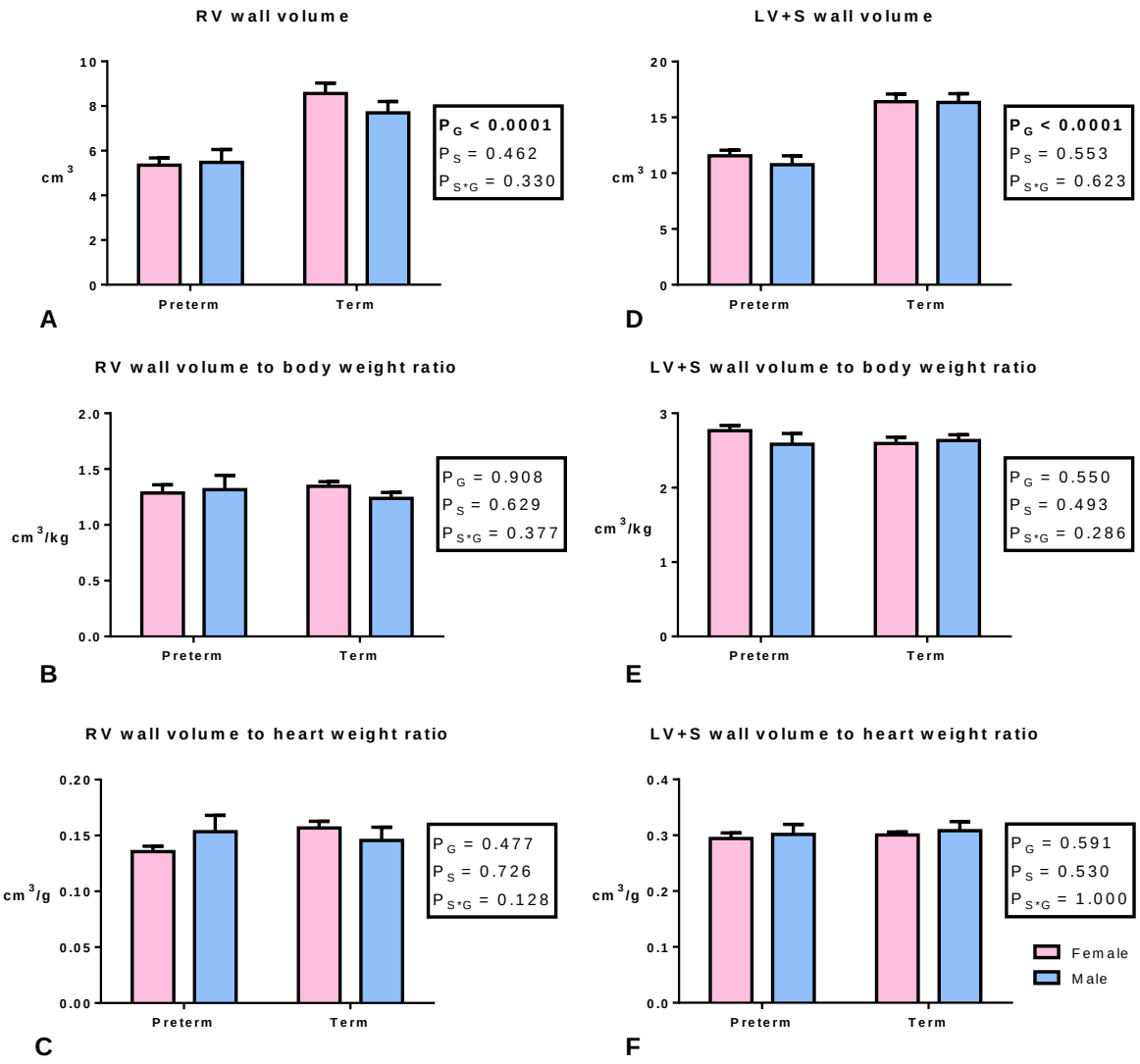

Figure 4.6. *Average absolute wall volumes and wall volumes relative to body weight and heart weight of the right ventricle (RV) free wall (A, B and C) and left ventricle plus septum (LV+S) (D, E and F) in preterm (n=7 female, n=6 male) and term (n=10 female, n=5 male) lambs 2 days after birth. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term) and sex (P_S; male or female) as factors. Values are means ± SEM.*

4.3.1.3 Ventricular chamber volume (Figure 4.7)

Right ventricle

Preterm lambs exhibited a significantly smaller (P_G=0.0001) RV chamber volume compared to term lambs (Figure 4.7A). When adjusted to body weight and heart weight, the RV chamber volume remained significantly smaller (P_G=0.029 and P_G=0.019, respectively) in preterm lambs compared to term controls (Figure 4.7B and 4.7C).

There were no significant differences between females and males in absolute RV chamber volume or when adjusted for body weight and heart weight at 2 days after birth.

Left ventricle

Similar to the RV chamber volume, the LV chamber volume of preterm lambs was significantly smaller (P_G=0.002) compared to term controls (Figure 4.7D). However, LV chamber volume relative to body weight and heart weight was not significantly different (Figure 4.7E and 4.7 F).

There were no significant differences in absolute LV chamber volume or when adjusted for body weight and heart weight between females and males.

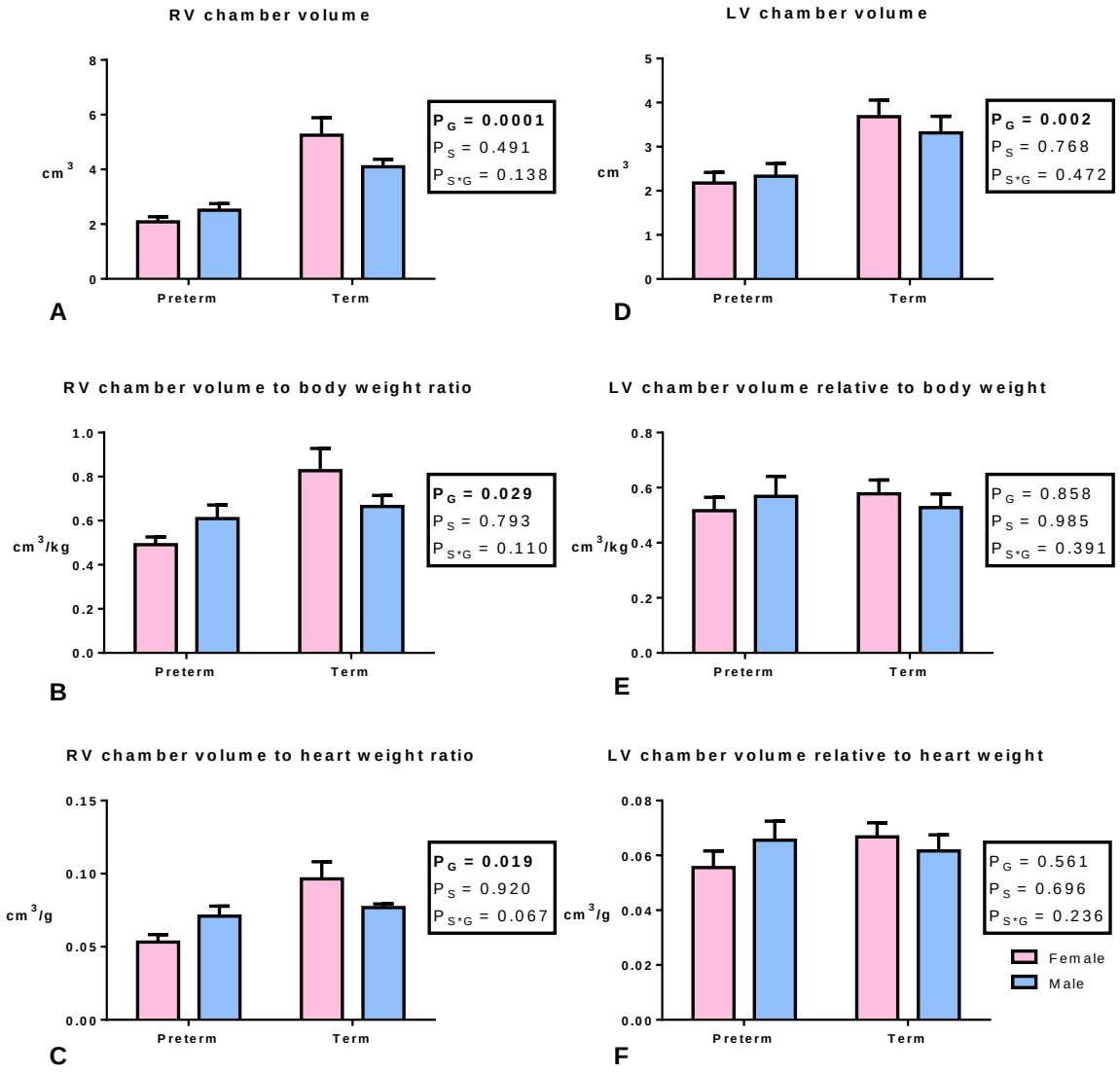

Figure 4.7. *Average absolute chamber volumes and chamber volumes relative to body weight and heart weight of the right ventricle (RV) (A, B and C) and left ventricle (LV) (D, E and F) in preterm (n=7 female, n=6 male) and term (n=10 female, n=5 male) lambs 2 days after birth. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term) and sex (P_S; male or female) as factors. Values are means ± SEM.*

4.3.1.4 Ventricular wall thickness (Table 4.3)

As reported in Table 4.3, the mean wall thickness of the RV and LV free walls and interventricular septum were all significantly thinner in preterm lambs compared to term

lambs at 2 days after birth. However, when adjusted for body weight and heart weight, the relative RV, LV and interventricular septum thickness were all significantly thicker in the preterm lambs compared to term controls. Furthermore, female lambs had significantly thinner septum walls compared to male lambs when adjusted for heart weight (P_G=0.046).

	Preterm		Term		P values
	Females n=7	**Males n=6**	**Females n=10**	**Males n=5**	
Absolute (mm)					
RV wall thickness	5.16 ± 0.19	5.01 ± 0.32	5.85 ± 0.18	6.02 ± 0.34	**P_G=0.002**
LV wall thickness	7.29 ± 0.26	7.17 ± 0.29	8.28 ± 0.26	8.28 ± 0.25	**P_G<0.001**
Interventricular septum wall	7.28 ± 0.29	7.20 ± 0.58	7.57 ± 0.27	8.64 ± 0.30	**P_G=0.030**
Relative to body weight (mm/kg)					
RV wall thickness	1.25 ± 0.055	1.21 ± 0.06	0.93 ± 0.05	0.97 ± 0.07	**P_G<0.0001**
LV wall thickness	1.76 ± 0.07	1.73 ± 0.07	1.31 ± 0.06	1.35 ± 0.08	**P_G<0.0001**
Interventricular septum wall	1.76 ± 0.08	1.73 ± 0.09	1.20 ± 0.07	1.40 ± 0.09	**P_G<0.001**
Relative to heart weight (mm/g)					
RV wall thickness	0.13 ± 0.007	0.14 ± 0.008	0.11 ± 0.006	0.12 ± 0.008	**P_G=0.002**
LV wall thickness	0.19 ± 0.009	0.20 ± 0.009	0.15 ± 0.007	0.16 ± 0.01	**P_G<0.0001**
Interventricular septum wall	0.19 ± 0.009	0.20 ± 0.01	0.14 ± 0.008	0.16 ± 0.01	**P_G<0.0001 P_S=0.046**

Table 4.3. *Average absolute wall thickness and wall thickness relative to body weight and heart weight of the right ventricle (RV) free wall; left ventricle (LV) free wall; and interventricular septum wall in preterm (female and male) and term (female and male) lambs 2 days after birth. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term) and sex (P_S; male or female) as factors. Values are means ± SEM.*

4.3.1.5 Cardiomyocyte number

In order to determine the total number of cardiomyocytes within the LV+S, the total number of cardiomyocyte nuclei were initially determined (using an optical disector/fractionator approach) and then adjusted for the proportion of mono-, bi-, tri- and tetra-nucleated cardiomyocytes.

4.3.1.5.1 Nuclearity (Figure 4.8)

Figure 4.8 shows the proportion of mono-, bi-, tri- and tetra-nucleated cardiomyocytes in the preterm and term hearts. Hearts of preterm lambs exhibited a significantly higher (P_G<0.0001) proportion of mononucleated cardiomyocytes but a significantly lower (P_G<0.0001) proportion of binucleated cardiomyocytes compared to term lambs. The proportion of trinucleated and tetranucleated cardiomyocytes was similar between the groups.

There were no significant differences in the percentages of mono-, bi-, tri- and tetra-nucleated cardiomyocytes between female and male lambs.

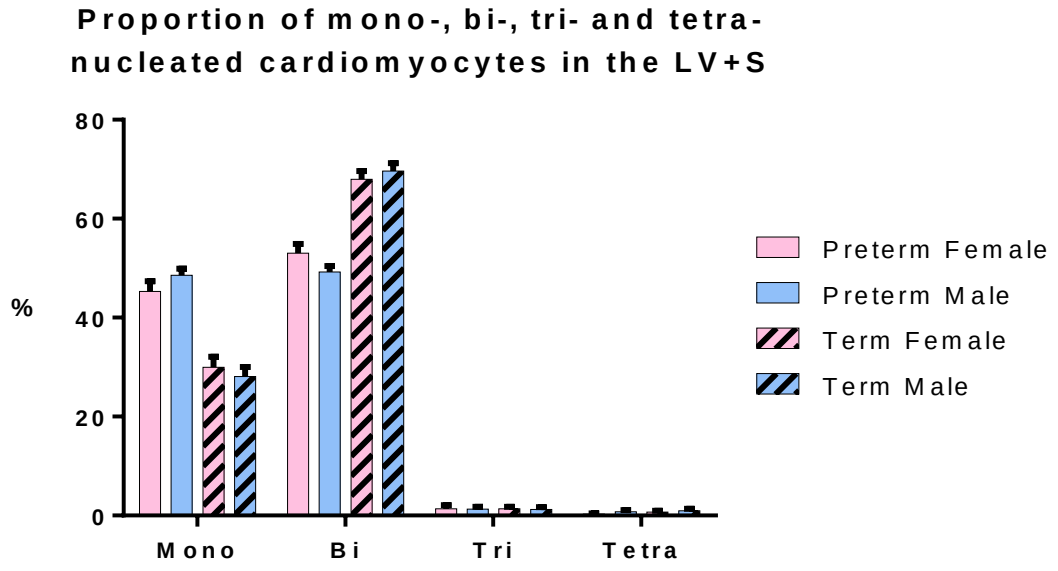

Figure 4.8. *Proportion of mono-, bi-, tri- and tetra-nucleated cardiomyocytes in the LV+S of preterm (n=7 female, n=6 male) and term (n=7 female, n=5 male) lambs at 2 days of age. Pink shading represents preterm females, blue shading represents preterm males, pink hashed represents term females and blue hashed represents term males. Values are means ± SEM.*

4.3.1.5.2 Total number of cardiomyocytes in the left ventricle plus septum (Figure 4.9)

At 2 days after birth, the total number of cardiomyocytes within the LV+S was not significantly different between preterm and term lambs (Figure 4.9A). When adjusted for body weight and heart weight, the preterm lambs had significantly more cardiomyocytes per kg of body and heart weight in the LV+S compared to term lambs ($P_G<0.0001$) (Figure 4.9B and 4.9C). Furthermore, there was also a trend for an interaction effect ($P_{S*G}=0.052$), with preterm males appearing to have significantly more cardiomyocytes compared to term males when adjusted for heart weight. There was no difference in total cardiomyocyte number in the LV+S between females and males.

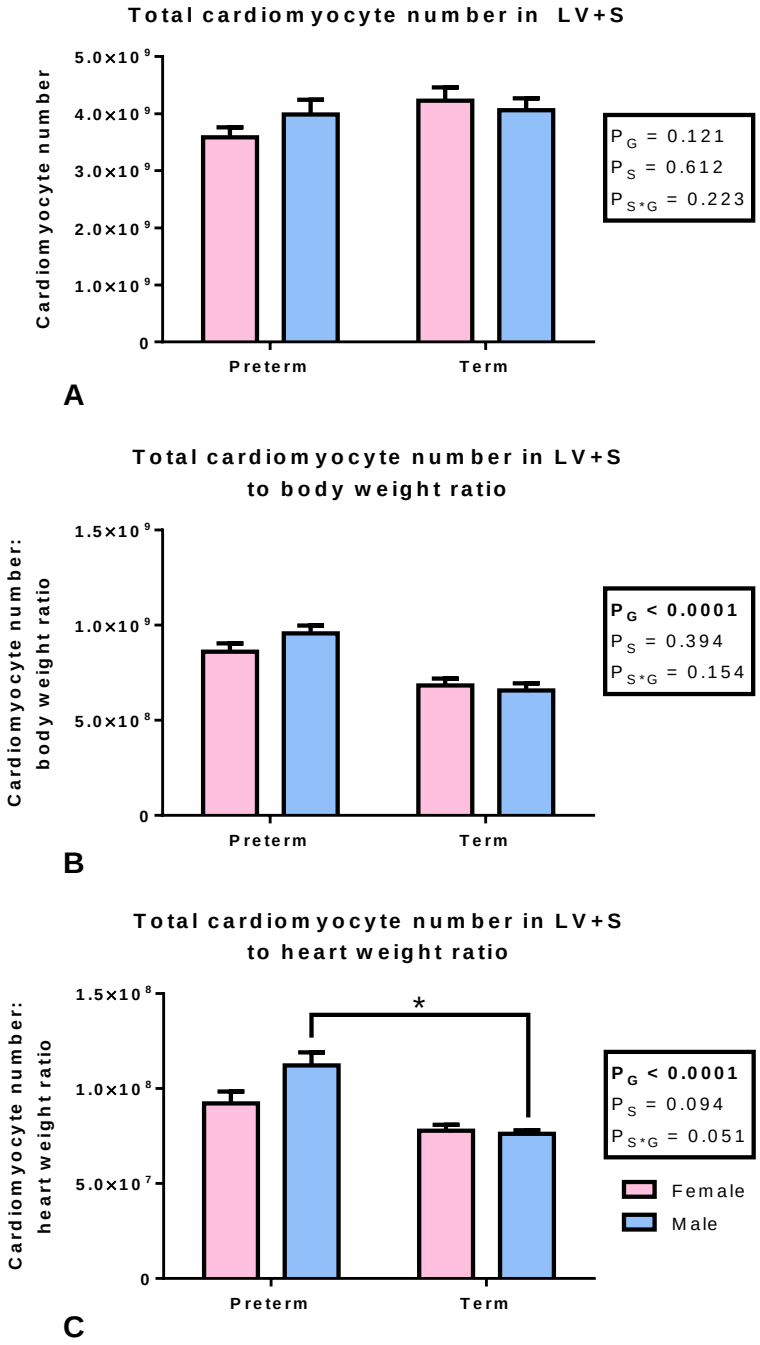

Figure 4.9. *The total number of cardiomyocytes (A) and number of cardiomyocytes relative to body weight (B) and heart weight (C) in the left ventricle plus septum (LV+S) in preterm (n=7 female, n=6 male) and term (n=7 female, n=5 male) lambs at 2 days of age. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term) and sex (P_S; male or female) as factors, followed by a Tukey's post-hoc test. *P<0.05 between preterm vs. term within the same sex. Values are means ± SEM.*

4.3.1.6 Total collagen content (Figure 4.10)

As previously mentioned (Section 4.2.7, page 123), some tissue samples were accidentally left out of the freezer and subsequently thawed. As a consequence, there were insufficient samples for analyses to be conducted in males. Hence, only data from female preterm lambs and female term lambs were compared using a student's t test.

Right ventricle

When assessed by hydroxyproline assay, the total collagen content (expressed as a percentage) in the RV free wall was not significantly different between female preterm and term lambs at 2 days of age (Figure 4.10A).

Left ventricle

Similar to the RV free wall, there was no significant difference in the total collagen content in the LV free wall between female preterm and term lambs at 2 days of age (Figure 4.10B).

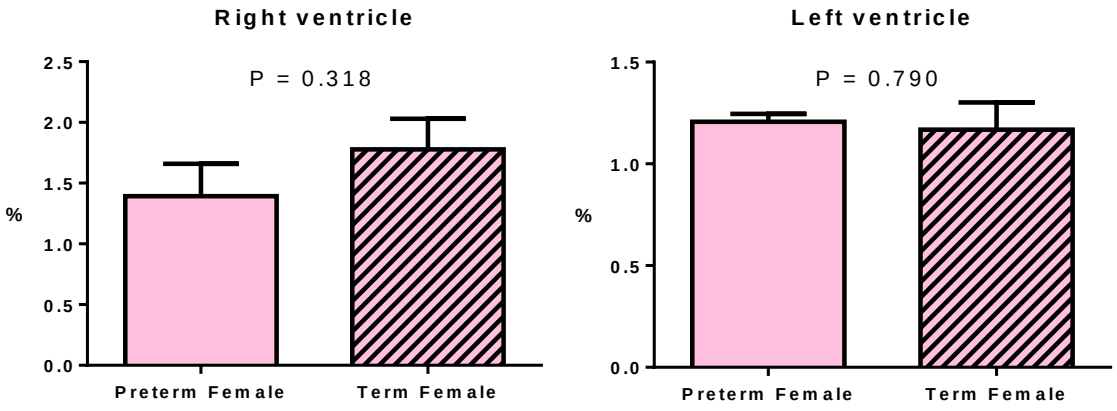

Figure 4.10. *Percentage of collagen in the right ventricle (A) and left ventricle (A) free walls of preterm female (n=6, pink) and term female (n=7, pink hashed) lambs at 2 days of age. Using a student's t-test, no statistical differences were observed. Values are means ± SEM.*

4.3.2 In adulthood (14.5 months of age)

4.3.2.1 Body weights and absolute and relative heart weights (Table 4.4)

In adulthood, there was a trend for preterm sheep to be lighter than term sheep (P_G=0.076); with preterm males lighter than term males (this did not quite reach statistical significance; P=0.065) but no difference between females (Table 4.4 and previously shown in Table 3.2, Chapter 3, Section 3.3.4.1, page 88). Heart weights were significantly reduced (P_G=0.014) in the preterm group compared to those born at term; this was also particularly evident between males whereas female heart weights were similar (Table 4.4 and previously shown in Table 3.4, Chapter 3, Section 3.3.4.4, page 93). However, when adjusted for body weight there was no significant difference between the groups (Table 4.4 and previously shown in Table 3.4, Chapter 3, Section 3.3.4.4, page 93).

There were no significant differences between sexes in body weights, absolute heart weights and relative heart weights.

Examination of the freshly excised hearts at necropsy, showed no evidence of gross structural abnormalities. Likewise, when the ventricles were sliced and sampled for analysis, there were no observable structural defects in the ventricles or valves.

	Preterm		**Term**		**P-values**
	Females n=7	**Males n=9**	**Females n=6**	**Males n=11**	
Body weight (kg)	54.36 ± 1.22	53.11 ± 2.92	55.75 ± 1.37	60.27 ± 2.11	P_G=0.076
Absolute heart weight (g)	265.75 ± 6.78	265.42 ± 14.95*	273.96 ± 2.98	318.95 ± 8.90	**P_G=0.014** P_S=0.069 P_{S*G}=0.066
Relative heart weight (g/kg)	4.89 ± 0.08	5.02 ± 0.16	4.90 ± 0.12	5.35 ± 0.22	NS

Table 4.4. *Body weights and absolute and relative heart weights of preterm (female and male) and term (female and male) sheep at 14.5 months of age. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors, followed by a Tukey's post-hoc test. *P<0.05 between preterm vs. term within the same sex. Values are means ± SEM. Abbreviations: NS – not significant.*

4.3.2.2 Ventricular measurements (Table 4.5)

Table 4.5 shows the mean absolute and relative LV+S and RV wall volumes, LV and RV chamber volumes and LV, RV and interventricular septum wall thickness of preterm and term sheep at 14.5 months of age.

Right ventricle

Absolute RV wall volumes were significantly reduced (P_G=0.023) in preterm sheep when compared to term sheep. This was mainly attributed to preterm male sheep exhibiting reduced (P=0.050) RV wall volume compared to term male sheep, whereas there were no differences between female preterm and term sheep. There were also no significant differences in absolute RV chamber volume or RV wall thickness between preterm and term sheep. However, when examining only males, preterm male sheep had significantly thinner (P=0.038) RV walls compared to term males, but no differences were observed in females. When adjusting for body weight, none of the RV parameters were found to be significantly different between the preterm and term sheep.

There were no significant differences between females and males in any of the absolute or relative to body weight RV measurements at 14.5 months of age. However, when adjusted to heart weight, female sheep exhibited thicker RV walls when compared to male sheep (P_S=0.002). All other RV measurements relative to heart weight were not significantly different between sexes.

Left ventricle

There was a trend for a smaller LV chamber volume (P_G=0.059) in preterm sheep compared with term sheep. Preterm sheep exhibited a significantly reduced LV+S wall volume (P_G<0.050) compared to terms; this was attributed to a marked reduction in LV+S wall volume in the preterm males compared to term males, but this difference between preterm and term sheep was not observed in females, such that there was a significant interaction effect (P_{S*G}=0.018). The interaction effect in LV+S wall volume persisted when adjusted for body weight (P_{S*G}=0.036). Preterm males also exhibited marked reductions in LV wall thickness compared to term male sheep, which was not seen between preterm female and term female sheep, hence there was a significant interaction effect (P_{S*G}=0.007). There were no significant

differences between preterm and term sheep in any of the LV parameters measured relative to body weight. However, when adjusted to heart weight, preterm sheep had significantly thicker LV walls compared to term sheep (P_G=0.003).

Absolute LV+S wall volume (P=0.007) was significantly increased in male sheep when compared to female sheep; no sex differences were observed in any of the other absolute or relative to body weight LV parameters measured. However, similar to the RV wall thickness, female sheep had significantly thicker LV walls compared to male sheep when adjusted for heart weight (P_S=0.020).

Interventricular septum

There were no significant differences observed in absolute interventricular septum thickness or when adjusted to body weight between preterm and term sheep or between female and male sheep. However, similar to the LV, when adjusted to heart weight, preterm sheep had significantly thicker septal walls compared to term sheep (P_G=0.012).

	Preterm		Term		P values
	Females n=7	Males n=7	Females n=6	Males n=7	
Absolute: (volume – cm^3; thickness – mm)					
RV wall volume	33.88 ± 1.89	33.88 ± 1.71*	37.10 ± 1.79	44.75 ± 4.69	**P_G=0.023**
LV+S wall volume	135.33 ± 5.10	137.82 ± 5.42*	132.18 ± 5.89	168.06 ± 8.76	**P_G<0.050 P_S=0.007 P_{S*G}=0.018**
RV chamber volume	32.42 ± 2.10	35.70 ± 0.07	31.11 ± 3.46	37.83 ± 2.93	P_S=0.080
LV chamber volume	32.73 ± 1.69	38.75 ± 2.31	41.15 ± 1.34	40.01 ± 3.24	P_G=0.059
RV wall thickness	7.21 ± 0.56	5.67 ± 0.56*	7.06 ± 0.61	7.39 ± 0.56	NS
LV wall thickness	15.70 ± 0.40	14.82 ± 0.43*	14.65 ± 0.43	16.23 ± 0.40	**P_{S*G}=0.007**
Septum thickness	7.28 ± 0.29	7.20 ± 0.58	7.57 ± 0.27	8.64 ± 0.30	P_{S*G}=0.089
Relative to body weight: (volume - cm^3/kg; thickness - mm/kg)					
RV wall volume	0.63 ± 0.04	0.62 ± 0.05	0.67 ± 0.03	0.73 ± 0.08	NS
LV+S wall volume	2.58 ± 0.04	2.38 ± 0.05*	2.37 ± 0.09	2.75 ± 0.21	**P_{S*G}=0.036**
RV chamber volume	0.60 ± 0.05	0.60 ± 0.05	0.57 ± 0.05	0.61 ± 0.05	NS
LV chamber volume	0.61 ± 0.05	0.67 ± 0.06	0.75 ± 0.06	0.66 ± 0.05	NS
RV wall thickness	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	NS
LV wall thickness	0.29 ± 0.01	0.28 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	P_G=0.081
Septum thickness	0.29 ± 0.01	0.29 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	P_G=0.088
Relative to heart weight: (volume - cm^3/g; thickness - mm/g)					
RV wall volume	0.13 ± 0.007	0.12 ± 0.008	0.14 ± 0.008	0.14 ± 0.01	NS
LV+S wall volume	0.51 ± 0.02	0.49 ± 0.02	0.49 ± 0.02	0.52 ± 0.02	NS
RV chamber volume	0.12 ± 0.009	0.13 ± 0.009	0.11 ± 0.02	0.12 ± 0.01	NS
LV chamber volume	0.12 ± 0.008	0.13 ± 0.02	0.14 ± 0.02	0.12 ± 0.008	NS

Relative to heart weight: (volume - cm^3/g; thickness - mm/g) continued.					
RV wall thickness	0.03 ± 0.001	0.02 ± 0.001	0.03 ± 0.001	0.02 ± 0.0005	**P_S=0.002**
LV wall thickness	0.06 ± 0.001	0.06 ± 0.002	0.05 ± 0.002	0.05 ± 0.002	**P_G=0.003** P_S=0.020
Septum thickness	0.06 ± 0.002	0.06 ± 0.002	0.05 ± 0.001	0.05 ± 0.002	P_G=0.012

Table 4.5. *Average absolute ventricular measurements of wall volume, chamber volume and wall thicknesses of the right ventricle (RV), left ventricle (LV) and interventricular septum and when adjusted to body weight and heart weight in preterm (male and female) and term (female and male) sheep at 14.5 months of age. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term) and sex (P_S; male or female) as factors, followed by a Tukey's post-hoc test. *P<0.05 between preterm vs. term within the same sex. Values are means ± SEM.*

4.3.2.3 Cardiomyocyte number in the LV+S (Figure 4.11 and 4.12)

At 14.5 months of age, it appears that preterm birth does affect the total number of LV+S cardiomyocytes. Figure 4.11A illustrates that preterm sheep had significantly fewer cardiomyocytes (P_G=0.039) in the LV+S when compared to those born at term. A significant interaction effect (P_{S*G}=0.047) shows that this is mostly attributed to the significant reduced number of cardiomyocytes in preterm male sheep compared to term male sheep, whereas there were no differences between female preterm and term sheep. However, when adjusted to body weight and heart weight, this reduction of cardiomyocytes within the preterm group was no longer evident.

Additionally, there were no significant differences in the absolute cardiomyocyte number and cardiomyocyte number relative to body weight or heart weight in the LV+S of female and male sheep as seen in Figure 4.11B and 4.11C.

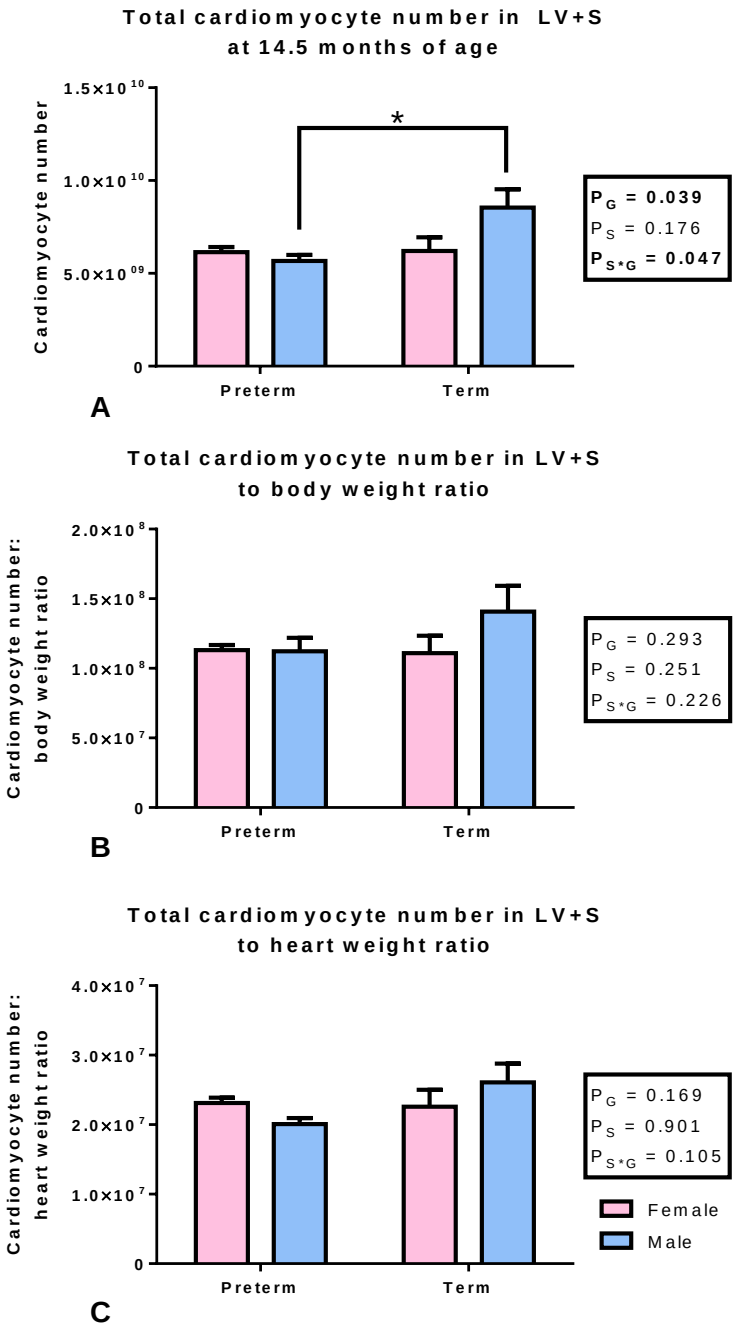

Figure 4.11. *The total number of cardiomyocytes (A) and number of cardiomyocytes relative to body weight (B) and heart weight (C) in the left ventricle plus septum (LV+S) in preterm (n=7 female, n=6 male) and term (n=7 female, n=7 male) lambs at 14.5 months of age. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term) and sex (P_S; male or female) as factors, followed by a Tukey's post-hoc test. *P<0.05 between preterm vs. term within the same sex. Values are means ± SEM.*

Furthermore, the total number of cardiomyocytes estimated at 14.5 months of age was found to be positively correlated with birth weight (regression analysis: P=0.0003, R^2=0.425) and heart weight at necropsy (regression analysis: P=0.0004, R^2=0.415), but not with body weight at necropsy (regression analysis: P=0.126, R^2=0.095) (Figure 4.12).

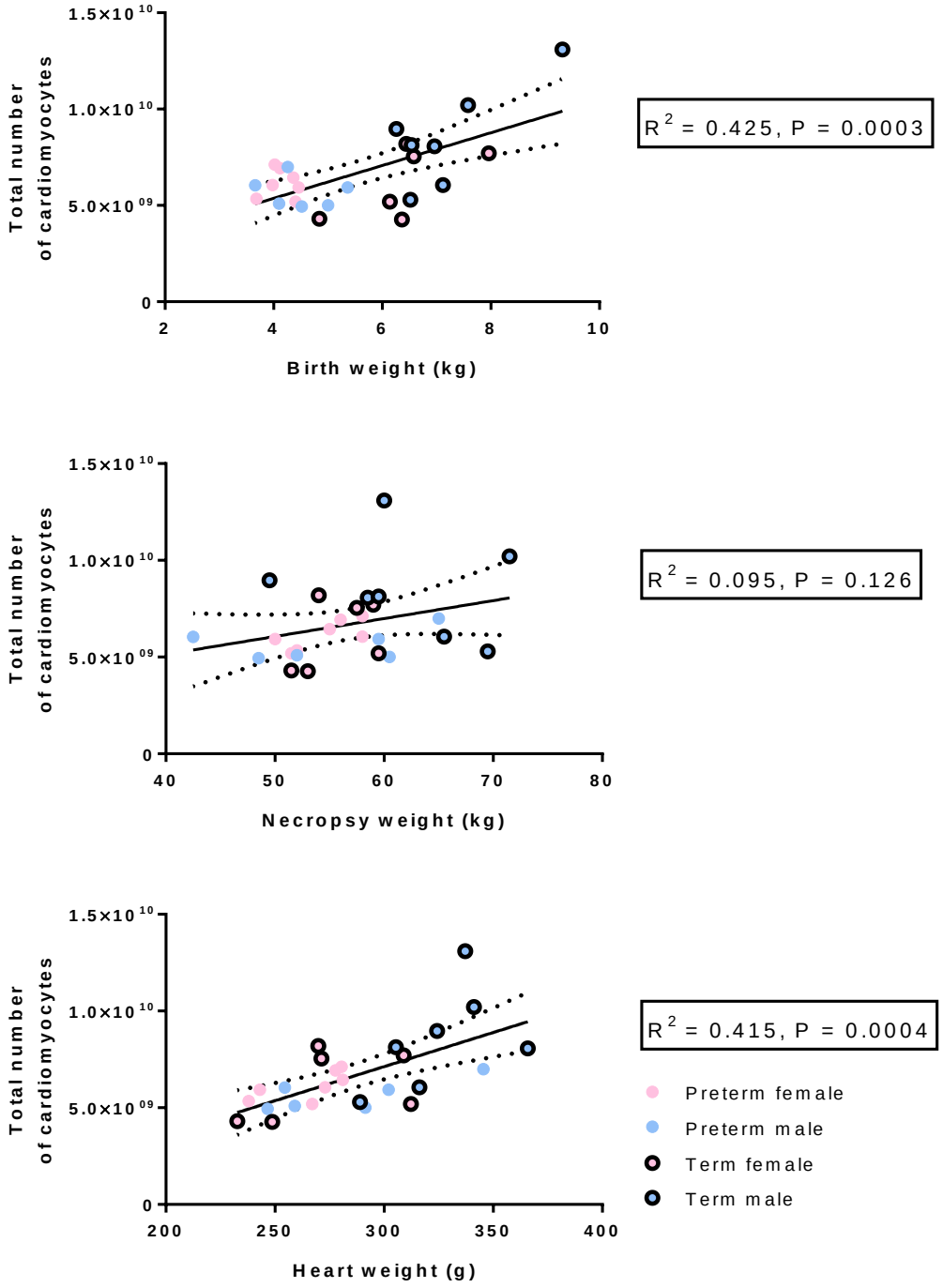

Figure 4.12. *The relationship between total number of cardiomyocytes in the left ventricle plus septum of all sheep at 14.5 months of age with birth weight (A), necropsy weight (B) and heart weight (C).*

4.3.2.4 Collagen content (Figure 4.13)

Right ventricle

The collagen content (expressed as a percentage) in the RV free wall was not significantly different between preterm and term sheep (Figure 4.13A).

There were also no significant differences in collagen content in the RV free wall between female and male sheep.

Left ventricle

Likewise, the percentage collagen content in the LV free wall was not significantly different between preterm and term sheep (Figure 4.13B). However, there was a trend for preterm males to have reduced collagen content compared to term males (P=0.071).

However, a sex difference was observed with male sheep exhibiting a significantly higher collagen content (P_S=0.010) in the LV free wall compared to females.

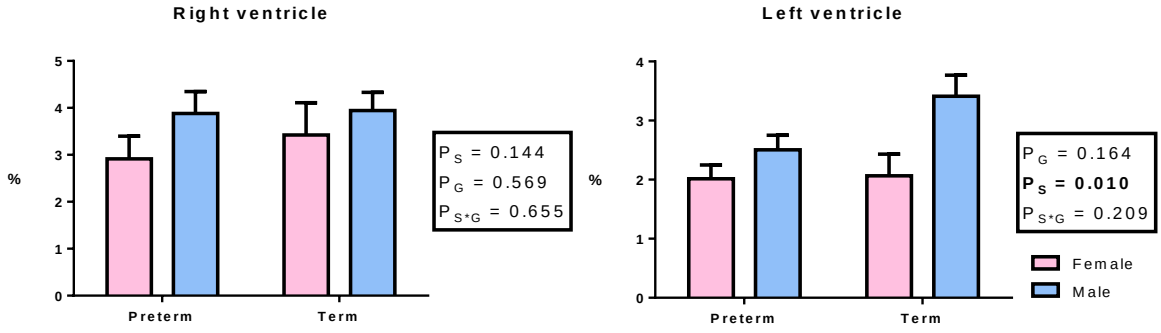

Figure 4.13. *Percentage of collagen in the RV (A) and LV (B) free walls of preterm (n=7 female, n=6 male) and term (n=9 female, n=11 male) sheep at 14.5 months of age. Pink represents females while blue represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term) and sex (P_S; male or female) as factors. Values are means ± SEM.*

4.4 Discussion

Using a sheep model, this study aimed to determine the effect of moderate preterm birth on heart structure and cardiomyocyte growth in the immediate period after birth and in early adulthood. In this study, hearts of preterm lambs were significantly lighter and smaller (in terms of RV and LV wall volume, chamber volume and wall thickness) compared to term lambs, in the immediate period after birth. However, when adjusted for body weight and heart weight, these reductions were no longer apparent; except for RV chamber volume; relative RV, LV and interventricular septum wall thickness were significantly increased in preterm lambs compared to term controls. The total number of cardiomyocytes in the LV+S of neonates was not different between groups; however, when adjusted for body weight and heart weight, preterm lambs had significantly more cardiomyocytes compared to term lambs. The LV+S of preterm lambs also had a higher proportion of mononucleated cardiomyocytes and lower proportion of binucleated cardiomyocytes compared to term lambs; thus, indicating immaturity of cardiomyocytes in the preterm hearts.

In adulthood, there was sexual dimorphism in the response to preterm birth with LV+S wall volume, LV wall thickness and total cardiomyocyte number in the LV+S of preterm males significantly reduced compared to terms; these differences in preterm and term adult hearts were not observed in females. The sex specific effects in males may relate to the greater impact of preterm birth on long-term body growth in males. Given that there was no difference in the absolute number of cardiomyocytes at 2 days of age in all groups, the observed reduction in cardiomyocyte number in the LV+S of male preterm sheep compared to male term sheep is likely due to their long-term attenuated body and/or heart size (as there was no differences relative to body weight or heart weight in adulthood).

In this study, I have chosen to include the IVS when analysing the effects on the left ventricular wall. After birth, the growth of the IVS matches the growth of the LV free wall, such that they are of a similar thickness. It is to be noted however, that the IVS is composed of a RV component and a LV component; these have different developmental origins and different postnatal growth patterns (Zaffran et al., 2004). Given that the measurements of cardiomyocyte number were based on a fractionator/optical disector approach, this does not affect the total number of cardiomyocytes in the LV+S. Therefore, comparisons can be legitimately made between different animals.

Prior to the commencement of this thesis, little was known of the effects of being born moderately preterm on the structure of the heart or cardiomyocyte growth. The final weeks of gestation are a critical period, whereby, the cardiomyocytes undergo a process of maturation in preparation for the haemodynamic transition at birth. Hence, it was considered imperative in this thesis, to gain an understanding of the effect of being born moderately preterm during this critical period of cardiomyocyte maturation; especially given that the majority of preterm infants are born moderately preterm. In my studies, it appears that there is greater heart growth relative to body weight in the preterm lambs compared to term lambs in the short-term cohort. It is unlikely that there was accelerated heart growth in the 2 days after birth in the preterm lambs, and so, it is likely to be a late gestation phenomenon where heart growth appears to be greater than body growth whilst *in utero*. This is also reflected in cardiomyocyte number where preterm lambs had significantly more cardiomyocytes when adjusted for body weight and heart weight. In addition, heart structure is likely to be different in the preterm lambs, given that wall thickness of the RV and LV+S is significantly thicker and RV chamber volume is significantly smaller, when adjusted for heart weight. In future studies, it would be of interest to further examine structural components of the heart wall at different gestational time points compared to term and postnatally. It is well known that heart growth is achieved in early gestation by hyperplasia of cardiomyocytes (Huttenbach et al., 2001), whereas in late gestation, cardiomyocytes undergo a process of maturation whereby they become mature and differentiated in preparation for the marked changes in haemodynamics at birth (Bubb et al., 2007). Sheep were considered an excellent model to use in my studies, given that the prenatal and postnatal cardiomyocyte growth trajectory closely resembles humans and immature and mature cardiomyocytes are readily identified; mature cardiomyocytes are binucleated and immature cardiomyocytes are mononucleated (Burrell et al., 2003, Jonker et al., 2007).

Interestingly, my findings suggest that there may have been enhanced cardiomyocyte proliferation in the preterm hearts in the perinatal period, given that postconceptionally the preterm lambs had 14 days less for cardiomyocyte proliferation to occur and when adjusted for body weight, there was a significant increase in cardiomyocyte number per kg body weight when compared to term hearts. This apparent increase in cardiomyocyte proliferation in the preterm hearts may be the result of exposure to betamethasone *in utero*. Indeed, it has been previously shown that cortisol chronically infused into the coronary artery of fetal sheep stimulates cell cycle activity of cardiomyocytes, thereby accelerating the proliferation of

cardiomyocytes and leading to an increase in fetal heart mass (Giraud et al., 2006). In contrast to these findings, however, other studies suggest that corticosteroid exposure leads to accelerated maturation of cardiomyocytes. For example, Kim et al. (2014) showed that a clinical dose of antenatal corticosteroids resulted in advanced cardiomyocyte maturation and improved cardiac function in very preterm piglets after birth. Indeed, the findings of my study do not support the concept that *in utero* exposure to betamethasone leads to accelerated maturation of cardiomyocytes in preterm hearts compared to term hearts, but instead support the concept that *in utero* exposure to betamethasone leads to enhanced cardiomyocyte proliferation.

In this regard, at 2 days of age, my findings clearly show that the myocardium of the moderately preterm lambs was more immature compared to terms, with a significant increase in the proportion of immature mononucleated cardiomyocytes (47 ± 1% in preterms and 29 ± 1% in terms) and a reduced proportion of mature binucleated cardiomyocytes (51 ± 1% in preterms and 69 ± 1% in terms) within the LV+S 2 days after birth. Overall, there was no evidence of an altered process of maturation in the preterm hearts. Based on previous studies, the relative proportion of mononucleated and binucleated cardiomyocytes in the hearts of preterm lambs in my study were at the levels expected for their postconceptional age. For instance, studies in fetal sheep, by Burrell et al. (2003) and Jonker et al. (2007), have demonstrated that at 135 days of gestation (the postconceptional age of the preterm lambs), approximately 50% of cardiomyocytes in the fetal lamb heart are mononucleated (immature); this is similar to the proportion of immature mononucleated cardiomyocytes observed in our preterm hearts.

It has been well described that males are more vulnerable to preterm birth, exhibiting both a higher morbidity and mortality compared to female infants in the neonatal period (Stevenson et al., 2000, Zisk et al., 2011). It is proposed that this male disadvantage may be the consequence of a developmental delay in males versus females (Peacock et al., 2012). Notably, a study by Lumbers et al. (2009) suggests that maturation of cardiomyocytes occurs earlier in females than in males; with a significantly increased proportion of binucleated cardiomyocytes in hearts of female sheep fetuses at 128 days of gestation compared to male fetuses. Interestingly, in my study, there was no evidence of this developmental delay of cardiomyocytes in the male neonatal heart; there was no difference in the total number of cardiomyocytes within the LV+S between preterm male and female lambs nor in the proportion of mononucleated (immature) or binucleated (mature) cardiomyocytes. Therefore,

there remains the possibility that there may have been accelerated maturation of cardiomyocytes in the male preterm lambs following exposure to betamethasone (although my findings in relation to cardiomyocyte number do not support this). Hence, in future studies, it is important to further clarify the role of antenatal corticosteroid exposure on cardiomyocyte proliferation and maturation. A good approach may be to examine a cohort of term lambs that are exposed to the same dose of antenatal betamethasone, in order to help distinguish the effects of preterm birth and corticosteroid exposure; however, interpretation of such studies would be confounded by the natural cortisol surge that occurs in term infants in the period prior to birth. Additionally, studies of preterm lambs that are not exposed to antenatal betamethasone would exclude the factor of antenatal corticosteroid exposure; however, these preterm lambs would require significantly greater neonatal intensive care, including greater ventilatory support, which would confound the findings. Alternatively, the effects on cardiomyocyte proliferation and maturation could be examined immediately at delivery.

Generally, infants born moderately preterm do not experience cardiovascular complications in the neonatal period; however, my findings clearly demonstrate that the myocardium is immature when compared to term lambs. In addition, the RV and LV walls were thinner and the ventricular chambers were smaller in preterm lambs compared to term lambs and this was attributed to the smaller body size of preterm lambs. Given that the preterm lambs exhibited a thinner and more immature LV wall, yet were exposed to the same increases in LV pressure as term lambs at birth, it is likely that this may lead to cardiac injury and to structural adaptations in the heart. The structural changes may be beneficial in the short-term but may lead to long-term deleterious consequences. In support of this idea, previous findings in our laboratory have shown that there are maladaptive changes in cardiac structure and cardiomyocyte growth in the postnatal period following moderate preterm birth at 9 weeks PTEA (Bensley et al., 2010). There was cardiomyocyte hypertrophy, altered cardiomyocyte nuclearity, induction of tetraploidy in mononucleated cardiomyocytes and increased collagen deposition within the myocardium of lambs born moderately preterm when compared to term lambs. In addition, in some preterm hearts, there was also infiltration of inflammatory cells into the myocardium, indicative of cardiac injury.

Although, cardiac remodelling was observed in these previous studies of moderately preterm lambs (at 9 weeks PTEA), the overall gross anatomical structure in the hearts of preterm females of my study appeared to have normalised by early adulthood; whereas there were

persistent long-term differences in preterm males compared to term males, such as reductions in LV wall volume and thickness and a concomitant reduction in total cardiomyocyte number within the LV+S. These long-term differences in cardiac growth in preterm males were likely attributed to the long-term attenuation of growth in preterm males, since these differences in heart growth were no longer evident when adjusted for body weight. Interestingly however, there was a significant difference in the LV+S wall thickness when adjusted for heart weight, with preterm sheep having thicker LV+S walls compared to term sheep; this is indicative of enhanced growth of the LV+S walls relative to size of the heart.

Of major concern, the overall reduction in absolute LV growth in preterm sheep was accompanied by a reduction in the number of cardiomyocytes within the LV+S compared to term male sheep. This is of major clinical significance, given the reduced proliferative capacity of cardiomyocytes postnatally; a reduced complement of cardiomyocytes is likely to impact on lifelong cardiac functional reserve and on the adaptive capabilities of the heart. Although the preterm adult male sheep were not hypertensive in my study, many studies have shown an association with preterm birth and hypertension in adulthood (Bonamy et al., 2005, Johansson et al., 2005, Doyle, 2008, Kerkhof et al., 2012). Hence, if these preterm male sheep do go on to develop elevated arterial pressure in later life, there may be deleterious consequences. Hypertension leads to left ventricular hypertrophy and the potential for physiological cardiac growth will be impaired when cardiomyocyte endowment is reduced. In future studies, it would be interesting to allow the moderately preterm sheep to reach old age to see if hypertension does ensue. Alternatively, it would be interesting to challenge the hearts of our preterm sheep in early adulthood by inducing hypertension and subsequently examine the cardiac pathological response.

In contrast to my findings, recent MRI studies in humans showed that individuals born very preterm (mean gestational age of 30.3 ± 2.5 weeks) had a significant increase in LV mass in adulthood compared to adults born at term (Lewandowski et al., 2013a). In these longitudinal imaging studies, these preterm individuals also exhibited an altered shape of the LV, such that the ventricle was shorter with a reduced internal diameter and a displaced apex when compared to adults born at term. In addition, preterm birth was associated with a smaller RV chamber and an increased RV mass in adulthood (Lewandowski et al., 2013b). The differences in the long-term cardiac growth described in these studies of human subjects compared to my studies in moderately preterm sheep are yet to be elucidated. Indeed, levels of adult arterial

pressure and the degrees of prematurity at birth are likely to be contributing factors. In the studies by Lewandowski and Leeson (2014), the long-term structural effects on the heart were increased by the severity of prematurity at birth; with decreases in gestational age at birth associated with a higher LV mass. In addition, these preterm subjects displayed elevated arterial pressure compared to those born at term and this may therefore account for the increased LV mass (Lewandowski et al., 2015).

In conclusion, this study gives insight to structural changes in the myocardium in the immediate period following moderate preterm birth and whether these differences persist into young adulthood. The findings of my thesis have shown that hearts of lambs born moderately preterm are significantly immature compared to term hearts 2 days after birth. By early adulthood, sheep born moderately preterm showed reduced cardiac growth proportional to body weight, which was most evident in males, such that preterm male sheep exhibited a reduction in cardiomyocyte number within the LV+S compared to term male sheep, whereas there were no differences between females. This may impact on the lifelong reserve of cardiomyocytes in preterm males and thus the functional capabilities of the heart. Therefore, it is envisaged that hearts of adult preterm males will be vulnerable, if exposed to secondary insults such as hypertension, and this may lead to further adverse cardiac complications. Further studies are required to elucidate this.

Chapter 5:

The Effect of Moderate Preterm Birth on the Large Conduit Arteries (Thoracic Aorta and Left Carotid Artery) in the Immediate Period after Birth and in Early Adulthood

5.1 Introduction

Preterm birth affects 9 - 12% of all live births worldwide (Beck et al., 2010). At the time of preterm birth, many organ systems are still immature, including the cardiovascular system. This is important because the immature cardiovascular system is exposed prematurely to the haemodynamic changes which accompany birth.

The haemodynamic transition at birth occurs as a result of the change from the fetal to postnatal circulatory configuration, when a baby takes it first breath and uses its lungs for the first time (Cuneo, 2013, Johnson et al., 2014). The transition involves the loss of the placenta, closure of shunts (i.e. ductus arteriosus, ductus venosus and foramen ovale) and alterations in cardiac output and blood flow and steep changes in circulating hormone levels, which in combination result in increased systemic arterial pressure and heart rate immediately following birth (Louey et al., 2000, Blackburn, 2006, Hillman et al., 2012). The conduit arteries close to the heart, particularly the aorta, are the first to be exposed to these very high systolic pressures immediately after birth. Hence, when birth comes early, vascular adaptions in immature arteries may occur in response to the premature haemodynamic transition; this may lead to maladaptive remodelling of arterial walls and lead to long-term vulnerability to cardiovascular disease.

There are three major components of the walls of the large conduit arteries: (1) elastin, (2) collagen and (3) smooth muscle. It is known that during the perinatal period, blood vessels undergo significant remodelling in dimensions and in the relative composition of the blood vessel wall to prepare for the haemodynamic transition (Langille, 1996). Normally, there is rapid laying down of elastin layers and collagen deposition in late gestation; so that blood vessels are prepared for the increased pressures at birth (Bendeck and Langille, 1991, Martyn and Greenwald, 1997). Of concern, preterm birth occurs during the critical period when the conduit arteries are undergoing the rapid laying down of elastin and collagen in the arterial wall. Hence, it is hypothesised that these immature blood vessels are ill-prepared for the haemodynamic transition at birth leading to maladaptive remodelling of the vasculature in the first days to months after birth and throughout life.

Maladaptive structural changes in the great vessels (aorta and pulmonary artery) have been previously reported in 9 week old sheep born moderately preterm (Bensley et al., 2012). In these studies, elastin deposition was significantly increased in the aorta and pulmonary artery, and smooth muscle content was significantly reduced in the aorta of preterm sheep compared

with term controls. In addition, and of major concern, there was also evidence of severe injury observed in the aortic walls of preterm lambs, along with thicker arterial walls and narrower lumen areas. The aortic injury and altered structure of the aorta and pulmonary artery are likely to be related to the marked rise in arterial pressure at the time of birth.

Additionally, studies in human subjects demonstrate altered vascular structure throughout life in individuals born preterm. For example, aortic and carotid artery growth was found to be altered after preterm birth, leading to arterial narrowing in the three-month follow up period of normotensive infants born very preterm (Schubert et al., 2011). In addition, imaging studies taken at rest, using MRI, conducted in hypertensive adolescents and adults born very preterm observed reductions in aortic size, such that there was a narrower lumen area in the thoracic and abdominal aorta, compared with those born at term (Bonamy et al., 2005, Edstedt Bonamy et al., 2008). Furthermore, an increased carotid artery intima-media wall thickness has been reported in hypertensive middle-aged adults born preterm; such that higher intima-media thickness was associated with higher systolic blood pressure (Lazdam et al., 2010, Hovi et al., 2011). Overall, these combined effects on the macrovasculature following preterm birth are likely to contribute to an increased risk for cardiovascular disease in later life (Simon et al., 2002).

In this chapter, the structure and composition of the thoracic aorta and left carotid artery, collected at necropsy in Chapters 2 and 3, are compared between moderately preterm (female and male) and term (female and male) sheep in the immediate period after birth and in young adulthood. At 2 days of age and 14.5 months of age, lumen area, media area, wall thickness, number of elastin layers and biochemical composition of the arterial wall (percentage of elastin, collagen and smooth muscle tissue area) were examined in the thoracic aorta and left carotid artery.

5.2 Methods

5.2.1 Vessel collection and fixation

At necropsy, several blood vessels were excised from the preterm and term sheep at 2 days of age (Chapter 2) and 14.5 months of age (Chapter 3). These included the left and right renal arteries, left and right carotid arteries, lower thoracic aorta (above the diaphragm), abdominal aorta (directly below where the left renal artery was attached), third-order mesenteric arteries and ascending aortic arch (attached to the heart when removing the heart). As described previously (Chapter 2, Section 2.2.6, page 48), once dissected, all vessels were placed in physiological saline with 1 ml of papaverine hydrochloride (1.2 mg/ml; Sigma-Aldrich, Missouri, USA) added for every 100 ml of saline to clear blood vessels of blood and to maximally dilate the blood vessels. Portions of the blood vessels were snap frozen in liquid nitrogen for Real-time Polymerase Chain Reaction and hydroxyproline analysis, or immersion fixed in 10% formalin for structural analysis. Frozen tissues were stored at -80^{o}C and fixed tissues were stored at 4^{o}C in 10% formalin. Due to time constraints, I have only conducted histological and hydroxyproline analysis on the thoracic aorta and left carotid artery, which will be presented in this thesis. Gene expression studies on the thoracic aorta and left carotid artery were performed concomitantly by research assistant, Ms. Tracey Flores; the other blood vessels will be analysed by other members of my laboratory at a later date.

5.2.2 Hydroxyproline assay

Collagen content (expressed as a percentage) was determined in the same manner as described in Chapter 4 (when myocardial collagen was measured; Section 4.2.7, page 119-120) in snap frozen tissues of the thoracic aorta and left carotid arteries of both short term and long term animals. These analyses were performed under the guidance of A/Prof. Chrishan Samuel from the Department of Pharmacology at Monash University. This assay determines the amount of collagen by measuring the hydroxyproline content in tissues (Samuel, 2009), since collagen is one of the few proteins that contain the amino acid hydroxyproline. Using a colorimetric-based assay and generating a standard curve against known hydroxyproline standards, the amount of hydroxyproline was determined in each sample and the total amount of collagen content was extrapolated from the standard curve.

Collagen content was then calculated by dividing the total collagen content by the dry weight of the tissues.

As mentioned in Chapter 4 (Section 4.2.7, page 120), some of the stored tissue samples from the short-term cohort were accidently left out of the freezer and subsequently thawed; the majority of the thawed samples were from male lambs and therefore analysis of males in the short term cohort was unable to be performed. Hence, only data from preterm and term females were compared (student's t test) for blood vessels collected at 2 days of age, whereas both sexes were able to be analysed in the long-term cohort.

5.2.3 Vessel preparation: processing, embedding, sectioning and staining

Fixed segments of the thoracic aorta and left carotid artery from preterm and term sheep from both the short term and long term cohorts were processed and embedded in paraffin wax for analysis of blood vessel wall structure. The processing of tissues to be embedded in paraffin was performed using an automated processing protocol (Leica Peloris II, Leica, New South Wales, Australia). An automated processing cycle was utilised, as described in Table 5.1.

Step	Reagent	Step time (minutes)	Temperature (^{O}C)
1	10% buffered formalin	10	45
2	80% Ethanol	1	Ambient
3	Ethanol	1	Ambient
4	Ethanol	1	Ambient
5	Ethanol	10	45
6	Ethanol	20	45
7	Ethanol	30	45
8	Xylene	1	Ambient
9	Xylene	10	45
10	Xylene	45	45
11	Wax	10	65
12	Wax	10	65
13	Wax	40	65

Table 5.1. *Automated processing protocol performed on the thoracic aorta and left carotid artery.*

Once processed, the blood vessels were embedded in paraffin. The blood vessels were placed flat at the bottom of the mould ensuring the lumen of the vessel was open and oriented in such a way that the blood vessels could be sectioned in a cross-sectional plane. The mould was then filled with wax and a processing cassette placed on top as a backing block. The moulds were left on a cold plate so the paraffin could harden.

When the wax was set, the blocks were sectioned at 5 µm and 3 sections per block collected and mounted onto SuperFrost Plus slides. Prior to staining, sections were dewaxed with 3 changes of xylene at 2 minutes each and 2 changes of EtOH at 2 minutes each before placing

in water. All sections were stained with Verhoeff van Gieson's stain in order to differentiate elastin, collagen and smooth muscle within the blood vessel walls; elastic fibers stained dark purple, collagen stained pink and smooth muscle stained brown. The staining reagents were prepared and applied according to the protocol (shown in Appendix, page 202).

This stain required careful assessment in the differentiating step in order to show the finest elastic fibres; therefore, only a small number of slides (approximately 5 slides) could be stained simultaneously. Additionally, fresh reagents had to be prepared for every staining session.

5.2.4 Capturing of images

Images of the stained sections of aortae and carotid arteries were digitally captured using Image Pro-Plus Version 6.2 software (Media Cybernetics, Bethesda, Maryland, USA). Sections of the blood vessel walls for analysis of the number of elastin layers and composition in the arterial wall (see Section 5.2.5) were captured at 10x, 20x and 40x magnifications, depending on the size of the blood vessel. Images of the whole cross-sectional view of the blood vessels for analysis of lumen area, medial area and wall thickness (see next section) were also captured at 10x and 20x magnifications, depending on the size of the blood vessel. For blood vessels that were too large, particularly those from adult animals, capturing of these sections required the entire slide to be scanned at 20x magnification using Aperio® ScanScope® AT Turbo (Leica Biosystems, New South Wales, Australia).

5.2.5 Image analysis measurements

Using a whole cross-sectional captured view of the vessels, lumen area, medial area and wall thickness were measured using Image Pro-Plus Version 6.2 as shown in Figure 5.1A, 5.1B and 5.1C. The lumenal area measurements were determined by measuring the internal perimeter to avoid any distortion of the lumen during embedding.

For the measurement of the number of elastin layers and analysis of arterial wall composition, images were captured at higher magnification (40x) and sampled across four areas (north, south, east and west) of the blood vessel wall (Figure 5.1D). To estimate the number of elastin layers, the layers were counted at each of the sampled areas and then averaged. Similarly,

when analysing the composition of the arterial wall, the percentages of collagen, elastin and smooth muscle were measured for each sampled area and then averaged.

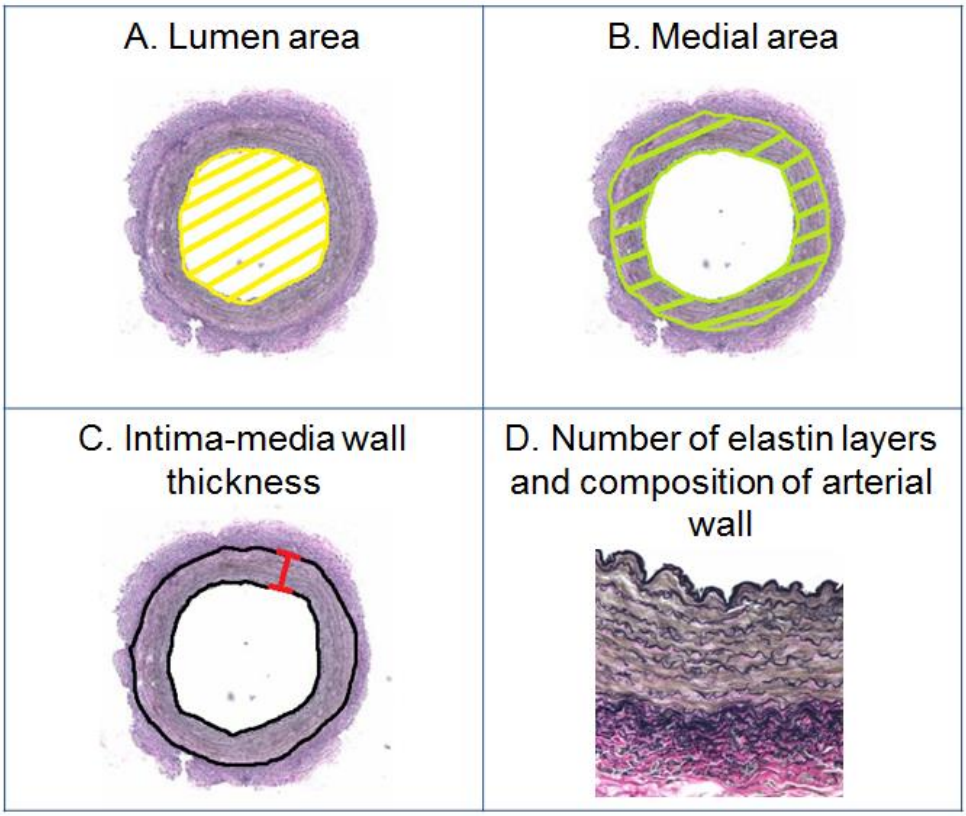

Figure 5.1. *Captured images of the thoracic aorta and left carotid artery to depict how the measurements of lumen area (A), media area (B), intima-media wall thickness (C) and number of elastin layers/composition of arterial wall (D) were investigated.*

5.2.6 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics Version 21 (IBM SPSS, Illinois, USA) and graphed using GraphPad Prism Version 6.0 (GraphPad Software, California, USA).

A student's t-test was used to analyse the hydroxyproline assay data; whereby a comparison of the percentage of collagen within the thoracic aorta and left carotid artery was compared between preterm and term female lambs at 2 days of age. Males were not analysed (see Section 5.2.2, page 147). All other parameters were analysed using a two-way ANOVA, with gestational age at birth (P_G; preterm or term) and sex (P_S; male or female) as factors.

Significant interaction effects detected in the 2-way ANOVA were further analysed by a Tukey's post-hoc test. Data is represented as the means ± SEM.

5.3 Results

5.3.1 Immediate period after birth (2 days of age)

When observed microscopically, there was no evidence of overt injury, such as evidence of breaks in the internal elastic lamina and/or infiltration of inflammatory cells in the blood vessel wall of the thoracic aorta or the left carotid artery.

5.3.1.1 Lumen area (Figure 5.2)

Thoracic aorta

The lumen area of the thoracic aorta was found to be significantly narrower (24.3% reduction; $P_G<0.0005$) in preterm lambs compared to term controls (Figure 5.2A). However, when adjusted to body weight, preterm lambs had a significantly larger lumen area (19.6% increase; $P_G=0.03$) compared to term lambs (Figure 5.2B).

There were no differences between the sexes in the absolute and relative lumen area measurements in the thoracic aorta.

Left carotid artery

Similar to that observed in the aorta, there was also a significant narrowing in the lumenal area of the left carotid artery (48.0% reduction; $P_G<0.0001$) in preterm lambs compared to term lambs (particularly in males), but not when expressed relative to body weight (Figure 5.2C).

Female lambs exhibited a significantly narrower lumen area in the left carotid artery compared to male lambs (28% reduction; $P_S<0.004$) and this remained when adjusted to body weight (Figure 5.2D).

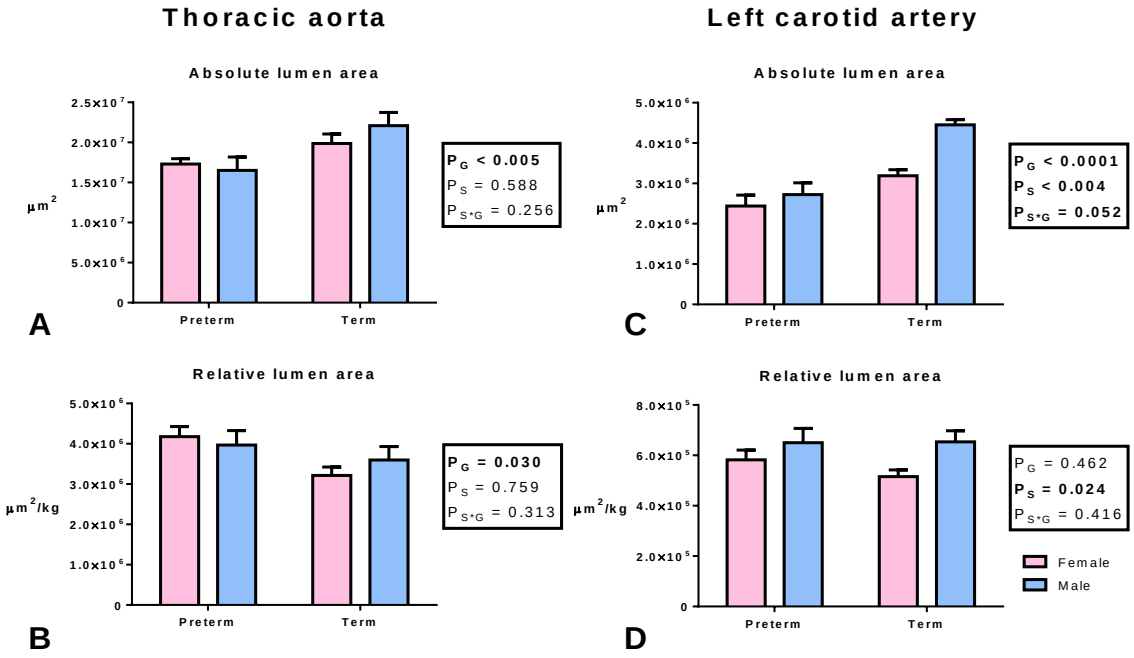

Figure 5.2. *Absolute (top panel) and relative (bottom panel) lumen area measurements for the thoracic aorta (A and B) and left carotid artery (C and D) in preterm (n= 7 female, n=6 male) and term (n=7 female, n=5 male) lambs 2 days after birth. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

5.3.1.2 Media area and media-to-lumen ratio (Figure 5.3)

Thoracic aorta

There were no significant differences observed in the medial cross-sectional area of the aorta in preterm lambs compared to term lambs, nor when adjusted to body weight (Figure 5.3A and 5.3B). Media-to-lumen ratio was also found to be similar between preterm and term lambs (Figure 5.3C).

There were also no differences found between sexes for absolute or relative medial area and media-to-lumen ratio in the thoracic aorta of female and male lambs at 2 days of age.

Left carotid artery

Absolute and relative medial area measurements of the left carotid artery were not significantly different between preterm and term lambs (Figure 5.3D and 5.3E). There was also no difference observed in media-to-lumen ratio of the left carotid artery between preterm and term lambs (Figure 5.3F).

Similarly, female and males lambs showed no differences in the absolute and relative medial area or media-to-lumen ratio of the left carotid artery at 2 days of age.

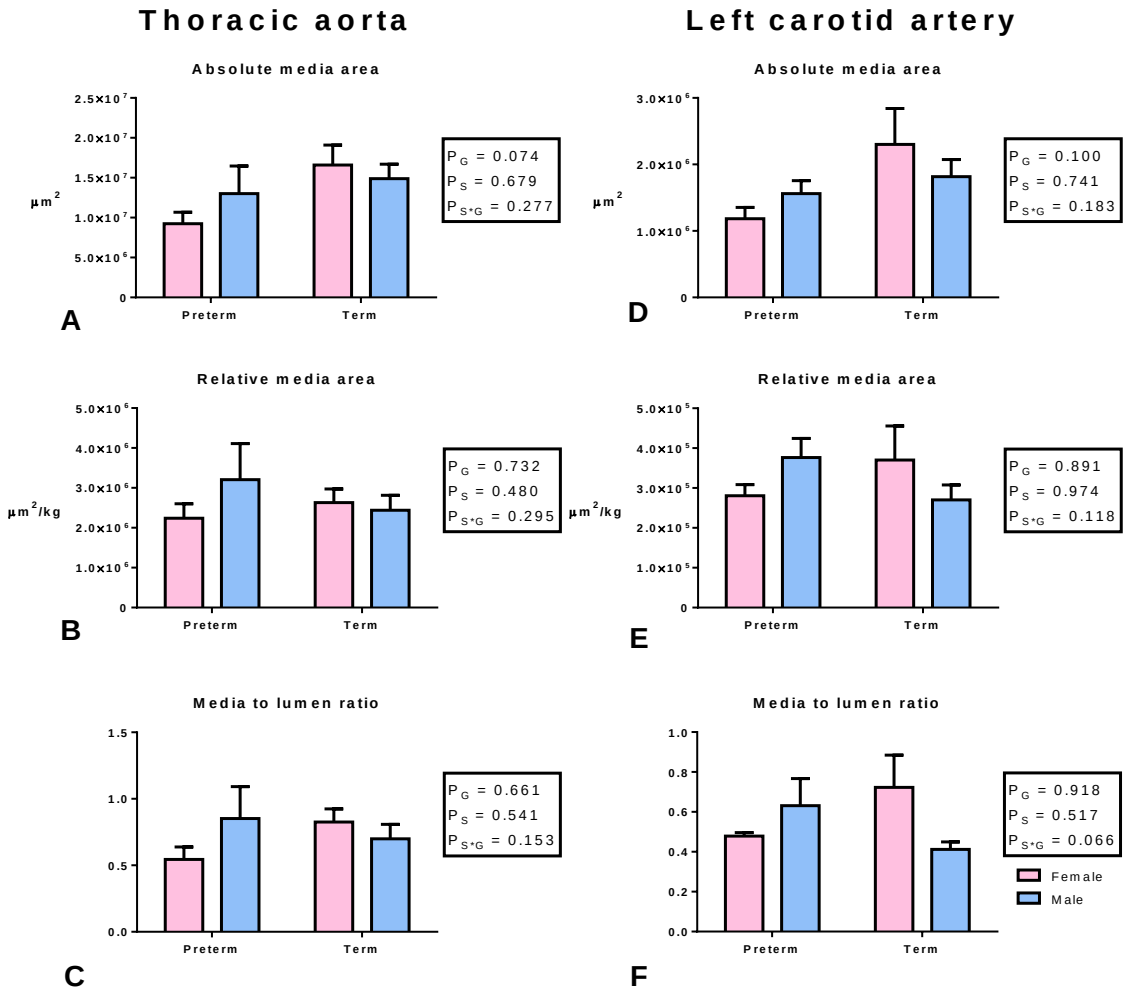

Figure 5.3. *Absolute (top panel) and relative (middle panel) medial area measurements and media-to-lumen ratio (bottom panel) of the thoracic aorta (A, B and C) and left carotid artery (D, E and F) in preterm (n=7 female, n=6 male) and term (n=7 female, n=5 male) lambs 2 days after birth. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

5.3.1.3 Wall thickness (Figure 5.4)

Thoracic aorta

Wall thickness of the thoracic aorta was found to be similar between preterm and term lambs at 2 days of age (Figure 5.4A). However, when adjusted to body weight, aortic walls were significantly thicker (P_G=0.005) in preterm lambs compared to term controls (Figure 5.4B).

There were no differences observed between female and male lambs.

Left carotid artery

The left carotid arterial wall was significantly thinner (31% reduction; P_G=0.049) in preterm lambs compared to term lambs (Figure 5.4C). When adjusted to body weight, this difference was no longer significant with preterm and term lambs having similar wall thicknesses in the left carotid artery (Figure 5.4D).

There were no differences in the wall thicknesses between female and male lambs.

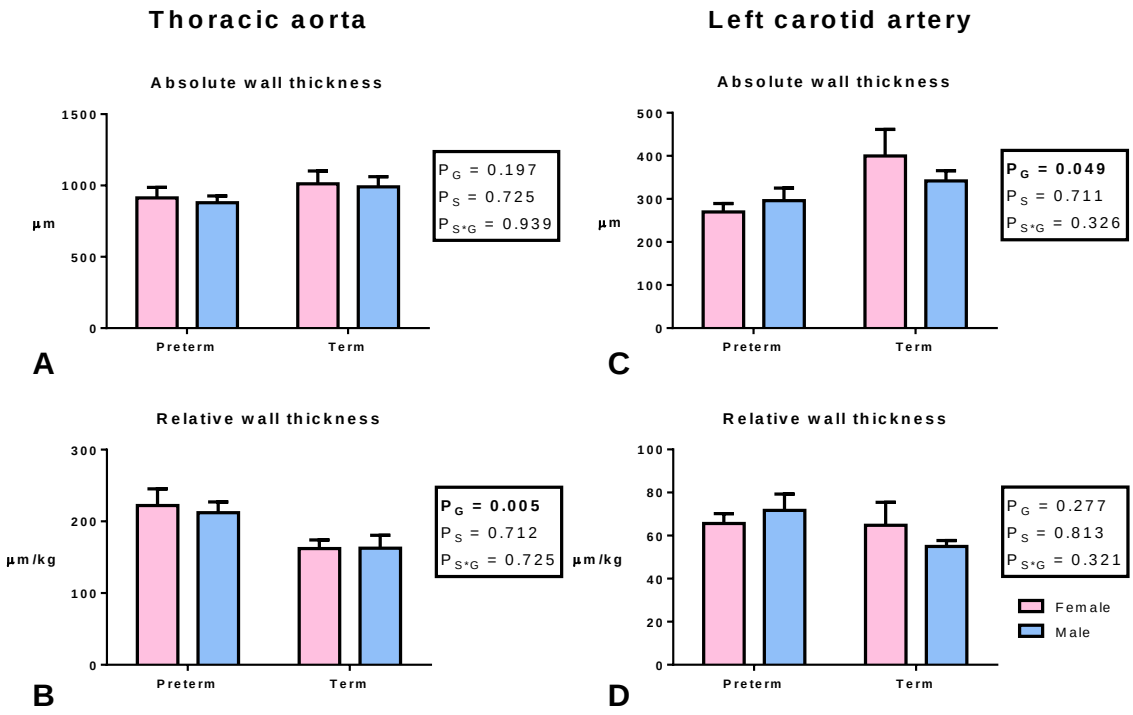

Figure 5.4. *Absolute (top panel) and relative (bottom panel) wall thickness measurements for the thoracic aorta (A and B) and left carotid artery (C and D) in preterm (n=7 female, n=6 male) and term (n=7 female, n=5 male) lambs 2 days after birth. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

5.3.1.4 Layers of elastin (Figure 5.5)

Thoracic aorta

There was no significant difference in the number of elastin layers within wall of the thoracic aorta between preterm and term lambs.

Similarly, there was no difference in the number of elastin layers between the sexes. The average number of elastin layers within the thoracic aorta for all groups was similar and ranged from 55 to 60 layers as seen in (Figure 5.5A).

Left carotid artery

The number of elastin layers in the left carotid artery between preterm and term lambs was not significantly different.

In comparing between males and females, there was also no significant difference in the number of elastin layers at 2 days of age. As seen in Figure 5.5B, there was much variation in the average number of layers for preterm and term female and male lambs.

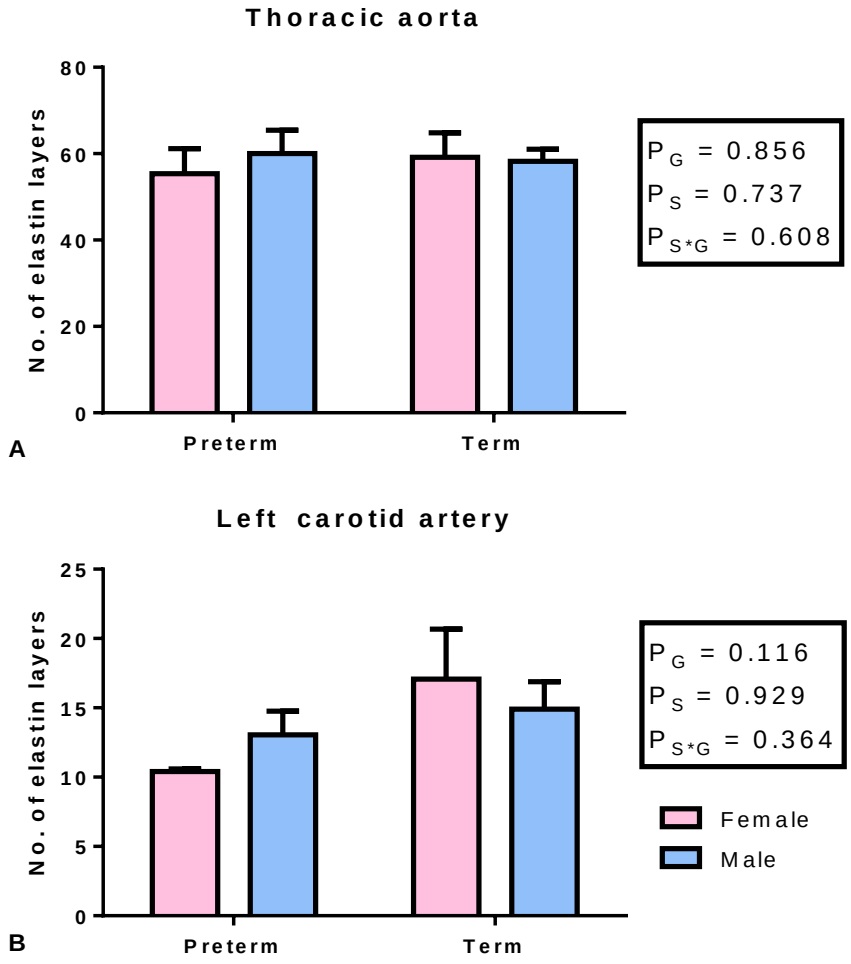

Figure 5.5. *Number of elastin layers in the arterial walls of the thoracic aorta (A) and left carotid artery (B) in preterm (n=7 female, n=6 male) and term (n=7 female, n=5 male) lambs 2 days after birth. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

5.3.1.5 Composition of arterial wall (Figure 5.6)

Thoracic aorta

The percentage of elastin and smooth muscle content in the thoracic aortic wall was not significantly different between preterm and term lambs (Figure 5.6A and 5.6C). However, there was a significantly lower amount of total collagen (P_G=0.048) in the preterm lambs compared to term controls; this was found to be particularly in males (Figure 5.6B).

Female lambs showed a strong trend (P_S=0.058) for lower total collagen deposition in the thoracic aortic wall compared to male lambs. However, elastin deposition and smooth muscle content were similar between female and male lambs.

Left carotid artery

The components of elastin, collagen and smooth muscle content in the left carotid arterial wall were not significantly different amongst preterm and term lambs (Figure 5.6D, 5.6E and 5.6F).

Similarly, there were no differences in the percentages of elastin, collagen and smooth muscle between female and males in the left carotid arterial wall.

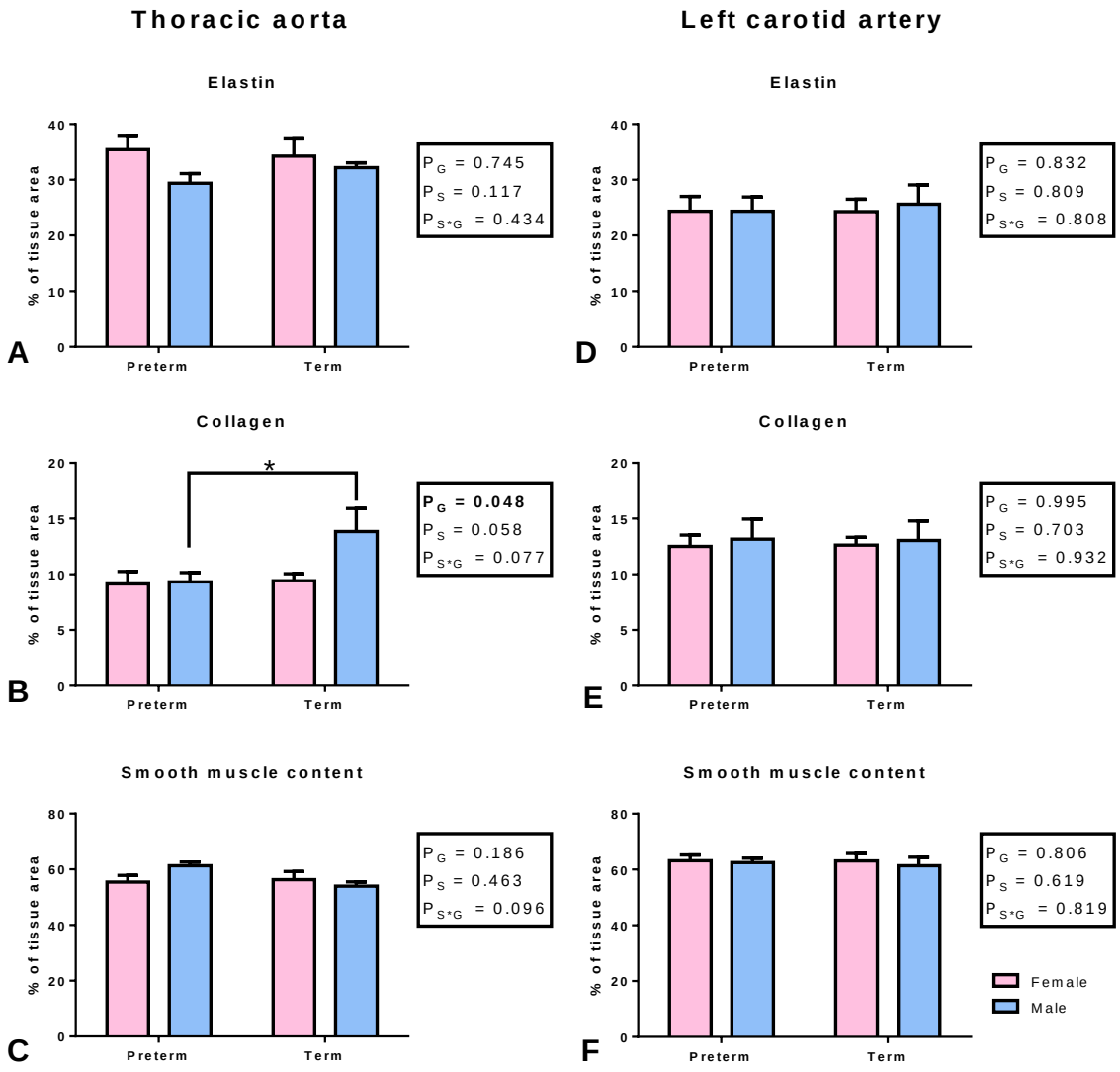

Figure 5.6. *Percentage of tissue area of elastin (top panel), collagen (middle panel) and smooth muscle content (bottom panel) within arterial walls of the thoracic aorta (A, B and C) and left carotid artery (D, E and F) in preterm (n=7 female, n=6 male) and term (n=7 female, n=5 male) lambs 2 days after birth. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors, followed by a Tukey's post-hoc test. *P<0.05 between preterm vs. term within the same sex. Values are means ± SEM.*

5.3.1.6 Collagen content (Figure 5.7)

To analyse percentage of collagen within the arterial walls via the hydroxyproline assay method, frozen tissue samples were utilised. Sex differences could not be examined as there were insufficient numbers of samples and therefore t-test analyses were performed to investigate differences between preterm female and term female lambs only.

Thoracic aorta

Figure 5.7A shows that the percentage of collagen was not significantly different in the thoracic aorta between preterm female and term female lambs 2 days after birth.

Left carotid artery

There was also no difference in the percentage of collagen in the left carotid artery between preterm and term female lambs (Figure 5.7B).

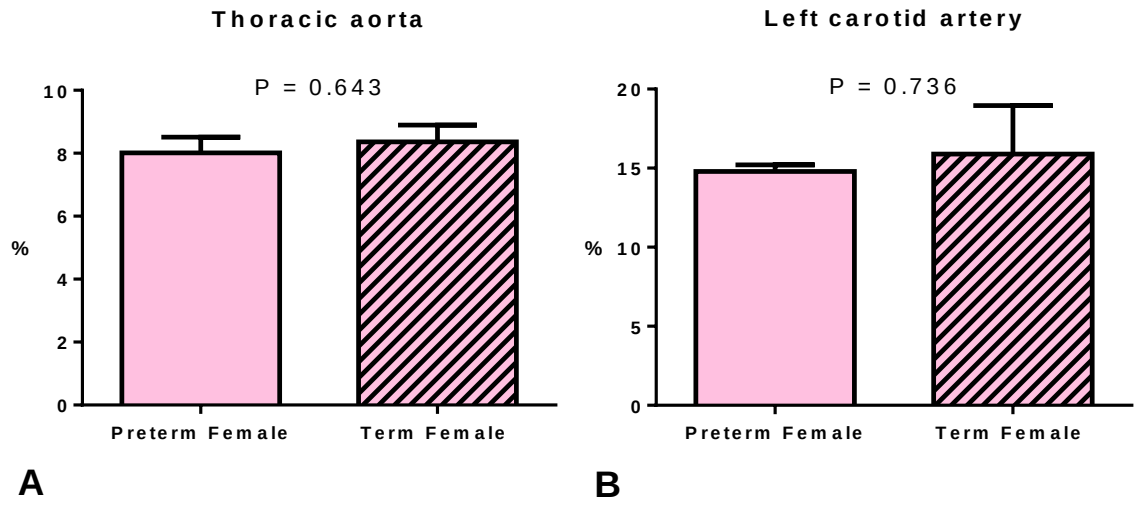

Figure 5.7. *Percentage of collagen in the walls of the thoracic aorta (A) and left carotid artery (B) of preterm female (n=6; pink) and term female (n=7, pink hashed) lambs 2 days after birth. Data was analysed using a student's t-test. Values are means ± SEM.*

5.3.2 In adulthood (14.5 months of age)

In the long-term cohort of sheep, there was no evidence of injury, such as overt lesions (that have been repaired) within the arterial walls of the thoracic aorta or the left carotid artery in any group. Vessels collected were excised from the same anatomical site in all sheep and also the same site as in the short-term cohort. Therefore, comparisons on the changes of morphometric and structural measurements can be discussed between lambs in the immediate period after birth and the sheep in young adulthood.

5.3.2.1 Lumen area (Figure 5.8A and 5.8D)

Thoracic aorta

There were no significant differences in the lumen area of the thoracic aorta between preterm and terms (Figure 5.8A). When adjusted for body weight, lumen area was also not significantly different between preterm and term lambs.

There were also no differences observed in the absolute or relative lumen area between females and males in adulthood.

In contrast, there was a significant interaction effect (P_{S*G}<0.01), where preterm males had a significantly narrower lumen area (P=0.041) when compared to term males only, whereas no difference was observed between females. However, when adjusted for body weight, the interaction effect no longer reached statistical significance (P_{S*G}=0.053).

Left carotid artery

There were no significant differences observed in lumen areas between preterm and term sheep, nor when adjusted for body weight (Figure 5.8D).

Similarly, female and male sheep had similar absolute and relative lumenal areas.

5.3.2.2 Media area (Figure 5.8B and 5.8E)

Thoracic aorta

Media area was not significantly different between preterm and term sheep in the thoracic aorta (Figure 5.8B). Media area relative to body weight was also not significantly different between preterm and term sheep.

Importantly, however, there was a significant interaction effect (P_{S*G}=0.042), whereby preterm males had significantly reduced medial areas (P=0.023) compared to term males, but medial areas between females remained similar.

Left carotid artery

Figure 5.8E shows preterm sheep had smaller medial areas in the left carotid artery compared to term sheep, but this did not quite reach statistical significance (P_G=0.063). There was no difference in the relative medial areas between preterm and term sheep.

Furthermore, there were no differences observed in the absolute and relative medial areas in the left carotid artery between female and male sheep.

5.3.2.3 Media-to-lumen ratio (Figure 5.8C and 5.8F)

Thoracic aorta

Figure 5.8C shows that media-to-lumen ratio was not significantly different in the thoracic aorta between preterm and term sheep.

There were also no differences in media-to-lumen ratio between female and male sheep.

Left carotid artery

Similar to the thoracic aorta, there was no significant difference in media-to-lumen ratio in the left carotid artery between preterm and term sheep (Figure 5.8F).

Media-to-lumen ratio of the left carotid artery in female and male sheep was also similar.

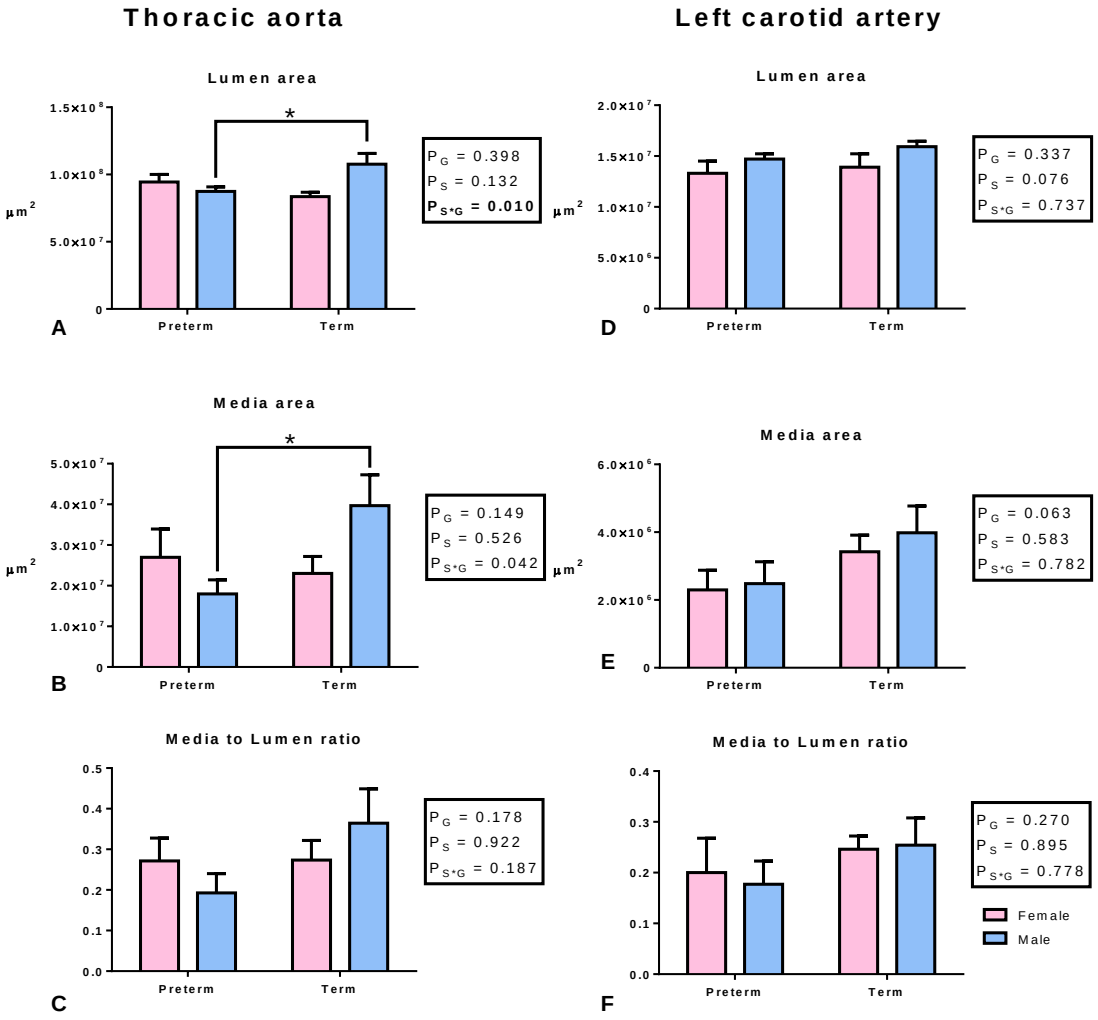

Figure 5.8. *Absolute lumen area (top panel), absolute media area (middle panel) and media-to-lumen area (bottom panel) measurements for the thoracic aorta (A, B and C) and left carotid artery (D, E and F) in preterm (n=7 female, n=7 male) and term (n=6 female, n=7 male) sheep at 14.5 months of age. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors, followed by a Tukey's post-hoc test. *P<0.05 between preterm vs. term within the same sex. Values are means ± SEM.*

5.3.2.4 Wall thickness (Figure 5.9)

Thoracic aorta

Overall, there was no significant difference in absolute (Figure 5.9A) or relative wall thickness (Figure 5.9B) of the thoracic aorta in preterm sheep compared to term sheep. However, there was a significant interaction effect (P_{S*G}=0.023) with a marked reduction in aortic wall thickness in preterm males versus term males, whereas there was no difference between preterm and term females. Preterm males had significantly thinner thoracic aortic walls (P=0.013) compared to term males, but when adjusted for body weight, there was no longer a significant difference.

Left carotid artery

There were no significant differences in absolute or relative wall thicknesses of the left carotid artery between preterm and term sheep in adulthood (Figure 5.9C and 5.9D).

Likewise, female and male sheep exhibited a similar absolute and relative wall thickness in the left carotid artery.

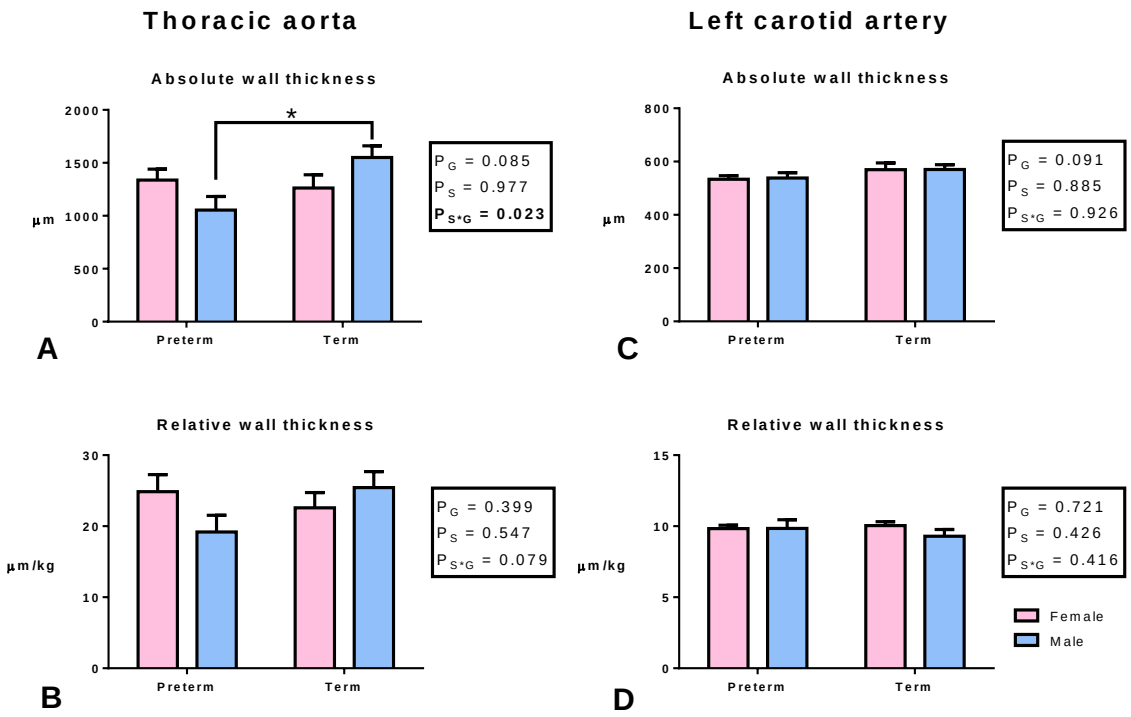

Figure 5.9. *Absolute (top panel) and relative (bottom panel) wall thickness measurements for the thoracic aorta (A and B) and left carotid artery (C and D) in preterm (n=7 female, n=7 male) and term (n=6 female, n=7 male lambs) at 14.5 months of age. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors, followed by a Tukey's post-hoc test. *P<0.05 between preterm vs. term within the same sex. Values are means ± SEM.*

5.3.2.5 Layers of elastin (Figure 5.10)

Thoracic aorta

Preterm birth impacted the number of elastin layers within the thoracic aorta in adulthood. Sheep born moderately preterm had significantly fewer elastin layers in the thoracic aorta (Figure 5.10A, P_G=0.027) compared to sheep born at term. This was attributed to marked effects specifically in preterm males with a significant interaction effect observed (P_{S*G}=0.032); preterm males had significantly fewer (P=0.008) elastin layers compared to term males but there was no difference between preterm and term female sheep.

When comparing between short-term and long-term cohorts, the number of elastin layers within the thoracic aortic wall increased significantly over time for sheep born at term (short-term: 59 ± 1 layers vs. long-term: 70 ± 2 layers; P_T=0.017). However, there was no difference in the number of elastin layers in the preterm group over time (short-term: 58 ± 2 layers vs. long-term: 60 ± 7 layers; P_T=0.667).

Left carotid artery

Preterm sheep had significantly fewer elastin layers in the left carotid artery (Figure 5.10B, P_G=0.012) compared to term sheep.

There were no significant differences found in the number of elastin layers in the left carotid arterial wall between female and male sheep.

When comparing between the short-term and long-term results, the number of elastin layers remained similar for both the preterm group and the term group in the short-term and long-term cohorts.

Overall, however, as the number of elastin layers was not significantly different in the short-term cohort, but significantly reduced by 14.5 months of age in both vessels of preterm sheep compared to term sheep; this shows that the development of elastin layers over time was affected by moderately preterm birth.

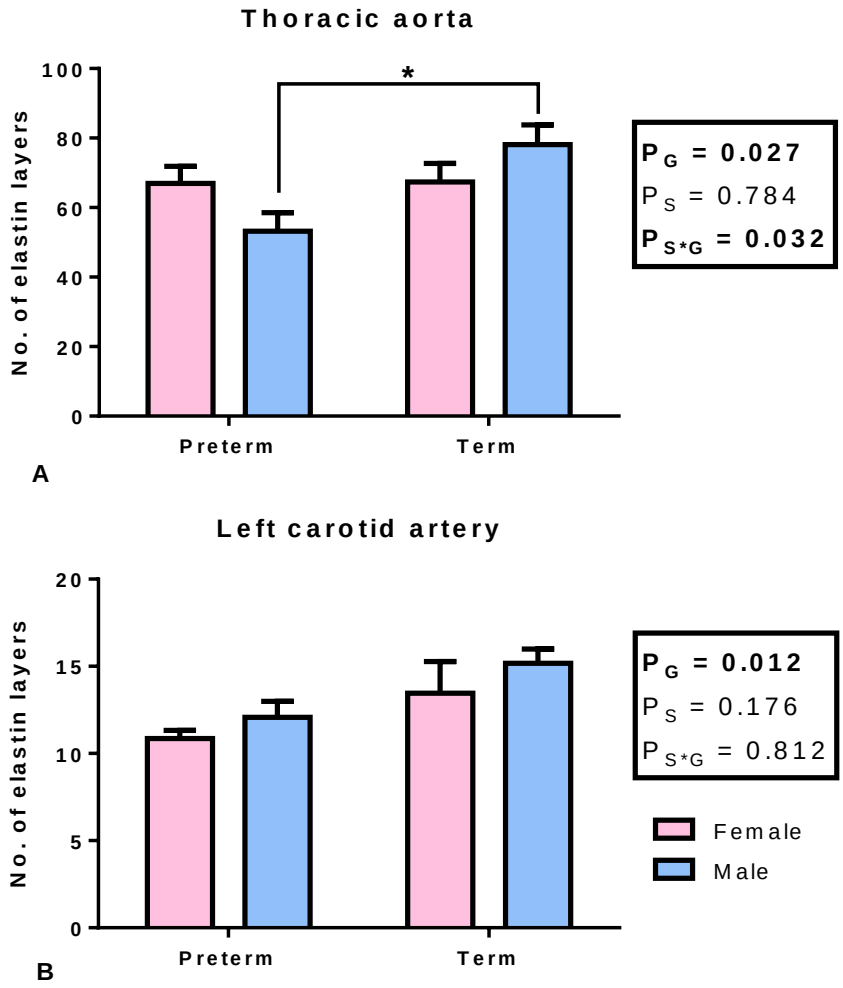

Figure 5.10. *Number of elastin layers in the thoracic aorta (A) and left carotid artery (B) in preterm (n=7 female, n=7 male) and term (n=6 female, n=7 male) sheep at 14.5 months of age. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors, followed by a Tukey's post-hoc test. *P<0.05 between preterm vs. term within the same sex. Values are means ± SEM.*

5.3.2.6 Composition of arterial wall (Figure 5.11)

Thoracic aorta

The percentage of elastin, collagen and smooth muscle area in the thoracic aorta was not significantly different between preterm and term sheep (Figure 5.11A, 5.11B and 5.11C).

Similarly, there were no significant differences in the composition of the thoracic aortic wall between female and male sheep.

In comparing the biochemical composition of the thoracic aortic wall over time, from short-term to long-term, there were no significant changes to the percentage of tissue area of elastin, collagen or smooth muscle content in the preterm animals. However, in the thoracic aorta of the term groups, percentage of collagen was significantly reduced over time (P_T=0.001) and there was a significant interaction effect (P_{S*T}=0.003), with term male lambs in the short-term having significantly higher (P<0.001) percentage of collagen compared to term male sheep in the long-term. Additionally, there was a strong trend for the percentage of elastin to be higher (P_T=0.063) in the long-term sheep compared to the short-term lambs in the term group.

Left carotid artery

Elastin deposition was found to be significantly reduced (P_G=0.0003) in the left carotid artery of preterm sheep compared to term sheep (Figure 5.11D). In contrast, smooth muscle content was found to be significantly greater (P_G=0.002) in the left carotid arterial walls in preterm sheep compared to term sheep (Figure 5.11F). However, no differences were observed in collagen deposition between preterm and term sheep (Figure 5.11E).

Overall, there were no differences in the percentage of elastin, collagen and smooth muscle area in the left carotid arterial wall between female and male sheep.

There was a strong trend for an interaction effect (P_{S*G}=0.055); with preterm females having significantly less (P=0.001) elastin compared to term females.

Over time, the percentage of elastin and collagen within the left carotid artery wall was significantly reduced in the long-term cohorts compared to the short-term cohorts (preterm

group: P_T<0.0001 (elastin), P_T=0.001 (collagen); term group: P_T=0.003 (elastin), P_T=0.006 (collagen)). However, smooth muscle content was significantly higher (preterm group: P_T<0.0001; term group: P_T<0.0001) in the left carotid artery over time in both preterm and term sheep.

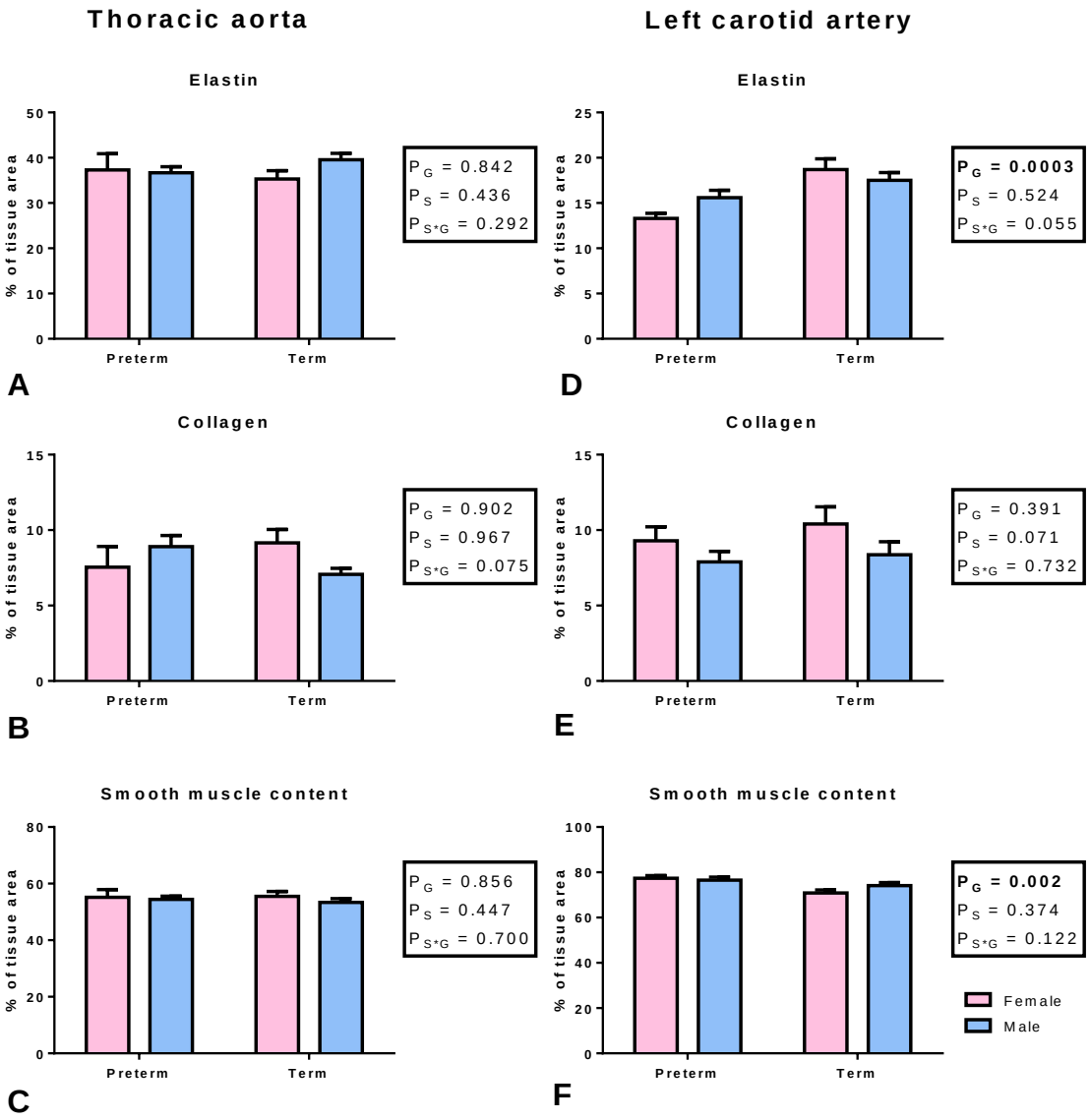

Figure 5.11. *Percentage of tissue area of elastin (top panel), collagen (middle panel) and smooth muscle content (bottom panel) within the arterial walls of the thoracic aorta (A, B and C) and left carotid artery (D, E and F) in preterm (n=7 female, n=7 male) and term (n=6 female, n=7 male) sheep at 14.5 months of age. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

5.3.2.7 Collagen content (Figure 5.12)

Thoracic aorta

The percentage of collagen, determined by the amount of hydroxyproline amino acid present within the blood vessel wall, was not significantly different in the thoracic aortic wall between preterm and term sheep at 14.5 months of age (Figure 5.12A).

Overall, the percentage of collagen was found to be significantly lower (P_S<0.005) in female sheep compared to male sheep.

Left carotid artery

The percentage of collagen was not significantly different in the left carotid artery between preterm and term sheep (Figure 5.12B).

There was also no difference in the percentage of collagen in the left carotid artery between female and male sheep.

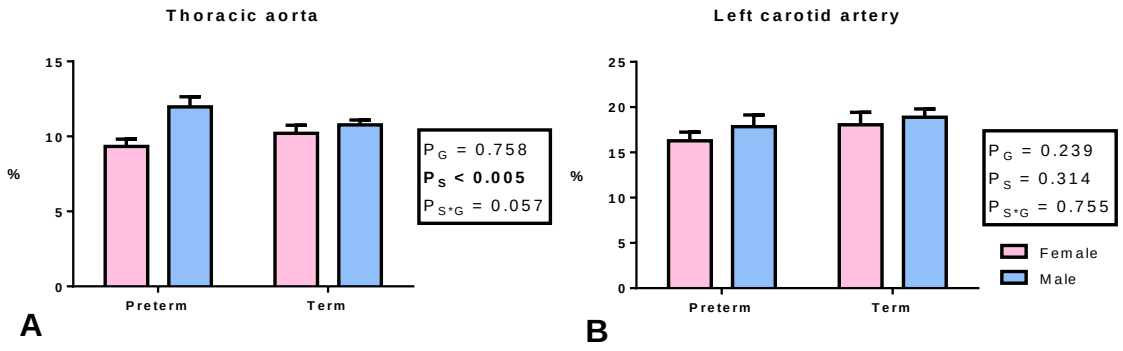

Figure 5.12. *Percentage of collagen in the thoracic aorta (A) and left carotid artery (B) of preterm (n=7 female, n=9 male) and term (n=6 female, n=11 male) sheep at 14.5 months of age. Pink shading represents females and blue shading represents males. Data were analysed using a 2-way ANOVA with gestational age at birth (P_G; preterm or term), sex (P_S; male or female) and their interaction (P_{S*G}) as factors. Values are means ± SEM.*

5.4 Discussion

5.4.1 Summary

This chapter investigates the effect of moderate preterm birth in an ovine model on the structure of the thoracic aorta and left carotid artery in the immediate period after birth and in early adulthood. In this study, there was no evidence of overt arterial injury to the aorta or left carotid artery in the immediate period following moderate preterm birth. At this time, the lumen of both the thoracic aorta and left carotid artery were narrower in preterm lambs compared to term lambs and there was a reduction in the intima-media thickness of the left carotid artery. These differences were not apparent when adjusted for body weight (with lumen area relative to body weight actually increased in the thoracic aorta) and therefore, were likely attributed to the reduced body size in the preterm lambs. Additionally, there were no differences in the composition of the blood vessel wall in either the thoracic aorta or left carotid artery between preterm and term lambs, except the proportion of collagen within the wall of the thoracic aorta was reduced in preterm males compared to terms. This difference was not observed between preterm and term females and was confirmed in the hydroxyproline analyses.

In adulthood, the number of elastin layers and thus overall elastin content were significantly reduced in the left carotid artery of preterm sheep, whereas the smooth muscle content increased. Importantly, there was sexual dimorphism in the long-term effects of preterm birth on aortic structure. There were significant reductions in lumen size, medial area, intima-media thickness and layers of elastin in the thoracic aorta in adult male preterm sheep compared to terms, whereas in adult females, there were no apparent differences in aortic structure between sheep born preterm and term.

5.4.2 No evidence of arterial injury in the immediate period after preterm birth

Previous studies in our laboratory have demonstrated that moderate preterm birth in sheep leads to injury in the ascending aorta (Bensley et al., 2012). Hence, at the commencement of my studies, it was considered likely that there may be widespread arterial injury to immature conduit arteries prematurely exposed to high postnatal arterial pressures. Encouragingly, however, we did not observe injury in the thoracic aorta or the left carotid artery of lambs 2

days after birth. Therefore, it is conceivable that the ascending aorta might be the only vascular region severely affected, as postnatally it is the region of highest dynamic pressures. Of concern, however, although there was no overt structural injury in the arterial walls of the conduit blood vessels examined, concomitant gene expression studies (performed by research assistant, Ms. Tracey Flores) demonstrated that there was increased mRNA expression of *P-selectin* (P=0.004) in the thoracic aorta and elevated mRNA expression of *Caspase-3* (P=0.032) and *IL-1β* (P=0.046) in the left carotid artery of preterm lambs compared to term lambs. *P-selectin* is a cell adhesion molecule on endothelial cells and platelets. It is a key marker of endothelial injury as it plays a major role in the initial recruitment of leukocytes at sites of injury during inflammation (Kansas, 1996, Blann et al., 2003, Manka et al., 2004). Activated *Caspase-3* is a key mediator of cellular apoptosis (Mallat and Tedgui, 2000), and *IL-1β* is a cytokine protein involved with the acute inflammatory response (Newton and Dixit, 2012). Hence, although there was no evidence of visible structural damage to the thoracic aorta or left carotid artery in my morphological analyses, there is clearly evidence of arterial injury at the molecular level.

5.4.3 Preterm birth impacts the biochemical composition and wall thickness of conduit arteries

In humans, it is well known that towards the end of gestation (near term), the large conduit arteries structurally prepare for the haemodynamic transition that occurs at birth (Berry et al., 1972, Martyn and Greenwald, 1997). In characterising the structural changes within the arterial wall that occur at this time, Bendeck and Langille (1991) reported significant and rapid accumulation of elastin and collagen in the thoracic and abdominal aorta of fetal sheep during the perinatal period (between 140 days gestation and 3 days after birth; term was 145 days gestation). In contrast, my findings suggest that by 0.9 of term (132 days gestation), the layers of elastin within the conduit artery wall have already been laid down, as there were no significant differences in the number of elastin layers, or proportion of elastin in the aorta or left carotid artery between preterm and term lambs at 2 days of age; likewise, the percentage smooth muscle content was not different. This difference in findings in relation to elastin deposition between my study and those reported previously by Bendeck and Langille (1991) may relate to the fact that in my study the fetal lambs were exposed to antenatal corticosteroids; this may have led to accelerated maturation and thus accelerated deposition of elastin within the conduit arteries of the preterm lambs.

Indeed, corticosteroids have been previously shown to stimulate elastin and collagen accumulation in the blood vessel wall (Leitman et al., 1984, Bendeck et al., 1994). For example, Bendeck et al. (1994) reported that administration of dexamethasone to fetal sheep upregulated (approximately 2 to 3-fold) mRNA expression of tropoelastin in the abdominal aorta, carotid and mesenteric arteries by 140 days gestation. These investigators therefore hypothesised that cortisol could stimulate elastin accumulation in late gestation. In addition, in the studies by Bensley et al. (2012), it was suggested that the increased elastin deposition in the aorta found in moderately preterm lambs at 9 weeks PTEA, may have been due to antenatal corticosteroid exposure of betamethasone rather than preterm birth *per se*. Moreover, exposure of blood vessels to corticosteroids has also been linked to impairment of endothelial and vascular smooth muscle function (Pulgar and Figueroa, 2006, Roghair et al., 2007). Hence, it may be the exposure of the fetus to betamethasone that has mediated the injurious gene expression changes (as described previously) to the thoracic aorta and left carotid artery in my preterm lambs at 2 days after birth. In future experiments, it would be beneficial to perform further studies to investigate a cohort of term lambs exposed to betamethasone or a cohort of preterm lambs not exposed to betamethasone; however, the latter option would require additional postnatal ventilation of the lambs in order for them to be viable.

Interestingly, unlike that observed in elastin deposition, collagen deposition was found to be significantly increased in the thoracic aortic wall of the term lambs in my study compared to preterm lambs; thus supporting the concept that collagen is laid down in the perinatal period and also suggesting that antenatal corticosteroid exposure does not affect vascular collagen deposition at this time. Given that, the function of collagen in the wall of the conduit artery is to provide strength and prevent elastic blood vessels from stretching beyond their physiological limits (Ooshima et al., 1974), the reduced collagen content in the thoracic aorta of preterm sheep has the potential to adversely impact aortic function in the neonatal period. Overall, the elasticity, flexibility and strength of the arterial wall are highly dependent on the ratio of elastin to collagen deposition. Hence, given that the percentage collagen was decreased in the preterm aorta, this may impact on the mechanical properties of the aortic wall and on function. Importantly, at the end of my experiment, segments of the abdominal aorta were freshly excised at necropsy for functional analyses using myography. These studies were performed by Prof. Helena Parkington and Dr. Marianne Tare from the Department of Physiology at Monash University, but data has not yet been analysed. It will be interesting to

see, in their studies, whether there are functional differences in the abdominal aorta of preterm lambs versus terms.

In my study, there was no difference in wall thickness of the thoracic aorta between preterm and term lambs in the immediate period after birth. Interestingly, however, the left carotid arterial wall was significantly thinner in preterm lambs compared to term lambs. The thinner arterial walls appear to be attributed to the smaller body size of the preterm lambs, given that wall thickness was not significantly different when adjusted to body weight. Nevertheless, as arterial pressure was not significantly different between preterm and term lambs in the immediate period after birth, exposure to the same arterial pressures as term lambs, when the wall is thinner in the preterm carotid artery, may lead to adaptive remodelling in the neonatal period and to potential adverse consequences in the long-term.

Human studies have shown that arterial wall thickening occurs in infants after preterm birth. For instance, ultrasound studies in the first 6 months of life of babies born very preterm have shown significantly higher aortic and carotid intima-media thickness in relation to vessel diameter compared to those born at term (Schubert et al., 2013). Furthermore, Skilton et al. (2011) reported increased carotid intima-media thickness in young adults born preterm (before 37 weeks of gestation) compared to those born at term. The thickening of the arterial walls is reportedly due to smooth muscle hypertrophy which results in thickening of the intima-media layer (Gaballa et al., 1998). This increased arterial wall thickness in subjects born preterm, may render the vasculature vulnerable to cardiovascular disease and could be a significant predictor for future cardiovascular risk (Berenson et al., 1998). Hence, it appears that vascular adaptations may occur in the vasculature of the preterm infant in the early neonatal period, which may lead to long-term adverse consequences.

In accordance with the human findings, Bensley et al. (2012) reported maladaptive remodelling of the aortic wall following moderate preterm birth, with increased elastin deposition, but reduced smooth muscle content in the aorta of preterm lambs at 9 weeks PTEA. In that study, the number of elastin layers within the aortic wall was not counted. Interestingly, in my study, when using a similar model of moderate preterm birth in sheep as Bensley et al. (2012) (except that the antenatal dose of betamethasone was higher), the number of elastin layers within both the thoracic aorta and left carotid artery was significantly less in preterm adult sheep, with an overall significantly reduced percentage elastin content in the left carotid artery compared to term adult sheep.

Collectively, my findings in the short-term and long-term cohorts showed that the number of elastin layers in the thoracic aorta (but not the left carotid artery) significantly increased from birth to adulthood in term sheep. Interestingly, however, the number of layers in the blood vessel wall was not significantly increased in either of the preterm vessels studied over time and as a result there were significantly fewer layers of elastin in both the aorta and left carotid artery in adulthood compared to terms. Notably, there was a gain in the number of aortic elastin layers in the term males (approximately +20 layers), whereas there was no significant change in the number of layers in the preterm males. In females, there was an intermediate increase in the number of aortic elastin layers (approximately +10 layers) for both groups. The findings in the preterm sheep suggest that the postnatal growth of the conduit arteries and deposition of elastin may be influenced by body growth, with the sheep born preterm remaining significantly smaller through to adulthood. The significantly fewer elastin layers in both conduit arteries in adulthood (and reduced percentage elastin in the left carotid artery) is clinically important because reductions in elastin properties have been shown to promote arterial stiffness, especially with advancing age (Izzo and Shykoff, 2001, Hamilton et al., 2007b). Of concern, arterial stiffness has been associated with the development of a number of cardiovascular complications such as hypertension, atherosclerosis, heart disease and stroke (de Groot et al., 2004, Mattace-Raso et al., 2006, Payne et al., 2010, Wagenseil and Mecham, 2012, Liu et al., 2015). For example, such changes in arterial wall structure can adversely impact the function of the arteries as they lose their elasticity and therefore have reduced compliance. The stiffer the artery, the more pressure that is required to distend the walls and this can lead to hypertension (Wagenseil and Mecham, 2012).

Interestingly, although there was a significant reduction in the number of elastin layers of the thoracic aorta in male preterm sheep compared to male term sheep in adulthood, the percentage tissue area of elastin was not significantly different between these two groups. This implies that the thickness of the elastin layers was greater in the male preterm sheep but further studies are required to confirm this.

5.4.4 Effect of preterm birth on lumen size in the conduit arteries

In this study, the lumen of the thoracic aorta and left carotid artery was significantly reduced in preterm lambs compared to term lambs in the immediate period following birth. This was

attributed to the smaller body size of the preterm lambs, given that lumen area when adjusted to body weight was not significantly different. My findings of a narrower arterial lumen size in the conduit arteries are in accordance with a narrower lumen area in the arteries of infants born preterm. A study investigating aortic and carotid artery growth in very preterm infants reported arterial narrowing in the first 6 months of life when compared to term infants (Schubert et al., 2011).

In the postnatal period, Bensley et al. (2012), using a similar sheep model of preterm birth, found that the lumen of the ascending aorta was significantly narrower in preterm lambs at 9 weeks PTEA when compared to terms, thus indicating that the narrowed aortic lumen persists into childhood. In accordance with these findings, imaging studies of the vasculature in adolescent girls and adults born preterm (mean gestational age of 29 weeks) also report a narrower lumen area in the thoracic aorta and abdominal aorta compared to those born at term; in conjunction with significantly higher arterial pressures (Bonamy et al., 2005, Edstedt Bonamy et al., 2008). In accordance with these findings, in my studies, I also found a significant reduction in the size of the lumen in the aorta of preterm male lambs in adulthood compared to terms; however, this was not evident in the aortae of preterm females and the lumen of the left carotid artery was not affected by preterm birth in either sexes.

Although there was no difference in arterial pressure in the preterm and term sheep at 14.5 months of age, given my findings of altered aortic wall structure, as well as narrowed lumen, in the aortae of male preterm lambs, it is conceivable that elevations in arterial pressure may ensue. In this regard, it will be important in future studies to compare the structure of the resistance vessels in my preterm and term sheep. It is well established that narrowing of the lumen of resistance vessels leads to a rise in total peripheral resistance and to a subsequent rise in arterial pressure (Korner et al., 1989, Intengan and Schiffrin, 2000, Taherzadeh et al., 2010). Hence, it is possible that it may be narrowing of the resistance vessels in subjects born preterm that render them vulnerable to hypertension. Importantly, in this regard, at the end of my experiments, in both the short-term and long-term studies, I did collect segments of resistance vessels (third-order mesenteric arteries) at necropsy. Examination of the structure and composition of these vessels will potentially provide valuable insight into the mechanisms leading to hypertension in preterm subjects.

5.4.5 Implication of findings to long-term arterial pressure

Certainly, there have been many recent epidemiological studies that have reported an association between preterm birth and the development of hypertension in later life (Johansson et al., 2005, Bonamy et al., 2007, Crump et al., 2011, Kerkhof et al., 2012, Sutherland et al., 2014, Gunay et al., 2014), with increases in arterial pressure in adulthood directly linked with decreasing gestational age at birth. For example, in a Swedish study of young men born preterm (average age of 18.2 years), there was a 0.31 mmHg increase of systolic blood pressure for every week born less than 37 completed weeks of gestation (Johansson et al., 2005). Unlike the studies described in humans, in my study the arterial pressure in my preterm sheep was not elevated at any time during the experimental period, and in early adulthood (14.5 months of age) there was no difference in arterial pressure between the preterm sheep and term sheep. Given that the sheep were born at 0.9 of gestation (moderately preterm), the human data would suggest that the effects on arterial pressure are likely to be subtle and they may not manifest until older age. Indeed, studies of the structure of the resistance vessels in my sheep model (described previously) will help to ascertain whether long-term elevations in arterial pressure are likely to occur and given my long-term vascular findings, it may be only males that develop long-term adverse consequences.

5.4.6 Sexual dimorphism in the effects of preterm birth on long-term vascular structure

In my long-term studies, there were significant reductions in lumen area, media area, intima-media thickness and number of elastin layers in the thoracic aorta of male sheep born preterm when compared to terms, but these differences were not apparent in females. This may relate to the 'male disadvantage' that is associated with preterm birth, where male infants have been shown to be particularly vulnerable to preterm birth and are at higher risk of developing health issues compared to females after preterm birth (Ingemarsson, 2003, Elsmén et al., 2004b). It is hypothesised that this is due to the delayed development of organ systems in males and so render the preterm male infants' vulnerable in early life (Peacock et al., 2012). Alternatively, there may be specific hormonal effects in preterm offspring (for example, relating to changes in testosterone in males and/or estrogen in females) that have led to the sexual dimorphism in long-term vascular structure.

Interestingly, in my findings, there was no observable structural immaturity in the thoracic aorta and left carotid arteries of preterm male lambs compared to term male lambs in the immediate period following birth; however, marked changes did develop by adulthood. Adaptations in vascular structure made by males born preterm may be beneficial in the short-term, but if they persist into adulthood such as the differences seen in my long-term study, they may lead to adverse cardiovascular consequences. It is known in the general population that hypertension more commonly affects males compared to females in early adulthood (Pemu and Ofili, 2008). The findings in my study showing structural differences in the thoracic aorta and left carotid artery of male preterm sheep may contribute to the underlying aetiology of hypertension in young men and their increased vulnerability compared to young women.

5.4.7 Conclusion

In conclusion, the findings of my study have clearly shown that there are structural differences in the thoracic aorta and left carotid structure following moderate preterm birth, particularly prevalent in adult male sheep. The reductions in lumen area, media area, wall thickness and number of elastin layers in the thoracic aorta of adult preterm male sheep compared to term male sheep are likely to render the adult preterm male vulnerable to secondary insults that may develop in later life. Interestingly, given that arterial pressure was similar between preterm and term sheep in early adulthood, thinner arterial walls and fewer elastin layers may also render adult preterm males more vulnerable to potential deleterious vascular consequences. Additionally, the narrowing of the lumen area in the thoracic aorta (but not in the left carotid artery) is interesting and suggests that other blood vessels should be explored. Importantly, it would be beneficial to investigate the structure of the resistance vessels of my preterm sheep as narrower resistance vessels are known to lead to a rise in total peripheral resistance and ultimately elevated arterial pressure.

Chapter 6:

General Discussion

The findings of this thesis provide important insight into how moderate preterm birth affects the structure of the cardiovascular system. The findings highlight differences in the structure of the heart and large conduit arteries in the immediate period following moderate preterm birth compared to terms and show evidence of altered structure by early adulthood, particularly in males born preterm.

6.1 Preterm birth: a major insult to immature organ systems

Prior to the studies conducted in our laboratory (studies by Bensley et al. (2010) and those described in this thesis), there have been very few studies that have looked specifically at the effects of moderate preterm birth on the cardiovascular system. Most studies to date, looking at the effects of preterm birth on immature organ systems, have focused on the effects of extremely and very preterm birth, as it is these infants that develop the most adverse complications and are at higher risk of morbidity and mortality (Basso and Wilcox, 2010, Stoll et al., 2010). Indeed, as gestational age at birth decreases the adverse consequences to neonatal health increases; the earlier a baby is born, the more immature the organ systems are, and the greater risk of injury and compromise in organ function. Regardless of gestational age at birth, all preterm survivors are at high risk of developing adverse health complications, with the adverse consequences to these very vulnerable babies numerous (Boyle et al., 2012). Males born preterm have been found to be particularly vulnerable to preterm birth exhibiting a heightened risk of death and disability after preterm birth when compared to females born preterm (Stevenson et al., 2000, Kent et al., 2012, Roy et al., 2014). As well, there is a higher incidence of preterm birth in male infants (Ingemarsson, 2003).

Much of the research to date has investigated the effect of preterm birth on the immature lungs and brain, as it is complications in these organs that have the greatest impact on neonatal survival and long-term quality of life. Clinical complications associated with preterm birth in the lungs include respiratory distress syndrome, bronchopulmonary dysplasia and asthma (Moss, 2006, Joshi and Kotecha, 2007, Mwansa-Kambafwile et al., 2010, Been et al., 2014), and in the brain, common complications are intraventricular haemorrhage, white matter injury and cerebral palsy (Counsell and Boardman, 2005, Ment and Vohr, 2008, Doesburg et al., 2011, Lindstrom et al., 2011, Douglas-Escobar and Weiss, 2012, Robinson, 2012, Boardman et al., 2014, Kemp, 2014, Tronnes et al., 2014). Other serious adverse

consequences to neonatal health following preterm birth include: feeding problems; necrotising enterocolitis (Lin et al., 2008, Gregory et al., 2011); infection due to a comprised immune system (Stoll et al., 2004, Levy, 2007, Strunk et al., 2007, Melville and Moss, 2013); renal dysfunction that can lead to renal failure when severe (Brenner and Mackenzie, 1997, Keller et al., 2003, Rodriguez et al., 2004, Hoy et al., 2008, Sutherland et al., 2011, Stelloh et al., 2012); hearing and vision deficits (Gabbard and Schryer, 2003, Hintz et al., 2005, Vohr and Allen, 2005, Vohr et al., 2005, Hellström et al., 2013) and cardiovascular problems such as patent ductus arteriosus and hypotension. For example, US data collected in the neonatal period from 9575 extremely preterm infants (22-28 weeks), born between 2003 to 2007, illustrate the severe range of neonatal complications; 93% had respiratory distress syndrome, 46% had a patent ductus arteriosus, 16% had severe intraventricular haemorrhage, 11% had necrotising enterocolitis and 36% had late-onset sepsis (Stoll et al., 2010). These extremely preterm babies also required many more clinical interventions neonatally to facilitate survival (Gilbert et al., 2003). Many of these neonatal morbidities and/or neonatal treatments of these morbidities can subsequently result in lifelong deleterious consequences to health. For instance, mechanical ventilation is necessary in many preterm infants that cannot breathe independently after preterm birth; yet this can lead to severe lung injury (Brew et al., 2013) and is consequently a major contributing factor to the development of bronchopulmonary dysplasia. Bronchopulmonary dysplasia is a chronic inflammatory lung disease in preterm infants which can lead to long-term problems in respiratory health (Moss, 2006, Laughon et al., 2011, Hacking et al., 2013).

The majority of the morbidities associated with preterm birth occur in extremely and very preterm births, and these infants represent a minority of overall preterm births (5.2% and 10.4% of all live preterm births, respectively) (Blencowe et al., 2012). Up until recently, data on the effects of moderate preterm birth has been limited, even though the majority of preterm births (84%) fall within this category (Shapiro-Mendoza and Lackritz, 2012). Infants born moderately preterm experience very few complications in the immediate period after birth and there is little requirement for medical assistance compared to those born at lower gestational ages. Therefore, it has been generally accepted that neonatal health outcomes of moderate preterm babies are not different to babies born at term. However, new research highlights that moderately preterm infants are at increased risk of long-term adverse health consequences, likely due to the fact that their organs are still relatively immature compared to term infants at the time of birth (Jaiswal et al., 2011, Leone et al., 2012, Brown et al., 2013).

For instance, Boyle et al. (2012) reported that in infants born from very preterm to full term (23 - 41 weeks of gestation), there was a gradient of increasing adverse health problems, including respiratory and gastrointestinal disorders, with decreasing gestational age at birth. Notably, however, this study showed that individuals born moderately preterm (32 - 33 weeks of gestation) and early term (37 - 38 weeks of gestation) at 3 and 5 years of age are the greatest contributors to the disease burden due to the large numbers of these subjects in these categories.

6.2 Impact of moderate preterm birth to an immature cardiovascular system

In the immediate period following preterm birth, the most common cardiovascular complications result from a patent ductus arteriosus and/or the presence of hypotension. The ductus arteriosus is a shunt from the pulmonary trunk to the aorta in the fetus that allows oxygenated blood to bypass the pulmonary circulation; this normally closes within 48 hours after birth (Schneider and Moore, 2006). In preterm infants, the shunt often remains open resulting in an inefficient circulation to organ systems and in severe circumstances can lead to heart failure (Dice and Bhatia, 2007). A patent ductus arteriosus has also been associated with increased risk of developing bronchopulmonary dysplasia, intraventricular haemorrhage in the brain and necrotising enterocolitis (Ohlsson and Shah, 2011).

Hypotension, low arterial pressure, is frequently observed in preterm infants in the immediate period after birth and is thought to be the result of immature cardiovascular control of arterial pressure; immaturity of the cardiac muscle is also likely to be a contributing factor (Harper, 2000, Kluckow and Evans, 2000, Dannevig et al., 2005). When hypotension is present, inotropic support, such as dopamine or dobutamine therapies are often required to raise arterial pressure levels (Ibrahim, 2008).

Although many preterm infants experience hypotension immediately after birth, there is a large body of evidence now showing that in later life, many subjects that are born preterm go on to develop hypertension. The initial hypotension in the neonatal period is in contrast to the many studies that report an association between preterm birth and the development of hypertension (elevated arterial pressure) in later life. Indeed, it is likely that the structural adaptations that occur in the immature cardiovascular system of preterm infants in early life may be beneficial in the short-term; however, if they persist into adulthood, they may result

in adverse cardiovascular consequences. In this regard, studies in animal models and in human subjects, are now emerging demonstrating significant alterations in the structure of the heart and blood vessels which is often accompanied by an elevation of arterial pressure in those born preterm (Bonamy et al., 2005, Lewandowski and Leeson, 2014, Sutherland et al., 2014, Lewandowski et al., 2015).

In an ovine model of moderate preterm birth, Bensley et al. (2010) showed that preterm birth results in maladaptive remodelling of the myocardium with evidence of cardiomyocyte hypertrophy, altered cardiomyocyte maturation and induction of cardiomyocyte polyploidy in lambs at 9 weeks of age when compared to term controls. In studies of human subjects, there have been reports of increased left and right ventricular mass and abnormal ventricular shape in the hearts of young adults born very preterm and this was accompanied by impaired ventricular function (Lewandowski et al., 2013a, Lewandowski et al., 2013b).

The findings of my thesis clearly show that the myocardium of the moderately preterm lambs is more immature compared to terms in the immediate period after birth, with a significantly increased proportion of immature cardiomyocytes and a reduced proportion of mature cardiomyocytes within the LV+S. Ventricular size (wall volume, chamber volume and wall thickness) was also found to be significantly reduced in the preterm lambs compared to term lambs; this appeared to be attributed to the smaller body size of preterm lambs. By early adulthood, these structural changes were no longer evident in preterm female sheep; however, preterm male sheep still exhibited significantly reduced LV wall volume and LV wall thickness and this was accompanied by a significant reduction in the complement of cardiomyocytes within the LV myocardium. This reduction in LV growth in the male preterm sheep in early adulthood is in contrast to the findings of Lewandowski et al. (2013a) in young human subjects. The differences in findings may be species related or may be due to the long-term attenuation of growth and normal arterial pressure in the preterm male sheep in my study.

There are also a number of studies now emerging reporting the impact of preterm birth on the structure of blood vessels during postnatal life. Studies by Bensley et al. (2012) examined the aorta and pulmonary artery of lambs born moderately preterm at 9 weeks of age PTEA and reported increased thickness and smaller lumenal areas of the ascending aorta compared to term lambs. These moderately preterm lambs also exhibited increased elastin deposition in both the aorta and pulmonary artery when compared to term lambs. Of concern, severe injury

to the blood vessel wall was also observed in the ascending aorta. This overt injury could be seen histologically as large intimal lesions which extended into the underlying media and repaired by deposition of collagen and smooth muscle.

Additionally, reports in human subjects have shown that children and adolescent girls born preterm exhibit a narrower aortic lumen area and adults born preterm also displayed an increased carotid intima-media thickness compared to term controls (Edstedt Bonamy et al., 2008, Polak et al., 2011, Schubert et al., 2013). In accordance with these findings, in my studies, in the immediate period following preterm birth I observed a significantly narrower lumen area in the thoracic aorta and left carotid artery and increased wall thickness relative to body weight in the thoracic aorta. There was no evidence of overt injury in the walls of these blood vessels; however, concomitant gene expression studies (conducted by a research assistant in our laboratory) showed upregulation of *P-selectin* in the thoracic aorta, and *Caspase-3* and *IL1β* in the left carotid artery of preterm lambs compared to term lambs; indicative of covert vascular injury.

In early adulthood, preterm sheep exhibited fewer layers of elastin and reduced elastin content in the left carotid artery compared to term sheep, whereas smooth muscle content was increased. Sexual dimorphism was evident in the thoracic aorta as there were significant reductions in lumen area, media area and intima-media thickness of male sheep born preterm when compared to terms, but this was not observed in females. However, similar to LV growth in adulthood, this is likely attributed to the long-term attenuation in body growth in preterm male sheep.

Given the persistent narrowing of the aorta in male sheep born preterm, it will be important in further studies to investigate the structure and postnatal growth of other blood vessels. In particular, the resistance vessels, as it has been well established that narrowing of the lumen of these blood vessels leads to a rise in total peripheral resistance and thus a subsequent rise in arterial pressure.

6.3 Impact of antenatal corticosteroid exposure

It is important to emphasise when interpreting the findings of my study that the fetal lambs were exposed to a clinical dose of betamethasone prior to delivery. This was in order to mimic the clinical scenario where pregnant women at risk of delivering prematurely are administered corticosteroids to facilitate survival of the infant if preterm delivery ensues

(Althabe et al., 2012, Roberts and Dalziel, 2013). Given that all preterm lambs in my study were exposed to antenatal betamethasone, it is not possible for me to definitively conclude that my findings were the result of moderate preterm birth; instead, my findings may have been the result of exposure to antenatal corticosteroids. Certainly, antenatal corticosteroids have been shown in many studies to influence organ development (Liggins, 1994, Huang et al., 2001, Morrison et al., 2012) including cardiomyocyte growth. In this regard, *in vivo* and *in vitro* studies have demonstrated that corticosteroids influence fetal cardiomyocyte growth, although the findings are conflicting. For example, a study in fetal sheep reported accelerated maturation of the cardiomyocytes (Kim et al., 2014), whilst another reported enhanced proliferation (Giraud et al., 2006). The difference in findings between studies may relate to the timing and dose of the antenatal corticosteroid administration. Corticosteroids have also been shown to influence the deposition of extracellular matrix within the blood vessel wall; both increased elastin and collagen accumulation have been observed in the blood vessel wall following exposure to corticosteroids (Leitman et al., 1984, Bendeck et al., 1994). In future studies, it is important to independently examine the effect of antenatal corticosteroids on fetal cardiac growth and on the structure of the vasculature.

Corticosteroids are routinely administered to pregnant women at risk of delivering preterm in order to accelerate the maturation of the lungs of the fetus, which in turn facilitates survival if preterm birth ensues. The current antenatal corticosteroid treatment protocols commonly used in the clinics are: (1) betamethasone 12 mg intramuscularly, 2 doses 24 hours apart or (2) dexamethasone 6 mg intramuscularly, 4 doses 12 hours apart (Hofmeyr, 2009, Surbek et al., 2012). Many trials have assessed the efficacy of different corticosteroids and regimens; for instance, the comparison of single versus repeated dosages and betamethasone versus dexamethasone (Brownfoot et al., 2008, Brownfoot et al., 2013). There are advantages and disadvantages to all the different protocols and they continue to be debated (Crowther and Harding, 2007). Indeed, further research is required to assess the benefits and risks in the short-term and long-term of the alternative corticosteroid therapies, including which drug, what dosage, administration and timing. Overall, however, there is a general consensus of opinion that corticosteroids reduce the risk of mortality and morbidities following preterm birth. It is well recognised that antenatal administration of corticosteroids reduce the incidence of not only respiratory distress syndrome but also intraventricular haemorrhage, necrotising enterocolitis, systemic infections and childhood development delay (Crowley, 2000, Chatterjee et al., 2007, Mwansa-Kambafwile et al., 2010, Brownfoot et al., 2013,

Roberts and Dalziel, 2013). Treatment between 26 and 35 weeks of gestation are reported to be the most beneficial in mitigating severe health outcomes of preterm birth (Hofmeyr, 2009).

As in clinical practice, it has been shown that administration of antenatal corticosteroids is necessary in preterm sheep studies in order to facilitate the survival of the lambs after birth. Hence, in the design of the studies in this thesis, we chose to treat the ewes that were assigned to deliver preterm with a clinically relevant dose of betamethasone (11.4 mg intramuscularly, 2 doses 24 hours apart), to reflect common practice in the clinic. An important finding of this study was that when compared to previous studies in moderately preterm lambs, where they were administered a much lower dose of betamethasone (3.7 mg) (De Matteo et al., 2010), there was no improvement in the survival rate of moderately preterm female lambs in my studies (with the higher dose of betamethasone; 22.8 mg) (76% survival 2 weeks after birth and 75% survival 2 weeks after birth, respectively). In contrast, there appeared to be improved survival in the male preterm lambs with the increased dose of antenatal betamethasone used in my studies when compared to the previous studies by De Matteo et al. (2010) (75% survival compared to 44% survival 2 weeks after birth). As follow up to these findings, further research is required to examine the differences in survival rates between the sexes following antenatal administration of corticosteroids, especially given that corticosteroid exposure can lead to adverse side effects in the mothers and offspring in later life (Gwathmey et al., 2011, Singh et al., 2012, Romejko-Wolniewicz et al., 2014, Weiss and Niemann, 2015). There may be the potential to lower the dose of corticosteroids if the mother is carrying a female fetus. Certainly, although the benefits far outweigh the negative side effects, it is well established that antenatal corticosteroid treatments can lead to adverse side effects in the mother and her offspring, particularly after administration of high dosages of corticosteroids. For instance, anaemia and leukocytosis has been reported in mothers administered a higher single corticosteroid dose prior to delivering prematurely (Romejko-Wolniewicz et al., 2014) and in offspring born preterm, a higher heart rate and elevated arterial pressure has been reported in those exposed to antenatal corticosteroids (Doyle et al., 2000, Gwathmey et al., 2011, Singh et al., 2012, Weiss and Niemann, 2015). Given my findings compared to De Matteo et al. (2010), in future studies it would be valuable to further explore the corticosteroid dosages administered antenatally with respect to the sex of the fetus.

6.4 Males are more vulnerable to the effects of preterm birth

It is now well recognised that male infants are more vulnerable to preterm birth than female infants, exhibiting higher rates of neonatal mortality and morbidity and long-term morbidity compared to females born at the same gestational ages (Stevenson et al., 2000, Hoyert et al., 2006, Zisk et al., 2011). In the immediate period after birth, it has been reported that complications, such as respiratory distress syndrome, jaundice and hypoglycaemia are more prevalent in preterm male babies compared to preterm female babies (Peacock et al., 2012, Tundidor et al., 2012, Lawn et al., 2014). Furthermore, preterm male neonates exhibit an increased risk of intraventricular haemorrhage and sepsis with poorer neurological outcomes by 2-3 years of age (Kent et al., 2012). This 'male disadvantage' is particular evident in infants born extremely and very preterm (Elsmén et al., 2004b, Zisk et al., 2011), but importantly, it has also been observed in moderately preterm, late preterm and term infants. For instance, the prevalence of respiratory distress syndrome is significantly higher in preterm males compared to preterm females following preterm birth up to 38 weeks of gestation (Anadkat et al., 2012, Altman et al., 2013). In addition, preterm males generally require more initial respiratory and circulatory support and neonatal care, which subsequently have the potential to lead to other adverse long-term outcomes compared to preterm females (Elsmén et al., 2004a). In addition, males born preterm exhibit a worse outcome in regards to long-term survival (Hoyert et al., 2006).

The male disadvantage has been linked to delayed development of organ systems in males compared to females and this is likely to be a contributing factor to the increased rates of mortality and morbidity in preterm males (Peacock et al., 2012). For example, the prevalence of respiratory complications is more common in preterm male infants than preterm female infants after birth and it has been shown that males have slower lung maturation compared to females (Fleisher et al., 1985, Torday and Nielsen, 1987), with females producing pulmonary surfactant at least one week earlier in gestation compared to males (Seaborn et al., 2010). Similarly, delayed maturation of the heart muscle has been described in the fetal heart of males when compared to females. In a study conducted in fetal lambs, Lumbers et al. (2009) found a significantly higher proportion of mature cardiomyocytes in the developing myocardium of female fetal sheep when compared to male fetal sheep at the same gestational age. Interestingly, however, in my study although there was a decreased proportion of mature (binucleated) cardiomyocytes in the LV+S of the preterm lambs in the

immediate following birth, I did not observe any differences in the proportion of mature and immature cardiomyocytes, between preterm male and preterm female; indicating no difference in the maturation of the cardiac muscle between sexes. Of course, there remains the possibility that there was accelerated maturation of the cardiomyocytes in the male hearts in the 2 days after birth in response to the increased functional demands after birth. Further studies are required to determine whether this is the case. Likewise, in my studies, there was no evidence of delayed maturation of the conduit arteries that I examined in male preterm lambs when compare to females. At 2 days after birth, there were no differences in the number of elastin layers or in the proportions of elastin, collagen and smooth muscle in the arterial wall of the thoracic aorta and left carotid artery between male and female preterm lambs.

Interestingly, in my study, the majority of long-term differences in cardiovascular structure following preterm birth (including cardiomyocyte growth and vascular structure) were observed in males; only a few differences were apparent in females. The long-term differences in cardiovascular structure between term and preterm males were not evident in the immediate period after birth; by adulthood, however, marked differences in cardiovascular structure were observed. In the heart, preterm male sheep exhibited a significantly thinner LV wall comprising of a reduced number of cardiomyocytes within the LV+S compared to term male sheep; this is likely attributed to the smaller body size of the preterm male sheep. Of concern, however, the significantly fewer cardiomyocytes within the wall of the LV+S is likely to impact both on cardiac functional reserve and the adaptive capabilities of the heart. Whilst the preterm sheep remain small in adult life, this may not be problematic but deleterious consequences are likely to ensue if there is enlargement of the heart (for example, following induction of hypertension or obesity) or if there is injury to the heart muscle (for example, following myocardial infarction). Given the very limited capacity for cardiomyocyte proliferation in adulthood, under such circumstances, the functional capacity and ability for physiological cardiac hypertrophy would be severely compromised when there is already a cardiomyocyte deficit. In future studies, it would be interesting to induce hypertension or obesity in male preterm and term sheep and investigate both the functional and structural cardiac response.

In the blood vessels, adult preterm male sheep exhibited a smaller lumenal area, medial area and arterial wall thickness and fewer elastin layers in the thoracic aorta compared to term male sheep. These differences are also likely to be attributed to the long-term attenuation in

body size of the preterm male sheep. Regardless of this, when taking into account that arterial pressure remained similar between preterm and term sheep in early adulthood, the differences in vascular structure are likely to render the preterm males more vulnerable to deleterious vascular complications. Given that the aortas of the preterm male sheep exhibit thinner walls and fewer layers of elastin, you would expect that they will be particularly vulnerable to aneurysm with age (Bloomer et al., 2012); aneurysm results from weakening of the aortic wall, which is due to the inadequate elastic components within the blood vessel (Isselbacher, 2005). In addition, it is expected that there would be adverse remodelling with age and especially if there is an increase in arterial pressure (which is often observed following preterm birth). Under these circumstances, when the aortic wall is required to thicken in response to increased wall stress, it is likely that there will be inadequate deposition of elastin within the aortic wall, given that the number of layers of elastin is markedly reduced in the preterm males compared to terms. Instead, it is likely that the adaptive thickening of the aortic wall would result from an accumulation of collagen; this in turn would lead to increased stiffness and reduced compliance of the aortic wall. The thinner aortic wall in the male preterm sheep may also render the aorta more vulnerable to an elevation in arterial pressure, which in turn, could lead to arterial injury, further weakening of the wall and vulnerability to atherosclerosis and aneurysm.

My findings of a narrower aortic lumen in offspring born preterm is in accordance with the clinical findings in subjects born preterm (Edstedt Bonamy et al., 2008, Schubert et al., 2011); however, the persistent narrowing of the aorta through to adulthood was only observed in the males born preterm. Importantly, the persistent narrowing of the aorta has the potential to adversely impact on LV function; especially given the observed cardiomyocyte deficit in the LV+S which has the potential to compromise cardiac contractility even further. In this study, I did not see narrowing of the lumen in the left carotid artery, suggesting that the vascular narrowing may be specific to the aorta. However, it will be of interest to explore this further in blood vessels throughout the arterial tree; other blood vessels were collected at necropsy in order to do this. It will be especially important to examine the resistance vessels, given that narrowing of resistance vessels can result in an increase in total peripheral resistance and subsequently lead to hypertension.

6.5 Further studies derived from this thesis

In this thesis, I have conducted two comprehensive sheep studies (in a short-term cohort and long-term cohort) and subsequently analysed cardiac structure and vascular structure of the thoracic aorta and left carotid artery in the immediate period following birth and in early adulthood. It is important to point out that these comprehensive sheep studies have not been limited to these analyses. Several experiments were performed concurrently with my own, including cardiovascular functional assessments. Additional organs/tissues from both the short-term and long-term cohorts were collected at necropsies, and these have been subsequently analysed or stored for future investigations.

Firstly, it is important to mention the accompanying PhD project of Mr. Paul Lombardo, from the Department of Medical Imaging and Radiation Sciences at Monash University (who is also under the co-supervision of Prof. Jane Black). Mr. Lombardo performed cardiac, vascular and renal ultrasounds on all the lambs in both the short-term and long-term cohorts at selected time-points. For the short-term group, ultrasounds were conducted on all lambs prior to necropsy at 2 days of age, and for the long-term group, they were conducted at 2 days after birth, 2 weeks of age for (preterm lambs only; term equivalent age), 3 months of age, 6 months of age, 12 months postnatal age and just prior to necropsy. The cardiac parameters measured included: systolic and diastolic LV anterior wall thickness, LV posterior wall thickness, LV chamber short axis internal diameter and interventricular septum thickness. Additional ultrasound measurements conducted were: length, width and thickness of the kidneys; resistive index in renal arteries; internal diameter and maximum blood flow of the aortic root, pulmonary arteries and carotid arteries; and external diameter, intima-media thickness and adventitia thickness of the carotid arteries. Overall, his study provides *in vivo* measurements of cardiovascular structure and function in all the lambs (short-term cohort) and sheep (long-term cohort) used in this thesis. Ultimately, his ultrasound analyses will complement the findings of my study and together, produce a series of comprehensive publications giving insight into the effect of moderate preterm birth on the cardiovascular system. In addition, there is currently an Honours student, working within our laboratory who is analysing the structure of the RV from the sheep in my studies. These findings will be of importance, considering the recent study by Lewandowski et al. (2013b) reported that the structural differences after preterm birth were greater in the RV compared to the LV.

Additionally, metabolic studies were performed on the sheep of the long-term cohort at approximately 14 months of age. Glucose tolerance and insulin secretion were measured by an intravenous glucose tolerance test and insulin sensitivity assessed using a hyperinsulinaemic euglycaemic clamp. These were performed by Dr. Robert De Matteo from the Department of Anatomy and Development Biology at Monash University, whilst collaborating with Dr. Kathy Gatford from the Robinson Research Institute at the University of Adelaide in South Australia. Fixed and frozen pancreas and muscle samples were also sent to the University of Adelaide, for morphology and gene expressive analyses by Honours student, Mr. Daniel Hodgson. These metabolic studies have been completed and are ready for publication.

On the day of necropsy for both short-term and long-term cohorts, myography analysis of vascular endothelial and smooth muscle function were performed in freshly excised blood vessels (inferior abdominal aorta, left descending coronary artery, basilar artery and third order mesenteric arteries) by Prof. Helena Parkington, Dr. Marianne Tare and Dr. Beth Allison. Data from these studies have not yet been analysed, but in due course will provide information on the differences in sensitivity to vasodilator and constrictor agents and give a measure of maximal response in these vessels. Once more, these vascular functional studies will complement the vascular structure analyses conducted in this thesis and ultimately lead to comprehensive publications of both vascular structure and function.

The remaining tissues that were snap frozen and/or fixed at necropsy included the brain, lungs, kidneys, gastrointestinal tract, liver, thymus, muscle tissues in the hind limb, pancreas and various blood vessels (abdominal aorta, aortic arch, renal arteries and third order mesenteric arteries). These have been stored for future analysis or already distributed to other researchers for current analysis. Ms. Tracey Flores (a research assistant in our laboratory) has been conducting gene expression studies, using Real-time Polymerase Chain Reaction, examining markers of endothelial injury, inflammation and apoptosis on frozen tissue samples of RV, LV, thoracic aorta, left carotid artery and third order mesenteric arteries. Histological analyses on the renal and third order mesenteric arteries are also currently being performed to investigate the structure and size of the resistance vessels. Additionally, Ms. Tracey Flores (during her Honours year in 2013) performed functional, immunohistochemistry and histological analyses on sections of the ileum collected from these animals. These studies have also been completed and are ready for publication.

Importantly, it is only by conducting studies like mine (in a clinically relevant animal model) that the effects of moderate preterm birth can be directly addressed, free of the many confounding factors associated with studies in human infants. Overall, my PhD project has drawn together a multi-faceted research team, which has enabled the gathering of data, not just for my cardiovascular structural studies, but from many organ systems. Ultimately, the collective findings from my preterm sheep experiments will lead to a much better understanding of the effects of moderate preterm birth in the immediate period after birth and in early adulthood. Together, the findings will provide insight into how different organs systems respond to being born early and thus identify potential strategies to alleviate the risk of adverse health outcomes in individuals born preterm.

6.6 Future directions

As briefly eluded to in the previous sections of this final discussion, the findings of this thesis have evoked multiple ideas for future directions of research. In particular, based on my findings, I consider that it is imperative to further explore: 1) the direct effects of antenatal corticosteroid exposure on the developing cardiovascular system (which was a confounder in my studies); 2) the impact of secondary cardiovascular insults in adulthood (in particular obesity, hypertension and myocardial infarction) 3) the short-term and long-term effects when preterm birth is combined with IUGR (which is a common co-morbidity of preterm birth) and finally 4) the short-term and long-term effects on the cardiovascular system when the severity of preterm birth is increased.

Betamethasone exposure

In my studies, it has not been possible to determine whether the cardiac and vascular structural differences observed between preterm and term sheep were due to preterm birth *per se* or due to exposure to antenatal corticosteroids, because administration of betamethasone to ewes delivering preterm was necessary to facilitate the survival of the preterm lambs after birth. It was not feasible to examine a preterm group without antenatal administration of corticosteroids to the ewes, as the majority of the lambs would not have survived and if they did, additional neonatal interventions (such as ventilation), would likely have been required in order for lambs to survive; thus introducing another confounding factor. However, given that corticosteroids have been shown to exert trophic effects on fetal

cardiomyocyte growth and on the composition of the arterial wall, it is important in future studies to differentiate the specific effects of antenatal exposure to corticosteroids on the immature cardiovascular system of preterm offspring, independent of preterm birth. A good way to approach this would be to administer the antenatal corticosteroids to the ewe and 48 hours later (or other nominated time) euthanise the fetus *in utero* and then subsequently examine the effects on the cardiac and vascular structure of the fetus. Using this approach, labour would not be induced in the ewes and the lambs would not be exposed to the haemodynamic transition that occurs at birth. Importantly, using this study design, it would be possible to explore the effects of antenatal corticosteroids in both sexes at different gestational ages (for example, equivalent to moderately preterm, very preterm and extremely preterm) and at varying doses.

Additionally, given that I showed improved survival of male preterm lambs in my studies (where I used an antenatal dose of corticosteroids of 11.4 mg) compared to the studies of De Matteo et al. (2010) (where they used a lower dose of antenatal corticosteroids) but no improvement was observed in the survival of female preterm lambs, I would like to explore this further. It would be beneficial to explore whether even higher doses of antenatal corticosteroids (>11.4 mg) can further improve survival in the male preterm lambs and alternatively, whether the dose of antenatal corticosteroids can be lowered even further (<3.7 mg) and not adversely affect the survival of preterm female lambs. These findings would have important implications for the treatment of women 'at risk' of preterm birth.

Response of the male adult preterm heart and aorta to secondary cardiovascular insults

The cardiomyocyte deficit within the LV+S of male adult preterm sheep compared to term sheep is of concern. As cardiomyocytes have limited proliferative capacity postnatally, the reduced complement of cardiomyocytes will impact on lifelong functional reserve and on the adaptive capabilities of the heart. Therefore, in future studies, it is imperative to gain an understanding of how the LV+S of the preterm male, that has a reduced complement of cardiomyocytes compared to terms, remodels in response to induction of obesity or hypertension. In particular, it is important to look at how the heart responds to induction of hypertension given that preterm birth is strongly associated with elevated arterial pressure in adulthood. It is expected that with fewer cardiomyocytes within the myocardium that when the LV+S is stimulated to hypertrophy there will be pathological remodelling leading to

greater deposition of extracellular collagen (fibrosis) and subsequent stiffening of the heart muscle. In addition, it is important to examine the extent of myocardial injury following myocardial infarction in adult preterm male sheep compared to adult term males. It is expected that the injury to the myocardium will be greater in the preterm males given that they have a reduced functional reserve.

Furthermore, it is important to better understand how the aortas of adult preterm males respond to the increased functional demands and arterial pressure associated with obesity and hypertension. Under these conditions, the thinner medial wall, with fewer layers of elastin, is likely to render the aorta vulnerable to injury, aneurysm and adverse remodelling.

IUGR combined with preterm birth

IUGR is a common co-morbidity of preterm birth (Zeitlin et al., 2000, Gardosi, 2005). Importantly, it has been shown that there is a reduction in the number of cardiomyocytes in the IUGR heart at birth (Corstius et al., 2005, Botting et al., 2012) and recent evidence in our laboratory has shown that this cardiomyocyte deficit persists into adulthood, with the number of cardiomyocytes in the adult heart directly proportional to birth weight (unpublished, Honours thesis: Stacey Vranas 2014). Given that my studies have shown that there are significantly fewer cardiomyocytes within the LV+S of adult preterm male sheep when compared to adult term sheep, it is likely that when IUGR is combined with preterm birth that there would be an additional adverse impact on the complement of cardiomyocytes. It is therefore imperative to conduct further animal studies where IUGR is combined with preterm birth to investigate the overall effects on cardiovascular structure and in particular on the growth of the cardiomyocytes.

Impact at early gestational ages

In my thesis, the preterm sheep did not show any evidence of developing hypertension in early adulthood, with no difference in arterial pressure between preterm and term sheep. This is contrary to the findings of many epidemiological studies which have reported that preterm birth is associated with the development of hypertension in adulthood. The absence of elevated arterial pressure in my adult preterm sheep compared to humans born preterm may be a species effect, age effect or the fact that they have been born moderately preterm.

Indeed, the degree of prematurity has been shown to influence the level of adult arterial pressure, with adult pressure inversely associated with gestational age at birth. Therefore, it would be beneficial to examine cohorts of sheep born at earlier gestational ages to see if hypertension develops by early adulthood and if the differences in the structure of the cardiovascular system are exacerbated. Of course these experiments would require neonatal intensive care facilities for the lambs in the period after birth.

Although hypertension was not evident in the preterm offspring in early adulthood, there were a number of cardiac and structural differences in the males that were born moderately preterm, which will likely render them vulnerable to cardiovascular insults and disease. It is expected that these adverse cardiovascular structural effects would have been far more severe, if the lambs had been delivered even earlier (as very or extremely preterm). It is imperative that this is explored in future studies.

6.7 Conclusions

In conclusion, using a clinically relevant ovine model of moderate preterm birth combined with antenatal corticosteroids, the findings of this study clearly demonstrate immaturity of the heart muscle and of the aorta and left carotid artery in the immediate period following preterm birth. In the long-term, the findings clearly show an impact on cardiovascular structure following moderate preterm birth, with these effects almost exclusively in males; likely due to their long-term attenuated growth. In particular, the cardiomyocyte deficit in the LV+S and thinner aortic wall with fewer layers of elastin is likely to render the cardiovascular system of adult preterm males vulnerable to secondary insults. As all offspring were exposed to antenatal corticosteroids, further studies are required to elucidate whether any of the adverse effects are directly attributed to the early life corticosteroid exposure.

Historically, interpretation of the findings in studies of preterm infants has been difficult due to differences in the etiology and degree of severity of preterm birth, as well as differences in their postnatal care. Using this animal model, we have eliminated many of these confounding factors to specifically examine the effects of moderate preterm birth on the immature cardiovascular system; however, there remained the need to use antenatal corticosteroids to facilitate survival after birth. Using the sheep model where the development of the cardiovascular system is similar to the human, the study design was beneficial in permitting the immediate and long-term follow-up of preterm born offspring. This approach enabled the

detection of the potential amplification or amelioration of changes in the cardiovascular system of preterm offspring over life. Importantly, both sexes were studied to investigate potential sexual dimorphism in the response to preterm birth. As is common to most experiments, there were limitations in my study design that need to be considered when interpreting the findings of this study; in particular, the sensitivity of arterial pressure measurements using an inflatable cuff, confounding effects of antenatal betamethasone and the inability to extrapolate findings in large elastic arteries to resistance blood vessels.

Nevertheless, the findings are of utmost clinical importance given the high number of individuals that are born moderately preterm; it is expected that with increasing severity of prematurity that there would be even greater adverse cardiovascular consequences. Overall, my findings highlight the long-term cardiovascular vulnerability in offspring born prematurely, even those born moderately preterm, and highlight the importance of long-term cardiovascular follow up in individuals born preterm.

Appendix

Verhoeff van Gieson's stain

Reagents:

1. 5% alcohol haematoxylin 20 ml

   A freshly prepared solution is desirable.

   Add to this in the order given:

   10% aqueous ferric chloride 8 ml

   Lugol's iodine solution 7 ml

   Prepare this mixture immediately before use.
2. 2% aqueous ferric chloride 10 ml
3. Van Gieson's stain

   Picric acid saturated aqueous 93 ml

   1% acid fuchsin 7 ml

Method:

1. Bring paraffin sections to water.
2. Stain on slide with reagent 1. 15 mins
3. Wash in water.
4. With a cloth wipe surplus water from around section.
5. Differentiate with reagent 2 (2% aqueous ferric chloride) a few seconds at a time, stop the process in tap water and examine microscopically until elastic fibres are defined on a slightly overstained background.
6. Wash in water.
7. Rinse in 95% ethanol.
8. Wash in water.
9. Counterstain in reagent 3 (Van Gieson's stain) 2 mins
10. Rinse briefly in water.
11. Blot section with No.1 Whatman paper
12. Dehydrate in absolute ethanol
13. Clear in xylene and mount.

References

ABDEL-HADY, H., NASEF, N., SHABAAN, A. E. & NOUR, I. 2013. Patent Ductus Arteriosus in Preterm Infants: Do We Have the Right Answers? *BioMed Research International,* 2013**,** 15.

ABOULGHAR, M. A. 2005. Perinatal complications of assisted reproduction. *Croatian Medical Journal,* 46**,** 751-758.

ADAMS, M. M., ELAM-EVANS, L. D., WILSON, H. G. & GILBERTZ, D. A. 2000. Rates of and factors associated with recurrence of preterm delivery. *Journal of the American Medical Association,* 283**,** 1591-1596.

AHERN, J., PICKETT, K. E., SELVIN, S. & ABRAMS, B. 2003. Preterm birth among African American and white women: a multilevel analysis of socioeconomic characteristics and cigarette smoking. *Journal of Epidemiology & Community Health,* 57**,** 606-611.

AHMAD, I., NEMET, D., ELIAKIM, A., KOEPPEL, R., GROCHOW, D., COUSSENS, M., GALLITTO, S., RICH, J., PONTELLO, A., LEU, S. Y., COOPER, D. M. & WAFFARN, F. 2010. Body composition and its components in preterm and term newborns: A cross-sectional, multimodal investigation. *American journal of human biology,* 22**,** 69-75.

AJE, T. O. & MILLER, M. 2009. Cardiovascular disease: A global problem extending into the developing world. *World Journal of Cardiology,* 1**,** 3-10.

AKINBAMI, L. J., SCHOENDORF, K. C. & KIELY, J. L. 2000. Risk of preterm birth in multiparous teenagers. *Archives of Pediatrics & Adolescent Medicine,* 154**,** 1101-1107.

ALDER, J., FINK, N., BITZER, J., HOSLI, I. & HOLZGREVE, W. 2007. Depression and anxiety during pregnancy: a risk factor for obstetric, fetal and neonatal outcome? A critical review of -the literature. *Journal of Maternal-Fetal & Neonatal Medicine,* 20**,** 189-209.

ALI, S. R., HIPPENMEYER, S., SAADAT, L. V., LUO, L., WEISSMAN, I. L. & ARDEHALI, R. 2014. Existing cardiomyocytes generate cardiomyocytes at a low rate after birth in mice. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** 8850-8855.

ALLEN, M. C., DONOHUE, P. K. & DUSMAN, A. E. 1993. The limit of viability - Neonatal outcome of infnats born at 22 to 25 weeks' gestation. *New England Journal of Medicine,* 329.

ALLISON, B., CROSSLEY, K., FLECKNOE, S., MORLEY, C., POLGLASE, G. & HOOPER, S. 2010. Pulmonary hemodynamic responses to in utero ventilation in very immature fetal sheep. *Respiratory Research,* 11**,** 111.

ALTHABE, F., BELIZAN, J. M., MAZZONI, A., BERRUETA, M., HEMINGWAY-FODAY, J., KOSO-THOMAS, M., MCCLURE, E., CHOMBA, E., GARCES, A., GOUDAR, S., KODKANY, B., SALEEM, S., PASHA, O., PATEL, A., ESAMAI, F., CARLO, W. A., KREBS, N. F., DERMAN, R. J., GOLDENBERG, R. L., HIBBERD, P., LIECHTY, E. A., WRIGHT, L. L., BERGEL, E. F., JOBE, A. H. & BUEKENS, P. 2012. Antenatal corticosteroids trial in preterm births to increase neonatal survival in developing countries: study protocol. *Reproductive Health,* 9**,** 22.

ALTIGANI, M., MURPHY, J. F., NEWCOMBE, R. G. & GRAY, O. P. 1989. Catch up growth in preterm infants. *Acta paediatrica Scandinavica. Supplement,* 357**,** 3-19.

ALTMAN, M., VANPÉE, M., CNATTINGIUS, S. & NORMAN, M. 2013. Risk Factors for Acute Respiratory Morbidity in Moderately Preterm Infants. *Paediatric and Perinatal Epidemiology,* 27**,** 172-181.

ALVERO, R. 2002. Assisted reproductive technologies: toward improving implantation rates and reducing high-order multiple gestations. *Obstetrical & Gynecological Survey,* 57**,** 519-529.

ALY, H., HAMMAD, T., NADA, A., MOHAMED, M., BATHGATE, S. & EL-MOHANDES, A. 2010. Maternal obesity, associated complications and risk of prematurity. *Journal of Perinatology,* 30**,** 447-451.

ANADKAT, J. S., KUZNIEWICZ, M. W., CHAUDHARI, B. P., COLE, F. S. & HAMVAS, A. 2012. Increased risk for respiratory distress among white, male, late preterm and term infants. *Journal of Perinatology,* 32**,** 780-785.

ANANTH, C. V. & SAVITZ, D. A. 1994. Vaginal bleeding and adverse reproductive outcomes: a meta-analysis. *Paediatric and Perinatal Epidemiology,* 8**,** 62-78.

ANANTH, C. V., SAVITZ, D. A., LUTHER, E. R. & BOWES, W. A., JR. 1997. Preeclampsia and preterm birth subtypes in Nova Scotia, 1986 to 1992. *American Journal of Perinatology,* 14**,** 17-23.

ANDERSON, B. L., SIMHAN, H. N., SIMONS, K. M. & WIESENFELD, H. C. 2007. Untreated asymptomatic group B streptococcal bacteriuria early in pregnancy and chorioamnionitis at delivery. *American Journal of Obstetrics & Gynecology,* 196**,** 524 e1-5.

ANDREWS, W. W., GOLDENBERG, R. L. & HAUTH, J. C. 1995. Preterm labor: emerging role of genital tract infections. *Infectious agents and disease,* 4**,** 196-211.

ANVERSA, P., LERI, A., ROTA, M., HOSODA, T., BEARZI, C., URBANEK, K., KAJSTURA, J. & BOLLI, R. 2007. Concise review: stem cells, myocardial regeneration, and methodological artifacts. *Stem Cells,* 25**,** 589-601.

APGAR, V. 1953. A proposal for a new method of evaluation of the newborn infant. *Current researches in anesthesia & analgesia,* 32**,** 260-267.

ASTOLFI, P. & ZONTA, L. A. 1999. Risks of preterm delivery and association with maternal age, birth order, and fetal gender. *Human Reproduction,* 14**,** 2891-2894.

AUGER, N., ABRAHAMOWICZ, M., WYNANT, W. & LO, E. 2014. Gestational age-dependent risk factors for preterm birth: associations with maternal education and age early in gestation. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 176**,** 132-136.

AUGER, N., SING, M. F., PARK, A. L., LO, E., TREMPE, N. & LUO, Z.-C. 2012. Preterm birth in the Inuit and First Nations populations of Québec, Canada, 1981–2008. *International Journal of Circumpolar Health,* 71**,** 10.3402/IJCH.v71i0.17520.

AXELSEN, S. M., HENRIKSEN, T. B., HEDEGAARD, M. & SECHER, N. J. 1995. Characteristics of vaginal bleeding during pregnancy. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 63**,** 131-134.

BARAD, D. H. & WITT, B. R. 2000. Multiple pregnancies and assisted reproductive technologies. *Journal of Women's Health and Gender-Based Medicine,* 9**,** 101-107.

BARBERA, A., GIRAUD, G. D., RELLER, M. D., MAYLIE, J., MORTON, M. J. & THORNBURG, K. L. 2000. Right ventricular systolic pressure load alters myocyte maturation in fetal sheep. *Am J Physiol Regul Integr Comp Physiol,* 279**,** R1157-64.

BARKER, D. J. 1995. Intrauterine programming of adult disease. *Molecular Medicine Today,* 1**,** 418-423.

BARKER, D. J., WINTER, P. D., OSMOND, C., MARGETTS, B. & SIMMONDS, S. J. 1989. Weight in infancy and death from ischaemic heart disease. *Lancet,* 2**,** 577-580.

BARKER, D. J. P. 2007. The origins of the developmental origins theory. *Journal of Internal Medicine,* 261**,** 412-417.

BARKER, D. J. P., OSMOND, C., FORSÉN, T. J., KAJANTIE, E. & ERIKSSON, J. G. 2005. Trajectories of Growth among Children Who Have Coronary Events as Adults. *New England Journal of Medicine,* 353**,** 1802-1809.

BARLOW, P., LEJEUNE, B., PUISSANT, F., ENGLERT, Y., VAN RYSSELBERGE, M., DEGUELDRE, M., VEKEMANS, M. & LEROY, F. 1988. Early pregnancy loss and obstetrical risk after in-vitro fertilization and embryo replacement. *Human Reproduction,* 3**,** 671-675.

BARON, D. M., YU, B., LEI, C., BAGCHI, A., BELOIARTSEV, A., STOWELL, C. P., STEINBICKER, A. U., MALHOTRA, R., BLOCH, K. D. & ZAPOL, W. M. 2012. Pulmonary hypertension in lambs transfused with stored blood is prevented by breathing nitric oxide. *Anesthesiology,* 116.

BARTON, L., HODGMAN, J. E. & PAVLOVA, Z. 1999. Causes of death in the extremely low birth weight infant. *Pediatrics,* 103**,** 446-451.

BASSO, O. & WILCOX, A. 2010. Mortality risk among preterm babies: immaturity versus underlying pathology. *Epidemiology,* 21**,** 521-527.

BATISTA, R. F. L., SILVA, A. A. M., BARBIERI, M. A., SIMÕES, V. M. F. & BETTIOL, H. 2012. Factors Associated with Height Catch-Up and Catch-Down Growth Among Schoolchildren. *PLoS ONE,* 7**,** e32903.

BEARZI, C., ROTA, M., HOSODA, T., TILLMANNS, J., NASCIMBENE, A., DE ANGELIS, A., YASUZAWA-AMANO, S., TROFIMOVA, I., SIGGINS, R. W., LECAPITAINE, N., CASCAPERA, S., BELTRAMI, A. P., D'ALESSANDRO, D. A., ZIAS, E., QUAINI, F., URBANEK, K., MICHLER, R. E., BOLLI, R., KAJSTURA, J., LERI, A. & ANVERSA, P. 2007. Human cardiac stem cells. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 14068-14073.

BECK, S., WOJDYLA, D., SAY, L., BETRAN, A. P., MERIALDI, M., REQUEJO, J. H., RUBENS, C., MENON, R. & LOOK, P. F. A. V. 2010. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bulletin of the World Health Organization,* 88**,** 31-38.

BEECKMAN, K., VAN DE PUTTE, S., PUTMAN, K. & LOUCKX, F. 2009. Predictive social factors in relation to preterm birth in a metropolitan region. *Acta Obstetricia et Gynecologica Scandinavica,* 88**,** 787-792.

BEEN, J. V., LUGTENBERG, M. J., SMETS, E., VAN SCHAYCK, C. P., KRAMER, B. W., MOMMERS, M. & SHEIKH, A. 2014. Preterm Birth and Childhood Wheezing Disorders: A Systematic Review and Meta-Analysis. *PLoS Medicine,* 11**,** e1001596.

BEIER, N. M. & BECKMAN, J. O. 1991. German Embryo Protection Act (October 24th, 1990): Gesetz zum Schutz von Embryonen (Embryonenschutzgesetz-ESchG). *Human Reproduction,* 6**,** 605-606.

BENDECK, M. P., KEELEY, F. W. & LANGILLE, B. L. 1994. Perinatal accumulation of arterial wall constituents: relation to hemodynamic changes at birth. *American Journal of Physiology,* 267**,** H2268-2279.

BENDECK, M. P. & LANGILLE, B. L. 1991. Rapid accumulation of elastin and collagen in the aortas of sheep in the immediate perinatal period. *Circulation Research,* 69**,** 1165-1169.

BENSLEY, J. G., DE MATTEO, R., HARDING, R. & BLACK, M. J. 2012. Preterm birth with antenatal corticosteroid administration has injurious and persistent effects on the structure and composition of the aorta and pulmonary artery. *Pediatr Res,* 71**,** 150-155.

BENSLEY, J. G., STACY, V. K., DE MATTEO, R., HARDING, R. & BLACK, M. J. 2010. Cardiac remodelling as a result of pre-term birth: implications for future cardiovascular disease. *Eur Heart J,* 31**,** 2058-2066.

BERENSON, G. S., SRINIVASAN, S. R., BAO, W., NEWMAN, W. P., 3RD, TRACY, R. E. & WATTIGNEY, W. A. 1998. Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. The Bogalusa Heart Study. *New England Journal of Medicine,* 338**,** 1650-1656.

BERGHELLA, V., HAYES, E., VISINTINE, J. & BAXTER, J. K. 2008. Fetal fibronectin testing for reducing the risk of preterm birth. *Cochrane Database of Systematic Reviews***,** CD006843.

BERKOWITZ, G. S. & PAPIERNIK, E. 1995. Working conditions, maternity legislation, and preterm birth. *Seminars in Perinatology,* 19**,** 272-278.

BERRY, C. L., LOOKER, T. & GERMAIN, J. 1972. Nucleic acid and scleroprotein content of the developing human aorta. *Journal of Pathology,* 108**,** 265-274.

BERRY, J. D., DYER, A., CAI, X., GARSIDE, D. B., NING, H., THOMAS, A., GREENLAND, P., VAN HORN, L., TRACY, R. P. & LLOYD-JONES, D. M. 2012. Lifetime Risks of Cardiovascular Disease. *New England Journal of Medicine,* 366**,** 321-329.

BHATTACHARYA, S., LOWIT, A., BHATTACHARYA, S., RAJA, E. A., LEE, A. J., MAHMOOD, T. & TEMPLETON, A. 2012. Reproductive outcomes following induced abortion: a national register-based cohort study in Scotland. *British Medical Journal Open,* 2.

BHATTACHARYA, S., RAJA, E. A., MIRAZO, E. R., CAMPBELL, D. M., LEE, A. J., NORMAN, J. E. & BHATTACHARYA, S. 2010. Inherited predisposition to spontaneous preterm delivery. *Obstetrics & Gynecology,* 115**,** 1125-1133.

BLAAK, E. 2001. Gender differences in fat metabolism. *Current Opinion in Clinical Nutrition and Metabolic Care,* 4**,** 499-502.

BLACK, M. J., SIEBEL, A. L., GEZMISH, O., MORITZ, K. M. & WLODEK, M. E. 2012. Normal lactational environment restores cardiomyocyte number after uteroplacental

insufficiency: implications for the preterm neonate. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* 302**,** R1101-R1110.

BLACKBURN, S. 2006. Placental, fetal, and transitional circulation revisited. *Journal of Perinatal & Neonatal Nursing,* 20**,** 290-294.

BLACKWELL, M. T., EICHENWALD, E. C., MCALMON, K., PETIT, K., LINTON, P. T., MCCORMICK, M. C. & RICHARDSON, D. K. 2005. Interneonatal Intensive Care Unit Variation in Growth Rates and Feeding Practices in Healthy Moderately Premature Infants. *Journal of Perinatology,* 25**,** 478-485.

BLANN, A. D., NADAR, S. K. & LIP, G. Y. H. 2003. The adhesion molecule P-selectin and cardiovascular disease. *European Heart Journal,* 24**,** 2166-2179.

BLENCOWE, H., COUSENS, S., CHOU, D., OESTERGAARD, M., SAY, L., MOLLER, A.-B., KINNEY, M. & LAWN, J. 2013. Born Too Soon: The global epidemiology of 15 million preterm births. *Reproductive Health,* 10**,** S2-S2.

BLENCOWE, H., COUSENS, S., OESTERGAARD, M. Z., CHOU, D., MOLLER, A. B., NARWAL, R., ADLER, A., VERA GARCIA, C., ROHDE, S., SAY, L. & LAWN, J. E. 2012. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet,* 379**,** 2162-2172.

BLOOMER, L. D. S., BOWN, M. J. & TOMASZEWSKI, M. 2012. Sexual dimorphism of abdominal aortic aneurysms: A striking example of “male disadvantage” in cardiovascular disease. *Atherosclerosis,* 225**,** 22-28.

BOARDMAN, J. P., WALLEY, A., BALL, G., TAKOUSIS, P., KRISHNAN, M. L., HUGHES-CARRE, L., ALJABAR, P., SERAG, A., KING, C., MERCHANT, N., SRINIVASAN, L., FROGUEL, P., HAJNAL, J., RUECKERT, D., COUNSELL, S. & EDWARDS, A. D. 2014. Common genetic variants and risk of brain injury after preterm birth. *Pediatrics,* 133**,** e1655-1663.

BOCCA-TJEERTES, I. F. A., KERSTJENS, J. M., REIJNEVELD, S. A., DE WINTER, A. F. & BOS, A. F. 2011. Growth and Predictors of Growth Restraint in Moderately Preterm Children Aged 0 to 4 Years. *Pediatrics,* 128**,** e1187-e1194.

BONAMY, A. K., BENDITO, A., MARTIN, H., ANDOLF, E., SEDIN, G. & NORMAN, M. 2005. Preterm birth contributes to increased vascular resistance and higher blood pressure in adolescent girls. *Pediatr Res,* 58**,** 845-849.

BONAMY, A. K., MARTIN, H., JORNESKOG, G. & NORMAN, M. 2007. Lower skin capillary density, normal endothelial function and higher blood pressure in children born preterm. *Journal of Internal Medicine,* 262**,** 635-642.

BOTTING, K. J., WANG, K. C., PADHEE, M., MCMILLEN, I. C., SUMMERS-PEARCE, B., RATTANATRAY, L., CUTRI, N., POSTERINO, G. S., BROOKS, D. A. & MORRISON, J. L. 2012. Early origins of heart disease: low birth weight and determinants of cardiomyocyte endowment. *Clinical and Experimental Pharmacology and Physiology,* 39**,** 814-823.

BOYLE, E. M., POULSEN, G., FIELD, D. J., KURINCZUK, J. J., WOLKE, D., ALFIREVIC, Z. & QUIGLEY, M. A. 2012. Effects of gestational age at birth on health outcomes at 3 and 5 years of age: population based cohort study. *British Medical Journal,* 344.

BRENNER, B. M. & MACKENZIE, H. S. 1997. Nephron mass as a risk factor for progression of renal disease. *Kidney International Supplements,* 63**,** S124-127.

BREW, N., HOOPER, S. B., ZAHRA, V., WALLACE, M. & HARDING, R. 2013. Mechanical ventilation injury and repair in extremely and very preterm lungs. *PLoS One,* 8**,** e63905.

BROWN, H. K., SPEECHLEY, K. N., MACNAB, J., NATALE, R. & CAMPBELL, M. K. 2013. Neonatal morbidity associated with late preterm and early term birth: the roles of gestational age and biological determinants of preterm birth. *International Journal of Epidemiology*.

BROWN, J. S., JR., ADERA, T. & MASHO, S. W. 2008. Previous abortion and the risk of low birth weight and preterm births. *Journal of Epidemiology & Community Health,* 62**,** 16-22.

BROWNFOOT, F. C., CROWTHER, C. A. & MIDDLETON, P. 2008. Different corticosteroids and regimens for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database of Systematic Reviews***,** CD006764.

BROWNFOOT, F. C., GAGLIARDI, D. I., BAIN, E., MIDDLETON, P. & CROWTHER, C. A. 2013. Different corticosteroids and regimens for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database of Systematic Reviews,* 8**,** CD006764.

BRUEL, A. & NYENGAARD, J. R. 2005. Design-based stereological estimation of the total number of cardiac myocytes in histological sections. *Basic Research in Cardiology,* 100**,** 311-319.

BRYAN, S. M. & HINDMARSH, P. C. 2006. Normal and Abnormal Fetal Growth. *Hormone Research in Paediatrics,* 65(suppl 3)**,** 19-27.

BUBB, K. J., COCK, M. L., BLACK, M. J., DODIC, M., BOON, W. M., PARKINGTON, H. C., HARDING, R. & TARE, M. 2007. Intrauterine growth restriction delays cardiomyocyte

maturation and alters coronary artery function in the fetal sheep. *Journal of Physiology,* 578**,** 871-881.

BUCHMAYER, S. M., SPAREN, P. & CNATTINGIUS, S. 2004. Previous pregnancy loss: risks related to severity of preterm delivery. *American Journal of Obstetrics & Gynecology,* 191**,** 1225-1231.

BUKOWSKI, R., GAHN, D., DENNING, J. & SAADE, G. 2001. Impairment of growth in fetuses destined to deliver preterm. *American Journal of Obstetrics & Gynecology,* 185**,** 463-467.

BURGUET, A., KAMINSKI, M., ABRAHAM-LERAT, L., SCHAAL, J. P., CAMBONIE, G., FRESSON, J., GRANDJEAN, H., TRUFFERT, P., MARPEAU, L., VOYER, M., ROZE, J. C., TREISSER, A. & LARROQUE, B. 2004. The complex relationship between smoking in pregnancy and very preterm delivery. Results of the Epipage study. *British Journal of Obstetrics and Gynaecology,* 111**,** 258-265.

BURKE, G. L., EVANS, G. W., RILEY, W. A., SHARRETT, A. R., HOWARD, G., BARNES, R. W., ROSAMOND, W., CROW, R. S., RAUTAHARJU, P. M. & HEISS, G. 1995. Arterial Wall Thickness Is Associated With Prevalent Cardiovascular Disease in Middle-Aged Adults : The Atherosclerosis Risk in Communities (ARIC) Study. *Stroke,* 26**,** 386-391.

BURNS, L., MATTICK, R. P. & COOKE, M. 2006. The use of record linkage to examine illicit drug use in pregnancy. *Addiction,* 101**,** 873-882.

BURRELL, J. H., BOYN, A. M., KUMARASAMY, V., HSIEH, A., HEAD, S. I. & LUMBERS, E. R. 2003. Growth and maturation of cardiac myocytes in fetal sheep in the second half of gestation. *Anat Rec A Discov Mol Cell Evol Biol,* 274**,** 952-961.

BUSCHMANN, I. & SCHAPER, W. 1999. Arteriogenesis Versus Angiogenesis: Two Mechanisms of Vessel Growth. *Physiology,* 14**,** 121-125.

CAI, W. & SCHAPER, W. 2008. Mechanisms of arteriogenesis. *Acta Biochimica et Biophysica Sinica,* 40**,** 681-692.

CARLSON, B. M. 2009. *Human Embryology and Developmental Biology,* Philadelphia, Mosby Elsevier.

CARMELIET, P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nature Medicine,* 6**,** 389-395.

CARMELIET, P. 2005. Angiogenesis in life, disease and medicine. *Nature,* 438**,** 932-936.

CARMELIET, P., FERREIRA, V., BREIER, G., POLLEFEYT, S., KIECKENS, L., GERTSENSTEIN, M., FAHRIG, M., VANDENHOECK, A., HARPAL, K., EBERHARDT, C., DECLERCQ, C., PAWLING, J., MOONS, L., COLLEN, D., RISAU, W. & NAGY, A. 1996. Abnormal blood

vessel development and lethality in embryos lacking a single VEGF allele. *Nature,* 380**,** 435-439.

CHAMBLESS, L. E., FOLSOM, A. R., CLEGG, L. X., SHARRETT, A. R., SHAHAR, E., NIETO, F. J., ROSAMOND, W. D. & EVANS, G. 2000. Carotid Wall Thickness is Predictive of Incident Clinical Stroke: The Atherosclerosis Risk in Communities (ARIC) Study. *American Journal of Epidemiology,* 151**,** 478-487.

CHAMBLESS, L. E., HEISS, G., FOLSOM, A. R., ROSAMOND, W., SZKLO, M., SHARRETT, A. R. & CLEGG, L. X. 1997. Association of Coronary Heart Disease Incidence with Carotid Arterial Wall Thickness and Major Risk Factors: The Atherosclerosis Risk in Communities (ARIC) Study, 1987–1993. *American Journal of Epidemiology,* 146**,** 483-494.

CHAMBLISS, L. R. 2008. Intimate partner violence and its implication for pregnancy. *Clinical Obstetrics and Gynecology,* 51**,** 385-397.

CHATTERJEE, J., GULLAM, J., VATISH, M. & THORNTON, S. 2007. The management of preterm labour. *Archives of Disease in Childhood. Fetal and Neonatal Edition,* 92**,** F88-F93.

CHAUHAN, S. P., SCARDO, J. A., HAYES, E., ABUHAMAD, A. Z. & BERGHELLA, V. 2010. Twins: prevalence, problems, and preterm births. *American Journal of Obstetrics & Gynecology,* 203**,** 305-315.

CHEONG, J. L. & DOYLE, L. W. 2012. Increasing rates of prematurity and epidemiology of late preterm birth. *Journal of Paediatrics and Child Health,* 48**,** 784-788.

CHOBANIAN, A. V., BAKRIS, G. L., BLACK, H. R. & ET AL. 2003. The seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure: The JNC 7 report. *Journal of the American Medical Association,* 289**,** 2560-2571.

CHRISTIE, B. 2000. Premature babies have high death and disability rate. *British Medical Journal,* 321**,** 467.

CLARK, R. H., THOMAS, P. & PEABODY, J. 2003. Extrauterine Growth Restriction Remains a Serious Problem in Prematurely Born Neonates. *Pediatrics,* 111**,** 986-990.

CLUBB, F. J., JR. & BISHOP, S. P. 1984. Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy. *Laboratory Investigation,* 50**,** 571-577.

CNATTINGIUS, S., VILLAMOR, E., JOHANSSON, S., EDSTEDT BONAMY, A. K., PERSSON, M., WIKSTROM, A. K. & GRANATH, F. 2013. Maternal obesity and risk of preterm delivery. *Journal of the American Medical Association,* 309**,** 2362-2370.

COLLINS, J. W., JR., DAVID, R. J., SIMON, D. M. & PRACHAND, N. G. 2007. Preterm birth among African American and white women with a lifelong residence in high-income Chicago neighborhoods: an exploratory study. *Ethnicity & Disease,* 17**,** 113-117.

COMMITTEE ON UNDERSTANDING PREMATURE BIRTH ASSURING HEALTHY OUTCOMES 2007. *Preterm Birth: Causes, Consequences, and Prevention*, The National Academies Press.

CONWAY, E. M., COLLEN, D. & CARMELIET, P. 2001. Molecular mechanisms of blood vessel growth. *Cardiovascular Research,* 49**,** 507-521.

COOKE, R. J., AINSWORTH, S. B. & FENTON, A. C. 2004. Postnatal growth retardation: a universal problem in preterm infants. *Archives of Disease in Childhood - Fetal and Neonatal Edition,* 89**,** F428-F430.

COOKE, R. J., RAWLINGS, D. J., MCCORMICK, K., GRIFFIN, I. J., FAULKNER, K., WELLS, J. C., SMITH, J. S. & ROBINSON, S. J. 1999. Body composition of preterm infants during infancy. *Arch Dis Child Fetal Neonatal Ed,* 80**,** F188-191.

COOPER, R., ATHERTON, K. & POWER, C. 2009. Gestational age and risk factors for cardiovascular disease: evidence from the 1958 British birth cohort followed to mid-life. *International Journal of Epidemiology,* 38**,** 235-244.

COOPERSTOCK, M. & CAMPBELL, J. 1996. Excess males in preterm birth: interactions with gestational age, race, and multiple birth. *Obstetrics & Gynecology,* 88**,** 189-193.

COPPER, R. L., GOLDENBERG, R. L., DAS, A., ELDER, N., SWAIN, M., NORMAN, G., RAMSEY, R., COTRONEO, P., COLLINS, B. A., JOHNSON, F., JONES, P. & MEIER, A. M. 1996. The preterm prediction study: maternal stress is associated with spontaneous preterm birth at less than thirty-five weeks' gestation. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. *American Journal of Obstetrics & Gynecology,* 175**,** 1286-1292.

CORSTIUS, H. B., ZIMANYI, M. A., MAKA, N., HERATH, T., THOMAS, W., VAN DER LAARSE, A., WREFORD, N. G. & BLACK, M. J. 2005. Effect of intrauterine growth restriction on the number of cardiomyocytes in rat hearts. *Pediatric Research,* 57**,** 796-800.

COUNSELL, S. J. & BOARDMAN, J. P. 2005. Differential brain growth in the infant born preterm: current knowledge and future developments from brain imaging. *Seminars in Fetal and Neonatal Medicine,* 10**,** 403-410.

COYLE, P. 1985. Interruption of the middle cerebral artery in 10-day-old rat alters normal development of distal collaterals. *Anatomical Record,* 212**,** 179-182.

CRAM, L. F., ZAPATA, M. I., TOY, E. C. & BAKER, B., 3RD 2002. Genitourinary infections and their association with preterm labor. *American Family Physician,* 65**,** 241-248.

CROSSLEY, K. J., ALLISON, B. J., POLGLASE, G. R., MORLEY, C. J., DAVIS, P. G. & HOOPER, S. B. 2009. Dynamic changes in the direction of blood flow through the ductus arteriosus at birth. *Journal of Physiology,* 587**,** 4695-4704.

CROSSLEY, K. J., MORLEY, C. J., ALLISON, B. J., POLGLASE, G. R., DARGAVILLE, P. A., HARDING, R. & HOOPER, S. B. 2007. Blood gases and pulmonary blood flow during resuscitation of very preterm lambs treated with antenatal betamethasone and/or Curosurf: effect of positive end-expiratory pressure. *Pediatric Research,* 62**,** 37-42.

CROWLEY, P. 2000. Prophylactic corticosteroids for preterm birth. *Cochrane Database of Systematic Reviews***,** CD000065.

CROWTHER, C. A. & HARDING, J. E. 2007. Repeat doses of prenatal corticosteroids for women at risk of preterm birth for preventing neonatal respiratory disease. *Cochrane Database of Systematic Reviews***,** CD003935.

CRUMP, C., WINKLEBY, M. A., SUNDQUIST, K. & SUNDQUIST, J. 2011. Risk of Hypertension Among Young Adults Who Were Born Preterm: A Swedish National Study of 636,000 Births. *American Journal of Epidemiology,* 173**,** 797-803.

CUNEO, B. 2013. Transition from Fetal to Neonatal Circulation. *In:* DA CRUZ, E. M., IVY, D. & JAGGERS, J. (eds.) *Pediatric and Congenital Cardiology, Cardiac Surgery and Intensive Care.* Springer London.

CZIKK, M. J., MCCARTHY, F. P. & MURPHY, K. E. 2011. Chorioamnionitis: from pathogenesis to treatment. *Clinical Microbiology and Infection,* 17**,** 1304-1311.

DAGLE, J. M., LEPP, N. T., COOPER, M. E., SCHAA, K. L., KELSEY, K. J., ORR, K. L., CAPRAU, D., ZIMMERMAN, C. R., STEFFEN, K. M., JOHNSON, K. J., MARAZITA, M. L. & MURRAY, J. C. 2009. Determination of genetic predisposition to patent ductus arteriosus in preterm infants. *Pediatrics,* 123**,** 1116-1123.

DANI, C., BERTINI, G., PEZZATI, M., CECCHI, A., CAVIGLIOLI, C. & RUBALTELLI, F. F. 2004. Early extubation and nasal continuous positive airway pressure after surfactant treatment for respiratory distress syndrome among preterm infants <30 weeks' gestation. *Pediatrics,* 113**,** e560-563.

DANNEVIG, I., DALE, H. C., LIESTOL, K. & LINDEMANN, R. 2005. Blood pressure in the neonate: three non-invasive oscillometric pressure monitors compared with invasively measured blood pressure. *Acta Paediatrica,* 94**,** 191-196.

DAS, U. G. & SYSYN, G. D. 2004. Abnormal fetal growth: intrauterine growth retardation, small for gestational age, large for gestational age. *Pediatric Clinics of North America,* 51**,** 639-654, viii.

DAUBMAN, S. 2010. MicroRNAs in Angiogenesis and Vascular Smooth Muscle Cell Function. *Circulation Research,* 106**,** 423-425.

DAVIS, P. H., DAWSON, J. D., RILEY, W. A. & LAUER, R. M. 2001. Carotid Intimal-Medial Thickness Is Related to Cardiovascular Risk Factors Measured From Childhood Through Middle Age. *Circulation,* 104**,** 2815-2819.

DAWSON, J. A., KAMLIN, C. O. F., WONG, C., TE PAS, A. B., VENTO, M., COLE, T. J., DONATH, S. M., HOOPER, S. B., DAVIS, P. G. & MORLEY, C. J. 2010. Changes in heart rate in the first minutes after birth. *Archives of Disease in Childhood - Fetal and Neonatal Edition,* 95**,** F177-F181.

DE GROOT, E., HOVINGH, G. K., WIEGMAN, A., DURIEZ, P., SMIT, A. J., FRUCHART, J.-C. & KASTELEIN, J. J. P. 2004. Measurement of Arterial Wall Thickness as a Surrogate Marker for Atherosclerosis. *Circulation,* 109**,** III-33-III-38.

DE MATTEO, R., BLASCH, N., STOKES, V., DAVIS, P. & HARDING, R. 2010. Induced Preterm Birth in Sheep: A Suitable Model for Studying the Developmental Effects of Moderately Preterm Birth. *Reprod Sci,* 17**,** 724-733.

DELNORD, M., BLONDEL, B. & ZEITLIN, J. 2015. What contributes to disparities in the preterm birth rate in European countries? *Current Opinion in Obstetrics & Gynecology,* 27**,** 133-142.

DELPISHEH, A., KELLY, Y., RIZWAN, S. & BRABIN, B. J. 2006. Socio-economic status, smoking during pregnancy and birth outcomes: an analysis of cross-sectional community studies in Liverpool (1993-2001). *Journal of Child Health Care,* 10**,** 140-148.

DEMARINI, S., DOLLBERG, S., HOATH, S. B., HO, M. & DONOVAN, E. F. 1999. Effects of antenatal corticosteroids on blood pressure in very low birth weight infants during the first 24 hours of life. *Journal of Perinatology,* 19**,** 419-425.

DEMICHEVA, E. & CRISPI, F. 2014. Long-Term Follow-Up of Intrauterine Growth Restriction: Cardiovascular Disorders. *Fetal Diagnosis and Therapy,* 36**,** 143-153.

DEMIR, R., KAYISLI, U. A., CAYLI, S. & HUPPERTZ, B. 2006. Sequential Steps During Vasculogenesis and Angiogenesis in the Very Early Human Placenta. *Placenta,* 27**,** 535-539.

DI STEFANO, I., KOOPMANS, D. R. & LANGILLE, B. L. 1998. Modulation of arterial growth of the rabbit carotid artery associated with experimental elevation of blood flow. *Journal of Vascular Research,* 35**,** 1-7.

DICE, J. E. & BHATIA, J. 2007. Patent Ductus Arteriosus: An Overview. *Journal of Pediatric Pharmacology and Therapeutics,* 12**,** 138-146.

DICKEY, R. P. 2007. The relative contribution of assisted reproductive technologies and ovulation induction to multiple births in the United States 5 years after the Society for Assisted Reproductive Technology/American Society for Reproductive Medicine recommendation to limit the number of embryos transferred. *Fertility and Sterility,* 88**,** 1554-1561.

DIGIULIO, D. B., GERVASI, M., ROMERO, R., VAISBUCH, E., MAZAKI-TOVI, S., KUSANOVIC, J. P., SEOK, K. S., GÓMEZ, R., MITTAL, P., GOTSCH, F., CHAIWORAPONGSA, T., OYARZÚN, E., KIM, C. J. & RELMAN, D. A. 2010. Microbial invasion of the amniotic cavity in pregnancies with small-for-gestational-age fetuses. *Journal of Perinatal Medicine,* 38**,** 495-502.

DOESBURG, S. M., RIBARY, U., HERDMAN, A. T., MILLER, S. P., POSKITT, K. J., MOISEEV, A., WHITFIELD, M. F., SYNNES, A. & GRUNAU, R. E. 2011. Altered long-range alpha-band synchronization during visual short-term memory retention in children born very preterm. *Neuroimage,* 54**,** 2330-2339.

DONOGHUE, D., LINCOLN, D., MORGAN, G. & BEARD, J. 2013. Influences on the degree of preterm birth in New South Wales. *Australian and New Zealand Journal of Public Health,* 37**,** 562-567.

DOUGLAS-ESCOBAR, M. & WEISS, M. D. 2012. Biomarkers of brain injury in the premature infant. *Frontiers in Neurology,* 3**,** 185.

DOYLE, L. W. 2008. Cardiopulmonary outcomes of extreme prematurity. *Semin Perinatol,* 32**,** 28-34.

DOYLE, L. W., FORD, G. W., DAVIS, N. M. & CALLANAN, C. 2000. Antenatal corticosteroid therapy and blood pressure at 14 years of age in preterm children. *Clinical Science,* 98**,** 137-142.

DULEY, L. 2009. The Global Impact of Pre-eclampsia and Eclampsia. *Seminars in Perinatology,* 33**,** 130-137.

DUPLESSIS, H. M., BELL, R. & RICHARDS, T. 1997. Adolescent pregnancy: understanding the impact of age and race on outcomes. *Journal of Adolescent Health,* 20**,** 187-197.

DUSICK, A. M., POINDEXTER, B. B., EHRENKRANZ, R. A. & LEMONS, J. A. 2003. Growth failure in the preterm infant: can we catch up? *Seminars in Perinatology,* 27**,** 302-310.

EDSTEDT BONAMY, A. K., BENGTSSON, J., NAGY, Z., DE KEYZER, H. & NORMAN, M. 2008. Preterm birth and maternal smoking in pregnancy are strong risk factors for aortic narrowing in adolescence. *Acta Paediatrica,* 97**,** 1080-1085.

EDWARDS, R. K. 2005. Chorioamnionitis and labor. *Obstetrics & Gynecology Clinics of North America,* 32**,** 287-296, x.

EISELT, M., CURZI-DASCALOVA, L., CLAIRAMBAULT, J., KAUFFMANN, F., MEDIGUE, C. & PEIRANO, P. 1993. Heart-rate variability in low-risk prematurely born infants reaching normal term: a comparison with full-term newborns. *Early Human Development,* 32**,** 183-195.

EKWO, E. E., GOSSELINK, C. A. & MOAWAD, A. 1993. Previous pregnancy outcomes and subsequent risk of preterm rupture of amniotic sac membranes. *British Journal of Obstetrics and Gynaecology,* 100**,** 536-541.

EL-METWALLY, D., VOHR, B. & TUCKER, R. 2000. Survival and neonatal morbidity at the limits of viability in the mid 1990s: 22 to 25 weeks. *Journal of Pediatrics,* 137**,** 616-522.

EL MALLAH, K. O., NARCHI, H., KULAYLAT, N. A. & SHABAN, M. S. 1997. Gestational and pre-gestational diabetes: comparison of maternal and fetal characteristics and outcome. *International Journal of Gynecology & Obstetrics,* 58**,** 203-209.

ELDER, D. E. & ZUCCOLLO, J. M. 2005. Autopsy after death due to extreme prematurity. *Archives of Disease in Childhood - Fetal and Neonatal Edition,* 90**,** F270-272.

ELSMÉN, E., PUPP, I. H. & HELLSTRÖM-WESTAS, L. 2004a. Preterm male infants need more initial respiratory and circulatory support than female infants. *Acta Paediatrica,* 93**,** 529-533.

ELSMÉN, E., STEEN, M. & HELLSTRÖM-WESTAS, L. 2004b. Sex and gender differences in newborn infants: why are boys at increased risk? *Journal of Men's Health & Gender,* 1**,** 303-311.

ERIKSSON, A. W. & FELLMAN, J. 2007. Temporal Trends in the Rates of Multiple Maternities in England and Wales. *Twin Research and Human Genetics,* 10**,** 626-632.

ERIKSSON, J. G., FORSEN, T., TUOMILEHTO, J., WINTER, P. D., OSMOND, C. & BARKER, D. J. 1999. Catch-up growth in childhood and death from coronary heart disease: longitudinal study. *British Medical Journal,* 318**,** 427-431.

ESCHENBACH, D. A., HILLIER, S., CRITCHLOW, C., STEVENS, C., DEROUEN, T. & HOLMES, K. K. 1988. Diagnosis and clinical manifestations of bacterial vaginosis. *American Journal of Obstetrics & Gynecology,* 158**,** 819-828.

ESCRIBA-AGUIR, V., CLEMENTE, I. & SAUREL-CUBIZOLLES, M. 2001a. Socio-economic factors associated with preterm delivery. Results of the European project in Spain. *Gaceta Sanitaria,* 15**,** 6-13.

ESCRIBA-AGUIR, V., PEREZ-HOYOS, S. & SAUREL-CUBIZOLLES, M. J. 2001b. Physical load and psychological demand at work during pregnancy and preterm birth. *International Archives of Occupational and Environmental Health,* 74**,** 583-588.

EUSER, A. M., DE WIT, C. C., FINKEN, M. J., RIJKEN, M. & WIT, J. M. 2008. Growth of preterm born children. *Hormone Research in Paediatrics,* 70**,** 319-328.

EVERS, J. L. 2002. Female subfertility. *Lancet.,* 360**,** 151-159.

FERRARA, N., CARVER-MOORE, K., CHEN, H., DOWD, M., LU, L., O'SHEA, K. S., POWELL-BRAXTON, L., HILLAN, K. J. & MOORE, M. W. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature.,* 380**,** 439-442.

FIELDS, D. A., KRISHNAN, S. & WISNIEWSKI, A. B. 2009. Sex differences in body composition early in life. *Gender Medicine,* 6**,** 369-375.

FISHMAN, M. C. & CHIEN, K. R. 1997. Fashioning the vertebrate heart: earliest embryonic decisions. *Development,* 124**,** 2099-2117.

FLEISHER, B., KULOVICH, M. V., HALLMAN, M. & GLUCK, L. 1985. Lung profile: sex differences in normal pregnancy. *Obstetrics & Gynecology,* 66**,** 327-330.

FONSECA, E. B., CELIK, E., PARRA, M., SINGH, M. & NICOLAIDES, K. H. 2007. Progesterone and the risk of preterm birth among women with a short cervix. *New England Journal of Medicine,* 357**,** 462-469.

FORTUNATO, S. J., MENON, R., VELEZ, D. R., THORSEN, P. & WILLIAMS, S. M. 2008. Racial disparity in maternal-fetal genetic epistasis in spontaneous preterm birth. *American Journal of Obstetrics & Gynecology,* 198**,** 666 e1-9.

FREEDMAN, S. B. & ISNER, J. M. 2002. Therapeutic angiogenesis for coronary artery disease. *Annals of Internal Medicine,* 136**,** 54-71.

FRENCH, J. I., MCGREGOR, J. A., DRAPER, D., PARKER, R. & MCFEE, J. 1999. Gestational bleeding, bacterial vaginosis, and common reproductive tract infections: risk for preterm birth and benefit of treatment. *Obstetrics & Gynecology,* 93**,** 715-724.

FUNG, Y. C. & LIU, S. Q. 1995. Determination of the mechanical properties of the different layers of blood vessels in vivo. *Proceedings of the National Academy of Sciences,* 92**,** 2169-2173.

FYFE, K. L., YIALLOUROU, S. R., WONG, F. Y. & HORNE, R. S. C. 2014. The development of cardiovascular and cerebral vascular control in preterm infants. *Sleep Medicine Reviews,* 18**,** 299-310.

GABALLA, M. A., JACOB, C. T., RAYA, T. E., LIU, J., SIMON, B. & GOLDMAN, S. 1998. Large artery remodeling during aging: biaxial passive and active stiffness. *Hypertension,* 32**,** 437-443.

GABBARD, S. A. & SCHRYER, J. 2003. Early amplification options. *Mental Retardation and Developmental Disabilities Research Reviews,* 9**,** 236-242.

GAGNON, R., CAMPBELL, K., HUNSE, C. & PATRICK, J. 1987. Patterns of human fetal heart rate accelerations from 26 weeks to term. *American Journal of Obstetrics & Gynecology,* 157**,** 743-748.

GAIRDNER, D. & PEARSON, J. 1971. A Growth Chart for Premature and Other Infants. *Archives of Disease in Childhood,* 46**,** 783-787.

GALLOP, P. M. & PAZ, M. A. 1975. Posttranslational protein modifications, with special attention to collagen and elastin. *Physiological Reviews,* 55**,** 418-487.

GARDNER, M. O., GOLDENBERG, R. L., CLIVER, S. P., TUCKER, J. M., NELSON, K. G. & COPPER, R. L. 1995. The origin and outcome of preterm twin pregnancies. *Obstetrics & Gynecology,* 85**,** 553-557.

GARDOSI, J. O. 2005. Prematurity and fetal growth restriction. *Early Human Development,* 81**,** 43-49.

GAVIN, A. R., HOLZMAN, C., SIEFERT, K. & TIAN, Y. 2009. Maternal depressive symptoms, depression, and psychiatric medication use in relation to risk of preterm delivery. *Womens Health Issues,* 19**,** 325-334.

GEORGIEFF, M. K., MILLS, M. M., GOMEZ-MARIN, O. & SINAIKO, A. R. 1996. Rate of change of blood pressure in premature and full term infants from birth to 4 months. *Pediatric Nephrology,* 10**,** 152-155.

GHODS, E., KREISSL, A., BRANDSTETTER, S., FUIKO, R. & WIDHALM, K. 2011. Head circumference catch-up growth among preterm very low birth weight infants: effect on neurodevelopmental outcome. *Journal of perinatal medicine,* 39**,** 579-86.

GIANNI, M. L., ROGGERO, P., LIOTTO, N., AMATO, O., PIEMONTESE, P., MORNIROLI, D., BRACCO, B. & MOSCA, F. 2012. Postnatal catch-up fat after late preterm birth. *Pediatric Research,* 72**,** 637-640.

GIBBS, R. S., ROMERO, R., HILLIER, S. L., ESCHENBACH, D. A. & SWEET, R. L. 1992. A review of premature birth and subclinical infection. *American Journal of Obstetrics & Gynecology,* 166**,** 1515-1528.

GILBERT, W. M., NESBITT, T. S. & DANIELSEN, B. 2003. The cost of prematurity: quantification by gestational age and birth weight. *Obstetrics & Gynecology,* 102**,** 488-492.

GILL, A., YU, V. Y., BAJUK, B. & ASTBURY, J. 1986. Postnatal growth in infants born before 30 weeks' gestation. *Archives of disease in childhood,* 61**,** 549-553.

GILLOGLEY, K. M., EVANS, A. T., HANSEN, R. L., SAMUELS, S. J. & BATRA, K. K. 1990. The perinatal impact of cocaine, amphetamine, and opiate use detected by universal intrapartum screening. *American Journal of Obstetrics & Gynecology,* 163**,** 1535-1542.

GIRAUD, G. D., LOUEY, S., JONKER, S., SCHULTZ, J. & THORNBURG, K. L. 2006. Cortisol Stimulates Cell Cycle Activity in the Cardiomyocyte of the Sheep Fetus. *Endocrinology,* 147**,** 3643-3649.

GLANTZ, J. C. 1997. Screening and treatment of bacterial vaginosis during pregnancy: a model for determining benefit. *American Journal of Perinatology,* 14**,** 487-490.

GOFFINET, F. 2005. Primary predictors of preterm labour. *British Journal of Obstetrics and Gynaecology,* 112 Suppl 1**,** 38-47.

GOH, J. M., BENSLEY, J. G., KENNA, K., SOZO, F., BOCKING, A. D., BRIEN, J., WALKER, D., HARDING, R. & BLACK, M. J. 2011. Alcohol exposure during late gestation adversely affects myocardial development with implications for postnatal cardiac function. *American Journal of Physiology - Heart and Circulatory Physiology,* 300**,** H645-H651.

GOLDENBERG, R. L., ANDREWS, W. W., FAYE-PETERSEN, O., CLIVER, S., GOEPFERT, A. R. & HAUTH, J. C. 2006. The Alabama Preterm Birth Project: placental histology in recurrent spontaneous and indicated preterm birth. *American Journal of Obstetrics & Gynecology,* 195**,** 792-796.

GOLDENBERG, R. L. & CULHANE, J. F. 2007. Low birth weight in the United States. *American Journal of Clinical Nutrition,* 85**,** 584S-590S.

GOLDENBERG, R. L., CULHANE, J. F., IAMS, J. D. & ROMERO, R. 2008. Epidemiology and causes of preterm birth. *Lancet,* 371**,** 75-84.

GOLDENBERG, R. L., HAUTH, J. C. & ANDREWS, W. W. 2000. Intrauterine infection and preterm delivery. *New England Journal of Medicine,* 342**,** 1500-1507.

GOLDENBERG, R. L., MAYBERRY, S. K., COPPER, R. L., DUBARD, M. B. & HAUTH, J. C. 1993. Pregnancy outcome following a second-trimester loss. *Obstetrics & Gynecology,* 81**,** 444-446.

GOLDENBERG, R. L., MERCER, B. M., MEIS, P. J., COPPER, R. L., DAS, A. & MCNELLIS, D. 1996. The preterm prediction study: fetal fibronectin testing and spontaneous preterm birth. NICHD Maternal Fetal Medicine Units Network. *Obstetrics & Gynecology,* 87**,** 643-648.

GONG, Y.-H., JI, C.-Y. & SHAN, J.-P. 2013. A Longitudinal Study on the Catch-Up Growth of Preterm and Term Infants of Low, Appropriate, and High Birth Weight. *Asia-Pacific Journal of Public Health*.

GRAVETT, M. G., HUMMEL, D., ESCHENBACH, D. A. & HOLMES, K. K. 1986. Preterm labor associated with subclinical amniotic fluid infection and with bacterial vaginosis. *Obstetrics & Gynecology,* 67**,** 229-237.

GREGORY, K. E., DEFORGE, C. E., NATALE, K. M., PHILLIPS, M. & VAN MARTER, L. J. 2011. Necrotizing Enterocolitis in the Premature Infant: Neonatal Nursing Assessment, Disease Pathogenesis, and Clinical Presentation. *Advances in neonatal care : official journal of the National Association of Neonatal Nurses,* 11**,** 155-166.

GRJIBOVSKI, A. M., BYGREN, L. O., YNGVE, A. & SJOSTROM, M. 2005. Large social disparities in spontaneous preterm birth rates in transitional Russia. *Public Health,* 119**,** 77-86.

GUNAY, F., ALPAY, H., GOKCE, I. & BILGEN, H. 2014. Is late-preterm birth a risk factor for hypertension in childhood? *Eur J Pediatr,* 173**,** 751-756.

GUNDERSEN, H. J., JENSEN, E. B., KIEU, K. & NIELSEN, J. 1999. The efficiency of systematic sampling in stereology--reconsidered. *Journal of Microscopy,* 193**,** 199-211.

GUYTON, J. R. & HARTLEY, C. J. 1985. Flow restriction of one carotid artery in juvenile rats inhibits growth of arterial diameter. *American Journal of Physiology,* 248**,** H540-546.

GWATHMEY, T. M., SHALTOUT, H. A., ROSE, J. C., DIZ, D. I. & CHAPPELL, M. C. 2011. Glucocorticoid-induced fetal programming alters the functional complement of angiotensin receptor subtypes within the kidney. *Hypertension,* 57**,** 620-626.

HACKING, D. F., GIBSON, A.-M., ROBERTSON, C., DOYLE, L. W. & FOR THE VICTORIAN INFANT COLLABORATIVE STUDY, G. 2013. Respiratory function at age 8–9 after extremely low birthweight or preterm birth in Victoria in 1997. *Pediatric Pulmonology,* 48**,** 449-455.

HALES, C. N. & OZANNE, S. E. 2003. The dangerous road of catch-up growth. *Journal of Physiology,* 547**,** 5-10.

HAMILTON, B., MININO, A., MARTIN, J., KOCHANEK, K., STROBINO, D. & GUYER, B. 2007a. Annual summary of vital statistics: 2005. *Pediatrics,* 119**,** 345-360.

HAMILTON, B. E., MARTIN, J. A. & VENTURA, S. J. 2012. Births: preliminary data for 2011. *National Vital Statistics Reports,* 61**,** 1-18.

HAMILTON, P. K., LOCKHART, C. J., QUINN, C. E. & MCVEIGH, G. E. 2007b. Arterial stiffness: clinical relevance, measurement and treatment. *Clinical Science,* 113**,** 157-170.

HARPER, R. M. 2000. Sudden infant death syndrome: a failure of compensatory cerebellar mechanisms? *Pediatric Research,* 48**,** 140-142.

HARVEY, R. P. & ROSENTHAL, N. 1999. *Heart Development,* San Diego, Academic Press.

HASSAN, M. I., ASCHNER, Y., MANNING, C. H., XU, J. & ASCHNER, J. L. 2003. Racial differences in selected cytokine allelic and genotypic frequencies among healthy, pregnant women in North Carolina. *Cytokine,* 21**,** 10-16.

HAY, W. W., JR. 2008. Strategies for feeding the preterm infant. *Neonatology,* 94**,** 245-254.

HEAMAN, M. I. 2005. Relationships between physical abuse during pregnancy and risk factors for preterm birth among women in Manitoba. *Journal of Obstetric, Gynecologic, & Neonatal Nursing,* 34**,** 721-731.

HEAMAN, M. I., BLANCHARD, J. F., GUPTON, A. L., MOFFATT, M. E. & CURRIE, R. F. 2005. Risk factors for spontaneous preterm birth among Aboriginal and non-Aboriginal women in Manitoba. *Paediatric and Perinatal Epidemiology,* 19**,** 181-193.

HEIL, M., EITENMÜLLER, I., SCHMITZ-RIXEN, T. & SCHAPER, W. 2006. Arteriogenesis versus angiogenesis: similarities and differences. *Journal of Cellular and Molecular Medicine,* 10**,** 45-55.

HELLSTRÖM, A., SMITH, L. E. H. & DAMMANN, O. 2013. Retinopathy of prematurity. *The Lancet,* 382**,** 1445-1457.

HERON, M., SUTTON, P. D., XU, J., VENTURA, S. J., STROBINO, D. M. & GUYER, B. 2010. Annual summary of vital statistics: 2007. *Pediatrics,* 125**,** 4-15.

HIDALGO, L. A., CHEDRAUI, P. A. & CHAVEZ, M. J. 2005. Obstetrical and neonatal outcome in young adolescents of low socio-economic status: a case control study. *Archives of Gynecology and Obstetrics,* 271**,** 207-211.

HILL, G. A., BRYAN, S., HERBERT, C. M., 3RD, SHAH, D. M. & WENTZ, A. C. 1990. Complications of pregnancy in infertile couples: routine treatment versus assisted reproduction. *Obstetrics & Gynecology,* 75**,** 790-794.

HILLIER, S. L., KROHN, M. A., CASSEN, E., EASTERLING, T. R., RABE, L. K. & ESCHENBACH, D. A. 1995a. The role of bacterial vaginosis and vaginal bacteria in amniotic fluid infection in women in preterm labor with intact fetal membranes. *Clinical Infectious Diseases,* 20 Suppl 2**,** S276-278.

HILLIER, S. L., MARTIUS, J., KROHN, M., KIVIAT, N., HOLMES, K. K. & ESCHENBACH, D. A. 1988. A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity. *New England Journal of Medicine,* 319**,** 972-978.

HILLIER, S. L., NUGENT, R. P., ESCHENBACH, D. A., KROHN, M. A., GIBBS, R. S., MARTIN, D. H., COTCH, M. F., EDELMAN, R., PASTOREK, J. G., 2ND, RAO, A. V. & ET AL. 1995b. Association between bacterial vaginosis and preterm delivery of a low-birth-weight

infant. The Vaginal Infections and Prematurity Study Group. *New England Journal of Medicine,* 333**,** 1737-1742.

HILLMAN, N., KALLAPUR, S. G. & JOBE, A. 2012. Physiology of Transition from intrauterine to Extrauterine Life. *Clinics in perinatology,* 39**,** 769-783.

HINTZ, S. R., KENDRICK, D. E., VOHR, B. R., POOLE, W. K. & HIGGINS, R. D. 2005. Changes in neurodevelopmental outcomes at 18 to 22 months' corrected age among infants of less than 25 weeks' gestational age born in 1993-1999. *Pediatrics,* 115**,** 1645-1651.

HOCHER, B. 2007. Fetal programming of cardiovascular diseases in later life - mechanisms beyond maternal undernutrition. *Journal of Physiology,* 579**,** 287-288.

HOERTER, J. A. & VASSORT, G. 1982. Participation of the sarcolemma in the control of relaxation of the mammalian heart during perinatal development. *Advances in myocardiology,* 3**,** 373-380.

HOFMEYR, G. 2009. *Antenatal corticosteroids for women at risk of preterm birth: RHL commentary* [Online]. Geneva: World Health Organization Reproductive Health Library. Available: http://apps.who.int/rhl/pregnancy_childbirth/complications/preterm_birth/cd004454_hofmeyrgj_com/en/index.html [Accessed 5 June 2015].

HOGUE, D. E., PROCTOR, J. F., WARNER, R. G. & LOOSLI, J. K. 1962. Relation of Selenium, Vitamin E and an Unidentified Factor to Muscular Dystrophy (Stiff-Lamb or White-Muscle Disease) in the Lamb. *J Anim Sci,* 21**,** 25-29.

HOLST, E., GOFFENG, A. R. & ANDERSCH, B. 1994. Bacterial vaginosis and vaginal microorganisms in idiopathic premature labor and association with pregnancy outcome. *Journal of Clinical Microbiology,* 32**,** 176-186.

HOOPER, S. B., POLGLASE, G. R. & TE PAS, A. B. 2014. A physiological approach to the timing of umbilical cord clamping at birth. *Archives of Disease in Childhood - Fetal and Neonatal Edition*.

HOOPER, S. B., TE PAS, A. B., LANG, J., VAN VONDEREN, J. J., ROEHR, C. C., KLUCKOW, M., GILL, A. W., WALLACE, E. M. & POLGLASE, G. R. 2015. Cardiovascular transition at birth: a physiological sequence. *Pediatric Research,* 77**,** 608-614.

HOVI, P., TURANLAHTI, M., STRANG-KARLSSON, S., WEHKALAMPI, K., JÄRVENPÄÄ, A.-L., ERIKSSON, J. G., KAJANTIE, E. & ANDERSSON, S. 2011. Intima-Media Thickness and Flow-Mediated Dilatation in the Helsinki Study of Very Low Birth Weight Adults. *Pediatrics,* 127**,** e304-e311.

HOY, W. E., BERTRAM, J. F., DENTON, R. D., ZIMANYI, M., SAMUEL, T. & HUGHSON, M. D. 2008. Nephron number, glomerular volume, renal disease and hypertension. *Current opinion in nephrology and hypertension,* 17**,** 258-265.

HOYERT, D. L., HERON, M. P., MURPHY, S. L. & KUNG, H. C. 2006. Deaths: Final Data for 2003. *National Vital Statistics Reports,* 54**,** 1-120.

HUANG, W. L., HARPER, C. G., EVANS, S. F., NEWNHAM, J. P. & DUNLOP, S. A. 2001. Repeated prenatal corticosteroid administration delays myelination of the corpus callosum in fetal sheep. *International Journal of Developmental Neuroscience,* 19**,** 415-425.

HULSEY, T. C., PATRICK, C. H., ALEXANDER, G. R. & EBELING, M. 1991. Prenatal care and prematurity: is there an association in uncomplicated pregnancies? *Birth,* 18**,** 146-150.

HUNTER, L. E. & SIMPSON, J. M. 2014. Prenatal screening for structural congenital heart disease. *Nature Reviews Cardiology,* 11**,** 323-334.

HUTTENBACH, Y., OSTROWSKI, M. L., THALLER, D. & KIM, H.-S. 2001. Cell proliferation in the growing human heart: MIB-1 immunostaining in preterm and term infants at autopsy. *Cardiovascular Pathology,* 10**,** 119-123.

IAMS, J. D., GOLDENBERG, R. L., MEIS, P. J., MERCER, B. M., MOAWAD, A., DAS, A., THOM, E., MCNELLIS, D., COPPER, R. L., JOHNSON, F. & ROBERTS, J. M. 1996. The length of the cervix and the risk of spontaneous premature delivery. National Institute of Child Health and Human Development Maternal Fetal Medicine Unit Network. *New England Journal of Medicine,* 334**,** 567-572.

IBANEZ, L., ONG, K., DUNGER, D. B. & DE ZEGHER, F. 2006. Early development of adiposity and insulin resistance after catch-up weight gain in small-for-gestational-age children. *Journal of Clinical Endocrinology & Metabolism,* 91**,** 2153-2158.

IBRAHIM, C. P. 2008. Hypotension in preterm infants. *Indian Pediatrics,* 45**,** 285-294.

INGEMARSSON, I. 2003. Gender aspects of preterm birth. *BJOG,* 110**,** 34-38.

INSTITUTE OF MEDICINE (US) COMMITTEE ON UNDERSTANDING PREMATURE BIRTH AND ASSURING HEALTHY OUTCOMES 2007. Mortality and Acute Complications in Preterm Infants. *In:* BEHRMAN, R. E. & BUTLER, A. S. (eds.) *Preterm Birth: Causes, Consequences, and Prevention.* Washington (DC): National Academies Press (US).

INTENGAN, H. D. & SCHIFFRIN, E. L. 2000. Structure and Mechanical Properties of Resistance Arteries in Hypertension: Role of Adhesion Molecules and Extracellular Matrix Determinants. *Hypertension,* 36**,** 312-318.

ISSELBACHER, E. M. 2005. Thoracic and Abdominal Aortic Aneurysms. *Circulation,* 111**,** 816-828.

IZZO, J. L., JR. & SHYKOFF, B. E. 2001. Arterial stiffness: clinical relevance, measurement, and treatment. *Reviews in Cardiovascular Medicine,* 2**,** 29-34, 37-40.

JADDOE, V. W., TROE, E. J., HOFMAN, A., MACKENBACH, J. P., MOLL, H. A., STEEGERS, E. A. & WITTEMAN, J. C. 2008. Active and passive maternal smoking during pregnancy and the risks of low birthweight and preterm birth: the Generation R Study. *Paediatric and Perinatal Epidemiology,* 22**,** 162-171.

JAISWAL, A., MURKI, S., GADDAM, P. & REDDY, A. 2011. Early neonatal morbidities in late preterm infants. *Indian Pediatrics,* 48**,** 607-611.

JOHANSSON, S., ILIADOU, A., BERGVALL, N., TUVEMO, T., NORMAN, M. & CNATTINGIUS, S. 2005. Risk of high blood pressure among young men increases with the degree of immaturity at birth. *Circulation,* 112**,** 3430-3436.

JOHNSON, R., RAFF, G., JOHENGEN, M., BENNETT, S., PITSILLIDES, K. & FINEMAN, J. 2014. The transition from fetal to postnatal life: feasibility of telemetric measurements of pressure and flow in the pulmonary and systemic circulations of lambs (862.8). *The FASEB Journal,* 28.

JONKER, S. S., ZHANG, L., LOUEY, S., GIRAUD, G. D., THORNBURG, K. L. & FABER, J. J. 2007. Myocyte enlargement, differentiation, and proliferation kinetics in the fetal sheep heart. *Journal of Applied Physiology,* 102**,** 1130-1142.

JOSHI, S. & KOTECHA, S. 2007. Lung growth and development. *Early Human Development,* 83**,** 789-794.

KAHAN, T. 2014. Focus on blood pressure as a major risk factor. *The Lancet,* 383**,** 1866-1868.

KANNEL, W. B. 1996. Blood pressure as a cardiovascular risk factor: Prevention and treatment. *Journal of the American Medical Association,* 275**,** 1571-1576.

KANSAS, G. S. 1996. Selectins and their ligands: current concepts and controversies. *Blood,* 88**,** 3259-3287.

KATAOKA, S., YAMADA, T., CHOU, K., NISHIDA, R., MORIKAWA, M., MINAMI, M., YAMADA, H., SAKURAGI, N. & MINAKAMI, H. 2006. Association between preterm birth and vaginal colonization by mycoplasmas in early pregnancy. *Journal of Clinical Microbiology,* 44**,** 51-55.

KATONA, P. G., FRASZ, A. & EGBERT, J. 1980. Maturation of cardiac control in full-term and preterm infants during sleep. *Early Human Development,* 4**,** 145-159.

KEATING, M. T. 2004. Genetic approaches to disease and regeneration. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences,* 359**,** 795-798.

KEIJZER-VEEN, M. G., DULGER, A., DEKKER, F. W., NAUTA, J. & VAN DER HEIJDEN, B. J. 2010. Very preterm birth is a risk factor for increased systolic blood pressure at a young adult age. *Pediatric Nephrology,* 25**,** 509-516.

KELLER, G., ZIMMER, G., MALL, G., RITZ, E. & AMANN, K. 2003. Nephron Number in Patients with Primary Hypertension. *New England Journal of Medicine,* 348**,** 101-108.

KEMP, C. 2014. Being born 4-6 weeks premature can affect brain structure, function: Study shows cognitive deficits, structural differences in brains of school-age children born late preterm. *AAP News*.

KENT, A. L., WRIGHT, I. M. & ABDEL-LATIF, M. E. 2012. Mortality and adverse neurologic outcomes are greater in preterm male infants. *Pediatrics,* 129**,** 124-131.

KERKHOF, G. F., BREUKHOVEN, P. E., LEUNISSEN, R. W. J., WILLEMSEN, R. H. & HOKKEN-KOELEGA, A. C. S. 2012. Does Preterm Birth Influence Cardiovascular Risk in Early Adulthood? *Journal of Pediatrics,* 161**,** 390-396.e1.

KIM, M. Y., EIBY, Y. A., LUMBERS, E. R., WRIGHT, L. L., GIBSON, K. J., BARNETT, A. C. & LINGWOOD, B. E. 2014. Effects of Glucocorticoid Exposure on Growth and Structural Maturation of the Heart of the Preterm Piglet. *PLoS ONE,* 9**,** e93407.

KIRCHENGAST, S. 2010. Gender Differences in Body Composition from Childhood to Old Age: An Evolutionary Point of View. *Life Science Journal,* 2**,** 1-10.

KISERUD, T. 2005. Physiology of the fetal circulation. *Seminars in Fetal and Neonatal Medicine,* 10**,** 493-503.

KLIEGMAN, R. M., MADURA, D., KIWI, R., EISENBERG, I. & YAMASHITA, T. 1994. Relation of maternal cocaine use to the risks of prematurity and low birth weight. *Journal of Pediatrics,* 124**,** 751-756.

KLUCKOW, M. & EVANS, N. 2000. Low superior vena cava flow and intraventricular haemorrhage in preterm infants. *Archives of Disease in Childhood - Fetal and Neonatal Edition,* 82**,** F188-F194.

KORNER, P. I., BOBIK, A., ANGUS, J. A., ADAMS, M. A. & FRIBERG, P. 1989. Resistance control in hypertension. *Journal of Hypertension. Supplement : official journal of the International Society of Hypertension,* 7**,** S125-134; discussion S135.

KROHN, M. A., HILLIER, S. L., NUGENT, R. P., COTCH, M. F., CAREY, J. C., GIBBS, R. S. & ESCHENBACH, D. A. 1995. The genital flora of women with intraamniotic infection. Vaginal Infection and Prematurity Study Group. *Journal of Infectious Diseases,* 171**,** 1475-1480.

KURDI, A. M., MESLEH, R. A., AL-HAKEEM, M. M., KHASHOGGI, T. Y. & KHALIFA, H. M. 2004. Multiple pregnancy and preterm labor. *Saudi Medical Journal,* 25**,** 632-637.

KUTZ, P., HORSCH, S., KUHN, L. & ROLL, C. 2009. Single-centre vs. population-based outcome data of extremely preterm infants at the limits of viability. *Acta Paediatrica,* 98**,** 1451-1455.

LANGHOFF-ROOS, J., KESMODEL, U., JACOBSSON, B., RASMUSSEN, S. & VOGEL, I. 2006. Spontaneous preterm delivery in primiparous women at low risk in Denmark: population based study. *British Medical Journal,* 332**,** 937-939.

LANGILLE, B. L. 1996. Arterial remodeling: relation to hemodynamics. *Canadian Journal of Physiology and Pharmacology,* 74**,** 834-841.

LANGILLE, B. L., BENDECK, M. P. & KEELEY, F. W. 1989. Adaptations of carotid arteries of young and mature rabbits to reduced carotid blood flow. *American Journal of Physiology - Heart and Circulatory Physiology,* 256**,** H931-H939.

LARSEN, W. J. 2001. *Human Embryology,* Philadelphia, Churchhill Livingstone.

LATAL-HAJNAL, B., VON SIEBENTHAL, K., KOVARI, H., BUCHER, H. U. & LARGO, R. H. 2003. Postnatal growth in VLBW infants: significant association with neurodevelopmental outcome. *Journal of Pediatrics,* 143**,** 163-170.

LAUGHON, M. M., LANGER, J. C., BOSE, C. L., SMITH, P. B., AMBALAVANAN, N., KENNEDY, K. A., STOLL, B. J., BUCHTER, S., LAPTOOK, A. R., EHRENKRANZ, R. A., COTTEN, C. M., WILSON-COSTELLO, D. E., SHANKARAN, S., MEURS, K. P. V., DAVIS, A. S., GANTZ, M. G., FINER, N. N., YODER, B. A., FAIX, R. G., CARLO, W. A., SCHIBLER, K. R., NEWMAN, N. S., RICH, W., DAS, A., HIGGINS, R. D. & WALSH, M. C. 2011. Prediction of Bronchopulmonary Dysplasia by Postnatal Age in Extremely Premature Infants. *American Journal of Respiratory and Critical Care Medicine,* 183**,** 1715-1722.

LAW, M., WALD, N. & MORRIS, J. 2003. Lowering blood pressure to prevent myocardial infarction and stroke: a new preventive strategy. *Health Technology Assessment,* 7**,** 1-94.

LAWLOR, D. A., HUBINETTE, A., TYNELIUS, P., LEON, D. A., SMITH, G. D. & RASMUSSEN, F. 2007. Associations of gestational age and intrauterine growth with systolic blood pressure in a family-based study of 386,485 men in 331,089 families. *Circulation,* 115**,** 562-568.

LAWN, J. E., BLENCOWE, H., DARMSTADT, G. L. & BHUTTA, Z. A. 2013. Beyond newborn survival: the world you are born into determines your risk of disability-free survival. *Pediatric Research,* 74**,** 1-3.

LAWN, J. E., BLENCOWE, H., OZA, S., YOU, D., LEE, A. C., WAISWA, P., LALLI, M., BHUTTA, Z., BARROS, A. J., CHRISTIAN, P., MATHERS, C. & COUSENS, S. N. 2014. Every Newborn: progress, priorities, and potential beyond survival. *Lancet,* 384**,** 189-205.

LAZDAM, M., DE LA HORRA, A., PITCHER, A., MANNIE, Z., DIESCH, J., TREVITT, C., KYLINTIREAS, I., CONTRACTOR, H., SINGHAL, A., LUCAS, A., NEUBAUER, S., KHARBANDA, R., ALP, N., KELLY, B. & LEESON, P. 2010. Elevated blood pressure in offspring born premature to hypertensive pregnancy: is endothelial dysfunction the underlying vascular mechanism? *Hypertension,* 56**,** 159-165.

LAZZARONI, F., BONASSI, S., MAGNANI, M., CALVI, A., REPETTO, E., SERRA, F., PODESTA, F. & PEARCE, N. 1993. Moderate maternal drinking and outcome of pregnancy. *European Journal of Epidemiology,* 9**,** 599-606.

LEEDS, K., GOURLEY, M., LAWS, P., ZHANG, J., AL-YAMAN, F. & SULLIVAN, E. A. 2007. Indigenous mothers and their babies, Australia 2001-2004. *Perinatal statistics series.* Canberra: Australian Institute of Health and Welfare.

LEITICH, H. 2005. Secondary predictors of preterm labour. *British Journal of Obstetrics and Gynaecology,* 112 Suppl 1**,** 48-50.

LEITICH, H., BODNER-ADLER, B., BRUNBAUER, M., KAIDER, A., EGARTER, C. & HUSSLEIN, P. 2003. Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis. *American Journal of Obstetrics & Gynecology,* 189**,** 139-147.

LEITICH, H. & KISS, H. 2007. Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. *Best Practice & Research Clinical Obstetrics & Gynaecology,* 21**,** 375-390.

LEITMAN, D. C., BENSON, S. C. & JOHNSON, L. K. 1984. Glucocorticoids stimulate collagen and noncollagen protein synthesis in cultured vascular smooth muscle cells. *Journal of Cell Biology,* 98**,** 541-549.

LEMIEUX, S., PRUD'HOMME, D., BOUCHARD, C., TREMBLAY, A. & DESPRES, J. P. 1993. Sex differences in the relation of visceral adipose tissue accumulation to total body fatness. *American Journal of Clinical Nutrition,* 58**,** 463-467.

LEONE, A., ERSFELD, P., ADAMS, M., SCHIFFER, P. M., BUCHER, H. U. & ARLETTAZ, R. 2012. Neonatal morbidity in singleton late preterm infants compared with full-term infants. *Acta Paediatrica,* 101**,** e6-10.

LEPERCQ, J., COSTE, J., THEAU, A., DUBOIS-LAFORGUE, D. & TIMSIT, J. 2004. Factors Associated With Preterm Delivery in Women With Type 1 Diabetes: A cohort study. *Diabetes Care,* 27**,** 2824-2828.

LEPPÄNEN, M., LAPINLEIMU, H., LIND, A., MATOMÄKI, J., LEHTONEN, L., HAATAJA, L., RAUTAVA, P. & ON BEHALF OF THE, P. S. G. 2013. Antenatal and Postnatal Growth and 5-Year Cognitive Outcome in Very Preterm Infants. *Pediatrics*.

LETTIERI, L., VINTZILEOS, A. M., RODIS, J. F., ALBINI, S. M. & SALAFIA, C. M. 1993. Does "idiopathic" preterm labor resulting in preterm birth exist? *American Journal of Obstetrics & Gynecology,* 168**,** 1480-1485.

LEVY, O. 2007. Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nature Reviews Immunology,* 7**,** 379-390.

LEWANDOWSKI, A. J., AUGUSTINE, D., LAMATA, P., DAVIS, E. F., LAZDAM, M., FRANCIS, J., MCCORMICK, K., WILKINSON, A. R., SINGHAL, A., LUCAS, A., SMITH, N. P., NEUBAUER, S. & LEESON, P. 2013a. Preterm heart in adult life: cardiovascular magnetic resonance reveals distinct differences in left ventricular mass, geometry, and function. *Circulation,* 127**,** 197-206.

LEWANDOWSKI, A. J., BRADLOW, W. M., AUGUSTINE, D., DAVIS, E. F., FRANCIS, J., SINGHAL, A., LUCAS, A., NEUBAUER, S., MCCORMICK, K. & LEESON, P. 2013b. Right ventricular systolic dysfunction in young adults born preterm. *Circulation,* 128**,** 713-720.

LEWANDOWSKI, A. J., DAVIS, E. F., YU, G., DIGBY, J. E., BOARDMAN, H., WHITWORTH, P., SINGHAL, A., LUCAS, A., MCCORMICK, K., SHORE, A. C. & LEESON, P. 2015. Elevated blood pressure in preterm-born offspring associates with a distinct antiangiogenic state and microvascular abnormalities in adult life. *Hypertension,* 65**,** 607-614.

LEWANDOWSKI, A. J. & LEESON, P. 2014. Preeclampsia, prematurity and cardiovascular health in adult life. *Early Human Development,* 90**,** 725-729.

LI, F., WANG, X., CAPASSO, J. M. & GERDES, A. M. 1996. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *Journal of Molecular and Cellular Cardiology,* 28**,** 1737-1746.

LIGGINS, G. C. 1994. The role of cortisol in preparing the fetus for birth. *Reproduction, Fertility & Development,* 6**,** 141-150.

LIN, H. C., HSU, C. H., CHEN, H. L., CHUNG, M. Y., HSU, J. F., LIEN, R. I., TSAO, L. Y., CHEN, C. H. & SU, B. H. 2008. Oral probiotics prevent necrotizing enterocolitis in very low birth weight preterm infants: a multicenter, randomized, controlled trial. *Pediatrics,* 122**,** 693-700.

LINDSTROM, K., LINDBLAD, F. & HJERN, A. 2011. Preterm birth and attention-deficit/hyperactivity disorder in school children. *Pediatrics,* 127**,** 858-865.

LIU, C.-Y., CHEN, D., BLUEMKE, D. A., WU, C. O., TEIXIDO-TURA, G., CHUGH, A., VASU, S., LIMA, J. A. C. & HUNDLEY, W. G. 2015. Evolution of Aortic Wall Thickness and Stiffness With Atherosclerosis: Long-Term Follow Up From the Multi-Ethnic Study of Atherosclerosis. *Hypertension,* 65**,** 1015-1019.

LLEVADOT, J. & ASAHARA, T. 2002. Effects of statins on angiogenesis and vasculogenesis. *Revista Española de Cardiología,* 55**,** 838-844.

LOCKWOOD, C. J., ARCURI, F., TOTI, P., FELICE, C. D., KRIKUN, G., GULLER, S., BUCHWALDER, L. F. & SCHATZ, F. 2006. Tumor Necrosis Factor-α and Interleukin-1β Regulate Interleukin-8 Expression in Third Trimester Decidual Cells : Implications for the Genesis of Chorioamnionitis. *American Journal of Pathology,* 169**,** 1294-1302.

LOCKWOOD, C. J. & KUCZYNSKI, E. 1999. Markers of risk for preterm delivery. *Journal of Perinatal Medicine,* 27**,** 5-20.

LOCKWOOD, C. J., SENYEI, A. E., DISCHE, M. R., CASAL, D., SHAH, K. D., THUNG, S. N., JONES, L., DELIGDISCH, L. & GARITE, T. J. 1991. Fetal fibronectin in cervical and vaginal secretions as a predictor of preterm delivery. *New England Journal of Medicine,* 325**,** 669-674.

LOCKWOOD, C. J. & WEINER, S. 1986. Assessment of fetal growth. *Clinics in Perinatology,* 13**,** 3-35.

LOGSDON, E. A., FINLEY, S. D., POPEL, A. S. & GABHANN, F. M. 2014. A systems biology view of blood vessel growth and remodelling. *Journal of Cellular and Molecular Medicine,* 18**,** 1491-1508.

LORENZ, J. M. 2011. Survival and long-term neurodevelopmental outcome of the extremely preterm infant. A systematic review. *Saudi Medical Journal,* 32**,** 885-894.

LOUEY, S., COCK, M. L., STEVENSON, K. M. & HARDING, R. 2000. Placental insufficiency and fetal growth restriction lead to postnatal hypotension and altered postnatal growth in sheep. *Pediatric Research,* 48**,** 808-814.

LOUEY, S., JONKER, S. S., GIRAUD, G. D. & THORNBURG, K. L. 2007. Placental insufficiency decreases cell cycle activity and terminal maturation in fetal sheep cardiomyocytes. *Journal of Physiology,* 580**,** 639-648.

LUMBERS, E. R., KIM, M. Y., BURRELL, J. H., KUMARASAMY, V., BOYCE, A. C., GIBSON, K. J., GATFORD, K. L. & OWENS, J. A. 2009. Effects of intrafetal IGF-I on growth of cardiac myocytes in late-gestation fetal sheep. *American Journal Of Physiology - Endocrinology And Metabolism,* 296**,** E513-519.

LUMLEY, J. 2003. Defining the problem: the epidemiology of preterm birth. *British Journal of Obstetrics and Gynaecology,* 110 Suppl 20**,** 3-7.

LUO, Z. C., WILKINS, R. & KRAMER, M. S. 2004. Disparities in pregnancy outcomes according to marital and cohabitation status. *Obstetrics & Gynecology,* 103**,** 1300-1307.

LUO, Z. C., WILKINS, R. & KRAMER, M. S. 2006. Effect of neighbourhood income and maternal education on birth outcomes: a population-based study. *Canadian Medical Association Journal,* 174**,** 1415-1420.

MA, C. C.-H. & MA, S. 2012. The Role of Surfactant in Respiratory Distress Syndrome. *Open Respiratory Medicine Journal,* 6**,** 44-53.

MADAN, A., JAN, J. E. & GOOD, W. V. 2005. Visual development in preterm infants. *Developmental Medicine & Child Neurology,* 47**,** 276-280.

MADAN, J., CHEN, M., GOODMAN, E., DAVIS, J., ALLAN, W. & DAMMANN, O. 2010. Maternal obesity, gestational hypertension, and preterm delivery. *Journal of Maternal - Fetal & Neonatal Medicine,* 23**,** 82-88.

MALLAT, Z. & TEDGUI, A. 2000. Apoptosis in the vasculature: mechanisms and functional importance. *British Journal of Pharmacology,* 130**,** 947-962.

MAMELLE, N. & MUNOZ, F. 1987. Occupational working conditions and preterm birth: a reliable scoring system. *American Journal of Epidemiology,* 126**,** 150-152.

MANKA, D., FORLOW, S. B., SANDERS, J. M., HURWITZ, D., BENNETT, D. K., GREEN, S. A., LEY, K. & SAREMBOCK, I. J. 2004. Critical role of platelet P-selectin in the response to arterial injury in apolipoprotein-E-deficient mice. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 24**,** 1124-1129.

MARTIN, J. A., HAMILTON, B. E., OSTERMAN, M. J., CURTIN, S. C. & MATTHEWS, T. J. 2015. Births: Final Data for 2013. *Natl Vital Stat Rep,* 64**,** 1-65.

MARTIN, J. A., HAMILTON, B. E., SUTTON, P. D., VENTURA, S. J., MENACKER, F., KIRMEYER, S. & MATHEWS, T. J. 2009. Births: Final Data for 2006. *National Vital Statistics Report.*

MARTIN, J. A., HAMILTON, B. E., SUTTON, P. D., VENTURA, S. J., MENACKER, F. & MUNSON, M. L. 2005. Births: Final Data for 2003. *National Vital Statistics Reports,* 54**,** 1-116.

MARTYN, C. N. & GREENWALD, S. E. 1997. Impaired synthesis of elastin in walls of aorta and large conduit arteries during early development as an initiating event in pathogenesis of systemic hypertension. *Lancet,* 350**,** 953-955.

MATHAI, S., DERRAIK, J. G., CUTFIELD, W. S., DALZIEL, S. R., HARDING, J. E., BIGGS, J., JEFFERIES, C. & HOFMAN, P. L. 2013. Increased adiposity in adults born preterm and their children. *PLoS One,* 8**,** e81840.

MATTACE-RASO, F. U. S., VAN DER CAMMEN, T. J. M., HOFMAN, A., VAN POPELE, N. M., BOS, M. L., SCHALEKAMP, M. A. D. H., ASMAR, R., RENEMAN, R. S., HOEKS, A. P. G.,

BRETELER, M. M. B. & WITTEMAN, J. C. M. 2006. Arterial Stiffness and Risk of Coronary Heart Disease and Stroke: The Rotterdam Study. *Circulation,* 113**,** 657-663.

MAUPIN, R., JR., LYMAN, R., FATSIS, J., PRYSTOWISKI, E., NGUYEN, A., WRIGHT, C., KISSINGER, P. & MILLER, J., JR. 2004. Characteristics of women who deliver with no prenatal care. *Journal of Maternal - Fetal & Neonatal Medicine,* 16**,** 45-50.

MAYHEW, T. M., PHARAOH, A., AUSTIN, A. & FAGAN, D. G. 1997. Stereological estimates of nuclear number in human ventricular cardiomyocytes before and after birth obtained using physical disectors. *Journal of Anatomy,* 191 (Pt 1)**,** 107-115.

MCGOVERN, P. G., LLORENS, A. J., SKURNICK, J. H., WEISS, G. & GOLDSMITH, L. T. 2004. Increased risk of preterm birth in singleton pregnancies resulting from in vitro fertilization-embryo transfer or gamete intrafallopian transfer: a meta-analysis. *Fertility and Sterility,* 82**,** 1514-1520.

MCMILLEN, I. C., ADAMS, M. B., ROSS, J. T., COULTER, C. L., SIMONETTA, G., OWENS, J. A., ROBINSON, J. S. & EDWARDS, L. J. 2001. Fetal growth restriction: adaptations and consequences. *Reproduction,* 122**,** 195-204.

MELVILLE, J. M. & MOSS, T. J. M. 2013. The immune consequences of preterm birth. *Frontiers in Neuroscience,* 7**,** 79.

MENON, R. 2008. Spontaneous preterm birth, a clinical dilemma: etiologic, pathophysiologic and genetic heterogeneities and racial disparity. *Acta Obstetricia et Gynecologica Scandinavica,* 87**,** 590-600.

MENON, R., VELEZ, D. R., SIMHAN, H., RYCKMAN, K., JIANG, L., THORSEN, P., VOGEL, I., JACOBSSON, B., MERIALDI, M., WILLIAMS, S. M. & FORTUNATO, S. J. 2006. Multilocus interactions at maternal tumor necrosis factor-alpha, tumor necrosis factor receptors, interleukin-6 and interleukin-6 receptor genes predict spontaneous preterm labor in European-American women. *American Journal of Obstetrics & Gynecology,* 194**,** 1616-1624.

MENT, L. R. & VOHR, B. R. 2008. Preterm birth and the developing brain. *Lancet Neurology,* 7**,** 378-379.

MERCER, B. M., GOLDENBERG, R. L., MOAWAD, A. H., MEIS, P. J., IAMS, J. D., DAS, A. F., CARITIS, S. N., MIODOVNIK, M., MENARD, M. K., THURNAU, G. R., DOMBROWSKI, M. P., ROBERTS, J. M. & MCNELLIS, D. 1999. The preterm prediction study: effect of gestational age and cause of preterm birth on subsequent obstetric outcome. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. *American Journal of Obstetrics & Gynecology,* 181**,** 1216-1221.

MERCOLA, M., RUIZ-LOZANO, P. & SCHNEIDER, M. D. 2011. Cardiac muscle regeneration: lessons from development. *Genes & Development,* 25**,** 299-309.

MIELKE, G. & BENDA, N. 2001. Cardiac Output and Central Distribution of Blood Flow in the Human Fetus. *Circulation,* 103**,** 1662-1668.

MINKOFF, H. 1983. Prematurity: infection as an etiologic factor. *Obstetrics & Gynecology,* 62**,** 137-144.

MOLLOVA, M., BERSELL, K., WALSH, S., SAVLA, J., DAS, L. T., PARK, S. Y., SILBERSTEIN, L. E., DOS REMEDIOS, C. G., GRAHAM, D., COLAN, S. & KUHN, B. 2013. Cardiomyocyte proliferation contributes to heart growth in young humans. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 1446-1451.

MONEY, D. 2005. The laboratory diagnosis of bacterial vaginosis. *The Canadian Journal of Infectious Diseases & Medical Microbiology,* 16**,** 77-79.

MOORE, K. L. & PERSAUD, T. V. N. 2008. *The Developing Human: Clinically Oriented Embryology,* Philadelphia, Saunders Elsevier.

MORRISON, J. L., BOTTING, K. J., SOO, P. S., MCGILLICK, E. V., HISCOCK, J., ZHANG, S., MCMILLEN, I. C. & ORGEIG, S. 2012. Antenatal Steroids and the IUGR Fetus: Are Exposure and Physiological Effects on the Lung and Cardiovascular System the Same as in Normally Grown Fetuses? *Journal of Pregnancy,* 2012**,** 15.

MOSS, T. J. 2006. Respiratory consequences of preterm birth. *Clinical and Experimental Pharmacology and Physiology,* 33**,** 280-284.

MOUTQUIN, J. M. 2003. Socio-economic and psychosocial factors in the management and prevention of preterm labour. *British Journal of Obstetrics and Gynaecology,* 110 Suppl 20**,** 56-60.

MUELLER-HEUBACH, E. & GUZICK, D. S. 1989. Evaluation of risk scoring in a preterm birth prevention study of indigent patients. *American Journal of Obstetrics & Gynecology,* 160**,** 829-835; discussion 835-837.

MWANSA-KAMBAFWILE, J., COUSENS, S., HANSEN, T. & LAWN, J. E. 2010. Antenatal steroids in preterm labour for the prevention of neonatal deaths due to complications of preterm birth. *International Journal of Epidemiology,* 39**,** i122-i133.

NESS, A. 2009. Prevention of preterm birth based on short cervix: symptomatic women with preterm labor or premature prelabor rupture of membranes. *Seminars in Perinatology,* 33**,** 343-351.

NEU, J. 2007. Gastrointestinal development and meeting the nutritional needs of premature infants. *American Journal of Clinical Nutrition,* 85**,** 629S-634S.

NEWTON, K. & DIXIT, V. M. 2012. Signaling in Innate Immunity and Inflammation. *Cold Spring Harbor Perspectives in Biology,* 4.

NG, P. C. 2009. Use of Oral Erythromycin for the Treatment of Gastrointestinal Dysmotility in Preterm Infants. *Neonatology,* 95**,** 97-104.

NGUYEN, V. B., PROBYN, M. E., CAMPBELL, F., YIN, K. V., SAMUEL, C. S., ZIMANYI, M. A., BERTRAM, J. F., BLACK, M. J. & MORITZ, K. M. 2014. Low-dose maternal alcohol consumption: effects in the hearts of offspring in early life and adulthood. *Physiological Reports,* 2.

NIKLASSON, A., ENGSTROM, E., HARD, A.-L., WIKLAND, K. A. & HELLSTROM, A. 2003. Growth in Very Preterm Children: A Longitudinal Study. *Pediatric Research,* 54**,** 899-905.

NINDL, B. C., SCOVILLE, C. R., SHEEHAN, K. M., LEONE, C. D. & MELLO, R. P. 2002. Gender differences in regional body composition and somatotrophic influences of IGF-I and leptin. *Journal of Applied Physiology,* 92**,** 1611-1618.

NKANSAH-AMANKRA, S., LUCHOK, K. J., HUSSEY, J. R., WATKINS, K. & LIU, X. 2010. Effects of maternal stress on low birth weight and preterm birth outcomes across neighborhoods of South Carolina, 2000-2003. *Maternal and Child Health Journal,* 14**,** 215-226.

NODEN, D. M. 1989. Embryonic Origins and Assembly of Blood Vessels. *American Journal of Respiratory and Critical Care Medicine,* 140**,** 1097-1103.

NORMAN, M. 2010. Preterm birth - an emerging risk factor for adult hypertension? *Seminars in Perinatology,* 34**,** 183-187.

O'LEARY, C. M., NASSAR, N., KURINCZUK, J. J. & BOWER, C. 2009. The effect of maternal alcohol consumption on fetal growth and preterm birth. *British Journal of Obstetrics and Gynaecology,* 116**,** 390-400.

OHLSSON, A. & SHAH, S. S. 2011. Ibuprofen for the prevention of patent ductus arteriosus in preterm and/or low birth weight infants. *Cochrane Database of Systematic Reviews***,** CD004213.

OLDFIELD, J. E., MUTH, O. H. & SCHUBERT, J. R. 1960. Selenium and vit. E as related to growth and white muscle disease in lambs. *Proc Soc Exp Biol Med,* 103**,** 799-800.

OLIVETTI, G., CIGOLA, E., MAESTRI, R., CORRADI, D., LAGRASTA, C., GAMBERT, S. R. & ANVERSA, P. 1996. Aging, cardiac hypertrophy and ischemic cardiomyopathy do not affect the proportion of mononucleated and multinucleated myocytes in the human heart. *Journal of Molecular and Cellular Cardiology,* 28**,** 1463-1477.

ONG, K. K. L., AHMED, M. L., EMMETT, P. M., PREECE, M. A. & DUNGER, D. B. 2000. Association between postnatal catch-up growth and obesity in childhood: prospective cohort study. *British Medical Journal,* 320**,** 967-971.

OOSHIMA, A., FULLER, G. C., CARDINALE, G. J., SPECTOR, S. & UDENFRIEND, S. 1974. Increased Collagen Synthesis in Blood Vessels of Hypertensive Rats and Its Reversal by Antihypertensive Agents. *Proceedings of the National Academy of Sciences,* 71**,** 3019-3023.

OPARIL, S., BISHOP, S. P. & CLUBB, F. J. 1984. Myocardial cell hypertrophy or hyperplasia. *Hypertension,* 6**,** III38.

ORVOS, H., HOFFMANN, I., FRANK, I., KATONA, M., PAL, A. & KOVACS, L. 2002. The perinatal outcome of pregnancy without prenatal care. A retrospective study in Szeged, Hungary. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 100**,** 171-173.

OSMOND, C. & BARKER, D. J. 2000. Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women. *Environmental Health Perspectives,* 108 Suppl 3**,** 545-553.

OWEN, J., GOLDENBERG, R. L., DAVIS, R. O., KIRK, K. A. & COPPER, R. L. 1990. Evaluation of a risk scoring system as a predictor of preterm birth in an indigent population. *American Journal of Obstetrics & Gynecology,* 163**,** 873-889.

PALLISER, H. K., KELLEHER, M. A., WELSH, T. N., ZAKAR, T. & HIRST, J. J. 2014. Mechanisms leading to increased risk of preterm birth in growth-restricted guinea pig pregnancies. *Reproductive Sciences,* 21**,** 269-276.

PARADIS, A. N., GAY, M. S., WILSON, C. G. & ZHANG, L. 2015. Newborn Hypoxia/Anoxia Inhibits Cardiomyocyte Proliferation and Decreases Cardiomyocyte Endowment in the Developing Heart: Role of Endothelin-1. *PLoS ONE,* 10**,** e0116600.

PATAN, S. 2000. Vasculogenesis and Angiogenesis as Mechanisms of Vascular Network Formation, Growth and Remodeling. *Journal of Neuro-Oncology,* 50**,** 1-15.

PATEL, A., FINE, B., SANDIG, M. & MEQUANINT, K. 2006. Elastin biosynthesis: The missing link in tissue-engineered blood vessels. *Cardiovascular Research,* 71**,** 40-49.

PATURAL, H., BARTHELEMY, J. C., PICHOT, V., MAZZOCCHI, C., TEYSSIER, G., DAMON, G. & ROCHE, F. 2004. Birth prematurity determines prolonged autonomic nervous system immaturity. *Clinical Autonomic Research,* 14**,** 391-395.

PATURAL, H., PICHOT, V., JAZIRI, F., TEYSSIER, G., GASPOZ, J. M., ROCHE, F. & BARTHELEMY, J. C. 2008. Autonomic cardiac control of very preterm newborns: a prolonged dysfunction. *Early Human Development,* 84**,** 681-687.

PAYNE, R. A., WILKINSON, I. B. & WEBB, D. J. 2010. Arterial Stiffness and Hypertension: Emerging Concepts. *Hypertension,* 55**,** 9-14.

PEACEMAN, A. M., ANDREWS, W. W., THORP, J. M., CLIVER, S. P., LUKES, A., IAMS, J. D., COULTRIP, L., ERIKSEN, N., HOLBROOK, R. H., ELLIOTT, J., INGARDIA, C. & PIETRANTONI, M. 1997. Fetal fibronectin as a predictor of preterm birth in patients with symptoms: a multicenter trial. *American Journal of Obstetrics & Gynecology,* 177**,** 13-18.

PEACOCK, J. L., BLAND, J. M. & ANDERSON, H. R. 1995. Preterm delivery: effects of socioeconomic factors, psychological stress, smoking, alcohol, and caffeine. *British Medical Journal,* 311**,** 531-535.

PEACOCK, J. L., MARSTON, L., MARLOW, N., CALVERT, S. A. & GREENOUGH, A. 2012. Neonatal and infant outcome in boys and girls born very prematurely. *Pediatric Research,* 71**,** 305-310.

PEJOVIC, B., PECO-ANTIC, A. & MARINKOVIC-ERIC, J. 2007. Blood pressure in non-critically ill preterm and full-term neonates. *Pediatric Nephrology,* 22**,** 249-257.

PEMU, P. I. & OFILI, E. 2008. Hypertension in Women: Part I. *Journal of Clinical Hypertension,* 10**,** 406-410.

PERELMAN, R. H., PALTA, M., KIRBY, R. & FARRELL, P. M. 1986. Discordance between male and female deaths due to the respiratory distress syndrome. *Pediatrics,* 78**,** 238-244.

PIELTAIN, C., DE CURTIS, M., GERARD, P. & RIGO, J. 2001. Weight gain composition in preterm infants with dual energy X-ray absorptiometry. *Pediatr Res,* 49**,** 120-124.

PIKE, I. L. 2005. Maternal stress and fetal responses: evolutionary perspectives on preterm delivery. *American Journal of Human Biology,* 17**,** 55-65.

POEHLMANN, J., SCHWICHTENBERG, A. J., BOLT, D. & DILWORTH-BART, J. 2009. Predictors of depressive symptom trajectories in mothers of preterm or low birth weight infants. *Journal of Family Psychology,* 23**,** 690-704.

POLAK, J. F., PENCINA, M. J., PENCINA, K. M., O'DONNELL, C. J., WOLF, P. A. & D'AGOSTINO, R. B. 2011. Carotid-Wall Intima–Media Thickness and Cardiovascular Events. *New England Journal of Medicine,* 365**,** 213-221.

PORRELLO, E. R., MAHMOUD, A. I., SIMPSON, E., HILL, J. A., RICHARDSON, J. A., OLSON, E. N. & SADEK, H. A. 2011. Transient Regenerative Potential of the Neonatal Mouse Heart. *Science,* 331**,** 1078-1080.

PORTER, T. F., FRASER, A. M., HUNTER, C. Y., WARD, R. H. & VARNER, M. W. 1997. The risk of preterm birth across generations. *Obstetrics & Gynecology,* 90**,** 63-67.

PRACTICE COMMITTEE OF AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE & PRACTICE COMMITTEE OF SOCIETY FOR ASSISTED REPRODUCTIVE TECHNOLOGY 2013. Criteria for number of embryos to transfer: a committee opinion. *Fertility and Sterility,* 99**,** 44-46.

PRIOR, B. M., YANG, H. T. & TERJUNG, R. L. 2004. What makes vessels grow with exercise training? *Journal of Applied Physiology,* 97**,** 1119-1128.

PULGAR, V. M. & FIGUEROA, J. P. 2006. Antenatal Betamethasone Administration Has a Dual Effect on Adult Sheep Vascular Reactivity. *Pediatric Research,* 60**,** 705-710.

PUNTIS, J. W. L. 2006. Nutritional support in the premature newborn. *Postgraduate Medical Journal,* 82**,** 192-198.

RAATIKAINEN, K., HEISKANEN, N. & HEINONEN, S. 2005. Marriage still protects pregnancy. *British Journal of Obstetrics and Gynaecology,* 112**,** 1411-1416.

RAJU, T. N. 2006. Epidemiology of late preterm (near-term) births. *Clinics in Perinatology,* 33**,** 751-763.

RAY, J. G., VERMEULEN, M. J., SHAPIRO, J. L. & KENSHOLE, A. B. 2001. Maternal and neonatal outcomes in pregestational and gestational diabetes mellitus, and the influence of maternal obesity and weight gain: the DEPOSIT study. Diabetes Endocrine Pregnancy Outcome Study in Toronto. *Quarterly Journal of Medicine,* 94**,** 347-356.

REGEV, R. H., LUSKY, A., DOLFIN, T., LITMANOVITZ, I., ARNON, S. & REICHMAN, B. 2003. Excess mortality and morbidity among small-for-gestational-age premature infants: a population-based study. *Journal of Pediatrics,* 143**,** 186-191.

REPKA, M. X. 2002. Ophthalmological problems of the premature infant. *Mental Retardation and Developmental Disabilities Research Reviews,* 8**,** 249-257.

RESNIK, R. 2002. Intrauterine growth restriction. *Obstetrics & Gynecology,* 99**,** 490-496.

RETNAKARAN, R., QI, Y., SERMER, M., CONNELLY, P. W., HANLEY, A. J. G. & ZINMAN, B. 2008. Glucose Intolerance in Pregnancy and Future Risk of Pre-Diabetes or Diabetes. *Diabetes Care,* 31**,** 2026-2031.

RIGO, J., NYAMUGABO, K., PICAUD, J. C., GERARD, P., PIELTAIN, C. & DE CURTIS, M. 1998. Reference values of body composition obtained by dual energy X-ray absorptiometry in preterm and term neonates. *J Pediatr Gastroenterol Nutr,* 27**,** 184-190.

RISAU, W. 1997. Mechanisms of angiogenesis. *Nature,* 386**,** 671-674.

ROACH, M. R. & BURTON, A. C. 1957. The Reason For The Shape Of The Distensibility Curves Of Arteries. *Canadian Journal of Biochemistry and Physiology,* 35**,** 681-690.

ROBERTS, D. & DALZIEL, S. 2013. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database of Systematic Reviews***,** CD004454.

ROBERTS, G., CHEONG, J., OPIE, G., CARSE, E., DAVIS, N., DUFF, J., LEE, K. J., DOYLE, L. & ON BEHALF OF THE VICTORIAN INFANT COLLABORATIVE STUDY, G. 2013. Growth of Extremely Preterm Survivors From Birth to 18 Years of Age Compared With Term Controls. *Pediatrics*.

ROBINSON, S. 2012. Neonatal posthemorrhagic hydrocephalus from prematurity: pathophysiology and current treatment concepts: A review. *Journal of Neurosurgery: Pediatrics,* 9**,** 10.3171/2011.12.PEDS11136.

RODRIGUES, T., ROCHA, L. & BARROS, H. 2007. Physical abuse during pregnancy and preterm delivery. *American Journal of Obstetrics & Gynecology*.

RODRIGUEZ, M. M., GOMEZ, A. H., ABITBOL, C. L., CHANDAR, J. J., DUARA, S. & ZILLERUELO, G. E. 2004. Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. *Pediatric and Developmental Pathology,* 7**,** 17-25.

ROGGERO, P., GIANNI, M. L., LIOTTO, N., TARONI, F., MORNIROLI, D. & MOSCA, F. 2011. Small for gestational age preterm infants: nutritional strategies and quality of growth after discharge. *Journal of Maternal - Fetal & Neonatal Medicine,* 24 Suppl 1**,** 144-146.

ROGHAIR, R. D., SEGAR, J. L., KILPATRICK, R. A., SEGAR, E. M., SCHOLZ, T. D. & LAMB, F. S. 2007. Murine aortic reactivity is programmed equally by maternal low protein diet or late gestation dexamethasone. *Journal of Maternal - Fetal & Neonatal Medicine,* 20**,** 833-841.

ROMEJKO-WOLNIEWICZ, E., TELIGA-CZAJKOWSKA, J. & CZAJKOWSKI, K. 2014. Antenatal steroids: can we optimize the dose? *Current Opinion in Obstetrics & Gynecology,* 26**,** 77-82.

ROMERO, R., AVILA, C., BREKUS, C. A. & MOROTTI, R. 1991. The role of systemic and intrauterine infection in preterm parturition. *Annals of the New York Academy of Sciences,* 622**,** 355-375.

ROMERO, R., MIRANDA, J., CHAIWORAPONGSA, T., CHAEMSAITHONG, P., GOTSCH, F., DONG, Z., AHMED, A. I., YOON, B. H., HASSAN, S. S., KIM, C. J., KORZENIEWSKI, S. J. & YEO, L. 2014. A novel molecular microbiologic technique for the rapid diagnosis of microbial invasion of the amniotic cavity and intra-amniotic infection in preterm labor with intact membranes. *American Journal of Reproductive Immunology,* 71**,** 330-358.

ROSEN, T., KUCZYNSKI, E., O'NEILL, L. M., FUNAI, E. F. & LOCKWOOD, C. J. 2001. Plasma levels of thrombin-antithrombin complexes predict preterm premature rupture of the fetal membranes. *Journal of Maternal - Fetal & Neonatal Medicine,* 10**,** 297-300.

ROSENBERG, A. 2008. The IUGR newborn. *Seminars in Perinatology,* 32**,** 219-224.

ROSENBERG, T. J., GARBERS, S., LIPKIND, H. & CHIASSON, M. A. 2005. Maternal obesity and diabetes as risk factors for adverse pregnancy outcomes: differences among 4 racial/ethnic groups. *American Journal of Public Health,* 95**,** 1545-1551.

ROY, P., KUMAR, A., KAUR, I. R. & FARIDI, M. M. 2014. Gender differences in outcomes of low birth weight and preterm neonates: the male disadvantage. *Journal of Tropical Pediatrics,* 60**,** 480-481.

RUDOLPH, A. M. 1979. Fetal and neonatal pulmonary circulation. *Annual Review of Physiology,* 41**,** 383-395.

RUDOLPH, A. M. 2000. Myocardial growth before and after birth: clinical implications. *Acta Paediatrica,* 89**,** 129-133.

RYSAVY, M. A., LI, L., BELL, E. F., DAS, A., HINTZ, S. R., STOLL, B. J., VOHR, B. R., CARLO, W. A., SHANKARAN, S., WALSH, M. C., TYSON, J. E., COTTEN, C. M., SMITH, P. B., MURRAY, J. C., COLAIZY, T. T., BRUMBAUGH, J. E. & HIGGINS, R. D. 2015. Between-Hospital Variation in Treatment and Outcomes in Extremely Preterm Infants. *New England Journal of Medicine,* 372**,** 1801-1811.

SAHNI, R., SCHULZE, K. F., KASHYAP, S., OHIRA-KIST, K., FIFER, W. P. & MYERS, M. M. 2000. Maturational changes in heart rate and heart rate variability in low birth weight infants. *Developmental Psychobiology,* 37**,** 73-81.

SAIGAL, S. & DOYLE, L. W. 2008. An overview of mortality and sequelae of preterm birth from infancy to adulthood. *Lancet,* 371**,** 261-269.

SAIGAL, S., STOSKOPF, B., STREINER, D., PANETH, N., PINELLI, J. & BOYLE, M. 2006. Growth trajectories of extremely low birth weight infants from birth to young adulthood: a longitudinal, population-based study. *Pediatric Research,* 60**,** 751-758.

SALADIN, K. S. 2012. *Anatomy & physiology : the unity of form and function,* New York McGraw-Hill

SAMUEL, C. S. 2009. Determination of collagen content, concentration, and sub-types in kidney tissue. *Methods in Molecular Biology,* 466**,** 223-235.

SANGILD, P. T. 2006. Gut Responses to Enteral Nutrition in Preterm Infants and Animals. *Experimental Biology and Medicine,* 231**,** 1695-1711.

SANGILD, P. T., THYMANN, T., SCHMIDT, M., STOLL, B., BURRIN, D. G. & BUDDINGTON, R. K. 2013. Invited Review: The preterm pig as a model in pediatric gastroenterology. *Journal of Animal Science,* 91**,** 4713-4729.

SARKAR, S., DECHERT, R., SCHUMACHER, R. E. & DONN, S. M. 2007. Is refractory hypotension in preterm infants a manifestation of early ductal shunting? *Journal of Perinatology,* 27**,** 353-358.

SAUREL-CUBIZOLLES, M. J., ZEITLIN, J., LELONG, N., PAPIERNIK, E., DI RENZO, G. C. & BREART, G. 2004. Employment, working conditions, and preterm birth: results from the Europop case-control survey. *Journal of Epidemiology & Community Health,* 58**,** 395-401.

SAVITZ, D. A., BLACKMORE, C. A. & THORP, J. M. 1991. Epidemiologic characteristics of preterm delivery: etiologic heterogeneity. *American Journal of Obstetrics & Gynecology,* 164**,** 467-471.

SAVITZ, D. A., DOLE, N., HERRING, A. H., KACZOR, D., MURPHY, J., SIEGA-RIZ, A. M., THORP, J. M., JR. & MACDONALD, T. L. 2005. Should spontaneous and medically indicated preterm births be separated for studying aetiology? *Paediatric and Perinatal Epidemiology,* 19**,** 97-105.

SAVITZ, D. A., KAUFMAN, J. S., DOLE, N., SIEGA-RIZ, A. M., THORP, J. M., JR. & KACZOR, D. T. 2004. Poverty, education, race, and pregnancy outcome. *Ethnicity & Disease,* 14**,** 322-329.

SCHNEIDER, D. J. & MOORE, J. W. 2006. Patent Ductus Arteriosus. *Circulation,* 114**,** 1873-1882.

SCHOLL, T. O., HEDIGER, M. L., SALMON, R. W., BELSKY, D. H. & ANCES, I. G. 1989. Association between low gynaecological age and preterm birth. *Paediatric and Perinatal Epidemiology,* 3**,** 357-366.

SCHOLL, T. O., MILLER, L. K., SHEARER, J., COFSKY, M. C., SALMON, R. W., VASILENKO, P., 3RD & ANCES, I. 1988. Influence of young maternal age and parity on term and preterm low birthweight. *American Journal of Perinatology,* 5**,** 101-104.

SCHUBERT, U., MÜLLER, M., ABDUL-KHALIQ, H., NORMAN, M. & BONAMY, A.-K. E. 2013. Relative intima-media thickening after preterm birth. *Acta Paediatrica,* 102**,** 965-969.

SCHUBERT, U., MÜLLER, M., EDSTEDT BONAMY, A. K., ABDUL-KHALIQ, H. & NORMAN, M. 2011. Aortic growth arrest after preterm birth: a lasting structural change of the vascular tree. *Journal of Developmental Origins of Health and Disease,* 2**,** 218-225.

SEABORN, T., SIMARD, M., PROVOST, P. R., PIEDBOEUF, B. & TREMBLAY, Y. 2010. Sex hormone metabolism in lung development and maturation. *Trends in Endocrinology & Metabolism,* 21**,** 729-738.

SECKL, J. R. & HOLMES, M. C. 2007. Mechanisms of disease: glucocorticoids, their placental metabolism and fetal 'programming' of adult pathophysiology. *Nature Clinical Practice Endocrinology & Metabolism,* 3**,** 479-488.

SEDMERA, D., PEXIEDER, T., VUILLEMIN, M., THOMPSON, R. P. & ANDERSON, R. H. 2000. Developmental patterning of the myocardium. *Anatomical Record,* 258**,** 319-337.

SEDMERA, D. & THOMPSON, R. P. 2011. Myocyte proliferation in the developing heart. *Developmental Dynamics,* 240**,** 1322-1334.

SENYO, S. E., LEE, R. T. & KÜHN, B. 2014. Cardiac regeneration based on mechanisms of cardiomyocyte proliferation and differentiation. *Stem Cell Research,* 13**,** 532-541.

SEOW, W. K. 1997. Effects of preterm birth on oral growth and development. *Australian Dental Journal,* 42**,** 85-91.

SEOW, W. K., YOUNG, W. G., TSANG, A. K. & DALEY, T. 2005. A study of primary dental enamel from preterm and full-term children using light and scanning electron microscopy. *Journal of Pediatric Dentistry,* 27**,** 374-379.

SHAPIRO-MENDOZA, C. K. & LACKRITZ, E. M. 2012. Epidemiology of late and moderate preterm birth. *Semin Fetal Neonatal Med,* 17**,** 120-125.

SHARAMI, S. H., FARAJI DARKHANEH, R., ZAHIRI, Z., MILANI, F., ASGHARNIA, M., SHAKIBA, M. & DIDAR, Z. 2013. The relationship between vaginal bleeding in the first and second trimester of pregnancy and preterm labor. *Iranian Journal of Reproductive Medicine,* 11**,** 385-390.

SIEGA-RIZ, A. M., SIEGA-RIZ, A. M. & LARAIA, B. 2006. The implications of maternal overweight and obesity on the course of pregnancy and birth outcomes. *Maternal and Child Health Journal,* 10**,** S153-156.

SIEWERT-DELLE, A. & LJUNGMAN, S. 1998. The impact of birth weight and gestational age on blood pressure in adult life: a population-based study of 49-year-old men. *American Journal of Hypertension,* 11**,** 946-953.

SILVER, H. M., SPERLING, R. S., ST CLAIR, P. J. & GIBBS, R. S. 1989. Evidence relating bacterial vaginosis to intraamniotic infection. *American Journal of Obstetrics & Gynecology,* 161**,** 808-812.

SIMON, A., GARIEPY, J., CHIRONI, G., MEGNIEN, J. L. & LEVENSON, J. 2002. Intima-media thickness: a new tool for diagnosis and treatment of cardiovascular risk. *Journal of Hypertension,* 20**,** 159-169.

SINGH, R. R., CUFFE, J. S. M. & MORITZ, K. M. 2012. Short- and long-term effects of exposure to natural and synthetic glucocorticoids during development. *Clinical and Experimental Pharmacology and Physiology,* 39**,** 979-989.

SINHA, S., MAILL, L. & JARDINE, L. 2012. *Essentials of neonatal medicine,* Oxford, John Wiley & Sons, Ltd.

SKILTON, M. R., VIIKARI, J. S. A., JUONALA, M., LAITINEN, T., LEHTIMÄKI, T., TAITTONEN, L., KÄHÖNEN, M., CELERMAJER, D. S. & RAITAKARI, O. T. 2011. Fetal Growth and Preterm Birth Influence Cardiovascular Risk Factors and Arterial Health in Young Adults: The Cardiovascular Risk in Young Finns Study. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 31**,** 2975-2981.

SMITH, G. C., PELL, J. P. & DOBBIE, R. 2003. Interpregnancy interval and risk of preterm birth and neonatal death: retrospective cohort study. *British Medical Journal,* 327**,** 313.

SMITH, G. C. S., SHAH, I., WHITE, I. R., PELL, J. P., CROSSLEY, J. A. & DOBBIE, R. 2006. Maternal and biochemical predictors of spontaneous preterm birth among nulliparous women: a systematic analysis in relation to the degree of prematurity. *International Journal of Epidemiology,* 35**,** 1169-1177.

SMITH, L. J., MCKAY, K. O., VAN ASPEREN, P. P., SELVADURAI, H. & FITZGERALD, D. A. 2010. Normal Development of the Lung and Premature Birth. *Paediatric Respiratory Reviews,* 11**,** 135-142.

SMITH, L. K., DRAPER, E. S., MANKTELOW, B. N., DORLING, J. S. & FIELD, D. J. 2007. Socioeconomic inequalities in very preterm birth rates. *Archives of Disease in Childhood - Fetal and Neonatal Edition,* 92**,** F11-14.

SMITH, L. M., ERVIN, M. G., WADA, N., IKEGAMI, M., POLK, D. H. & JOBE, A. H. 2000. Antenatal Glucocorticoids Alter Postnatal Preterm Lamb Renal and Cardiovascular Responses to Intravascular Volume Expansion. *Pediatric Research,* 47**,** 622-627.

SMYLIE, J., CRENGLE, S., FREEMANTLE, J. & TAUALII, M. 2010. Indigenous Birth Outcomes in Australia, Canada, New Zealand and the United States – an Overview. *The Open Women's Health Journal***,** 7-17.

SOKOL, R. J., JANISSE, J. J., LOUIS, J. M., BAILEY, B. N., AGER, J., JACOBSON, S. W. & JACOBSON, J. L. 2007. Extreme prematurity: an alcohol-related birth effect. *Alcoholism: Clinical and Experimental Research,* 31**,** 1031-1037.

SOONPAA, M. H., KIM, K. K., PAJAK, L., FRANKLIN, M. & FIELD, L. J. 1996. Cardiomyocyte DNA synthesis and binucleation during murine development. *American Journal of Physiology - Heart and Circulatory Physiology,* 271**,** H2183-H2189.

SPENCE, M. R., WILLIAMS, R., DIGREGORIO, G. J., KIRBY-MCDONNELL, A. & POLANSKY, M. 1991. The relationship between recent cocaine use and pregnancy outcome. *Obstetrics & Gynecology,* 78**,** 326-329.

SRIVASTAVA, D. & OLSON, E. N. 2000. A genetic blueprint for cardiac development. *Nature,* 407**,** 221-226.

STACY, V., DE MATTEO, R., BREW, N., SOZO, F., PROBYN, M. E., HARDING, R. & BLACK, M. J. 2009. The influence of naturally occurring differences in birthweight on ventricular cardiomyocyte number in sheep. *Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology,* 292**,** 29-37.

STELLOH, C., ALLEN, K. P., MATTSON, D. L., LERCH-GAGGL, A., REDDY, S. & EL-MEANAWY, A. 2012. Prematurity in mice leads to reduction in nephron number, hypertension, and proteinuria. *Translational Research,* 159**,** 80-89.

STEVENS-SIMON, C., BARRETT, J., MCGREGOR, J. A., FRENCH, J. & PERSUTTE, W. 2000. Short cervix: a cause of preterm delivery in young adolescents? *Journal of Maternal - Fetal & Neonatal Medicine,* 9**,** 342-347.

STEVENSON, D., VERTER, J., FANAROFF, A., OH, W., EHRENKRANZ, R., SHANKARAN, S., DONOVAN, E., WRIGHT, L., LEMONS, J., TYSON, J., KORONES, S., BAUER, C., STOLL, B. & PAPILE, L. 2000. Sex differences in outcomes of very low birthweight infants: the newborn male disadvantage. *Archives of Disease in Childhood. Fetal and Neonatal Edition,* 83**,** F182-F185.

STOLL, B. J., HANSEN, N. I., ADAMS-CHAPMAN, I., FANAROFF, A. A., HINTZ, S. R., VOHR, B. & HIGGINS, R. D. 2004. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. *Journal of the American Medical Association,* 292**,** 2357-2365.

STOLL, B. J., HANSEN, N. I., BELL, E. F., SHANKARAN, S., LAPTOOK, A. R., WALSH, M. C., HALE, E. C., NEWMAN, N. S., SCHIBLER, K., CARLO, W. A., KENNEDY, K. A., POINDEXTER, B. B., FINER, N. N., EHRENKRANZ, R. A., DUARA, S., SÁNCHEZ, P. J., O'SHEA, T. M., GOLDBERG, R. N., VAN MEURS, K. P., FAIX, R. G., PHELPS, D. L., FRANTZ, I. D.,

WATTERBERG, K. L., SAHA, S., DAS, A., HIGGINS, R. D., FOR THE EUNICE KENNEDY SHRIVER NATIONAL INSTITUTE OF CHILD, H. & HUMAN DEVELOPMENT NEONATAL RESEARCH, N. 2010. Neonatal Outcomes of Extremely Preterm Infants From the NICHD Neonatal Research Network. *Pediatrics*.

STROBINO, B. & PANTEL-SILVERMAN, J. 1989. Gestational vaginal bleeding and pregnancy outcome. *American Journal of Epidemiology,* 129**,** 806-815.

STRUNK, T., RICHMOND, P., SIMMER, K., CURRIE, A., LEVY, O. & BURGNER, D. 2007. Neonatal immune responses to coagulase-negative staphylococci. *Current Opinion in Infectious Diseases,* 20**,** 370-375.

SUBHEDAR, N. V. 2003. Treatment of hypotension in newborns. *Seminars in Neonatology,* 8**,** 413-423.

SUCOV, H. M. 1998. Molecular insights into cardiac development. *Annual Review of Physiology,* 60**,** 287-308.

SURBEK, D., DRACK, G., IRION, O., NELLE, M., HUANG, D. & HOESLI, I. 2012. Antenatal corticosteroids for fetal lung maturation in threatened preterm delivery: indications and administration. *Archives of Gynecology and Obstetrics,* 286**,** 277-281.

SUTHERLAND, M. R., BERTAGNOLLI, M., LUKASZEWSKI, M.-A., HUYARD, F., YZYDORCZYK, C., LUU, T. M. & NUYT, A. M. 2014. Preterm Birth and Hypertension Risk: The Oxidative Stress Paradigm. *Hypertension,* 63**,** 12-18.

SUTHERLAND, M. R., GUBHAJU, L., MOORE, L., KENT, A. L., DAHLSTROM, J. E., HORNE, R. S. C., HOY, W. E., BERTRAM, J. F. & BLACK, M. J. 2011. Accelerated Maturation and Abnormal Morphology in the Preterm Neonatal Kidney. *J Am Soc Nephrol,* 22**,** 1365-1374.

SWEENEY, J. K. & BLACKBURN, S. 2013. Neonatal physiological and behavioral stress during neurological assessment. *Journal of Perinatal & Neonatal Nursing,* 27**,** 242-252.

TAHERZADEH, Z., BREWSTER, L. M., VAN MONTFRANS, G. A. & VANBAVEL, E. 2010. Function and structure of resistance vessels in black and white people. *Journal of Clinical Hypertension* 12**,** 431-438.

TANNER, K., SABRINE, N. & WREN, C. 2005. Cardiovascular malformations among preterm infants. *Pediatrics,* 116**,** e833-838.

TAYLOR, C. R., ALEXANDER, G. R. & HEPWORTH, J. T. 2005. Clustering of U.S. women receiving no prenatal care: differences in pregnancy outcomes and implications for targeting interventions. *Maternal and Child Health Journal,* 9**,** 125-133.

THOMSEN, A. C., MORUP, L. & HANSEN, K. B. 1987. Antibiotic elimination of group-B streptococci in urine in prevention of preterm labour. *Lancet,* 1**,** 591-593.

THORNBURG, K., JONKER, S., O'TIERNEY, P., CHATTERGOON, N., LOUEY, S., FABER, J. & GIRAUD, G. 2011. Regulation of the cardiomyocyte population in the developing heart. *Progress in biophysics and molecular biology,* 106**,** 289-99.

THORNTON, C., DAHLEN, H., KORDA, A. & HENNESSY, A. 2013. The incidence of preeclampsia and eclampsia and associated maternal mortality in Australia from population-linked datasets: 2000-2008. *American Journal of Obstetrics & Gynecology,* 208**,** 476.e1-476.e5.

THUREEN, P. J. 2007. The Neonatologist's Dilemma: Catch-up Growth or Beneficial Undernutrition in Very Low Birth Weight Infants-What Are Optimal Growth Rates? *Journal of Pediatric Gastroenterology and Nutrition,* 45.

TITA, A. T. & ANDREWS, W. W. 2010. Diagnosis and management of clinical chorioamnionitis. *Clinics in Perinatology,* 37**,** 339-354.

TORDAY, J. S. & NIELSEN, H. C. 1987. The Sex Difference in Fetal Lung Surfactant Production. *Experimental Lung Research,* 12**,** 1-19.

TORLONI, M. R., BETRAN, A. P., DAHER, S., WIDMER, M., DOLAN, S. M., MENON, R., BERGEL, E., ALLEN, T. & MERIALDI, M. 2009. Maternal BMI and preterm birth: A systematic review of the literature with meta-analysis. *Journal of Maternal - Fetal & Neonatal Medicine***,** 1-14.

TRACY, S. K., TRACY, M. B., DEAN, J., LAWS, P. & SULLIVAN, E. 2007. Spontaneous preterm birth of liveborn infants in women at low risk in Australia over 10 years: a population-based study. *British Journal of Obstetrics and Gynaecology: an International Journal of Obstetrics and Gynaecology,* 114**,** 731-735.

TRICHE, E. W. & HOSSAIN, N. 2007. Environmental factors implicated in the causation of adverse pregnancy outcome. *Seminars in Perinatology,* 31**,** 240-242.

TRONNES, H., WILCOX, A. J., LIE, R. T., MARKESTAD, T. & MOSTER, D. 2014. Risk of cerebral palsy in relation to pregnancy disorders and preterm birth: a national cohort study. *Developmental Medicine & Child Neurology,* 56**,** 779-785.

TUCKER, J. & MCGUIRE, W. 2004. Epidemiology of preterm birth. *British Medical Journal,* 329**,** 675-678.

TUNDIDOR, D., GARCÍA-PATTERSON, A., MARÍA, M. A., UBEDA, J., GINOVART, G., ADELANTADO, J. M., DE LEIVA, A. & CORCOY, R. 2012. Perinatal Maternal and

Neonatal Outcomes in Women With Gestational Diabetes Mellitus According to Fetal Sex. *Gender Medicine,* 9**,** 411-417.

UCUZIAN, A. A., GASSMAN, A. A., EAST, A. T. & GREISLER, H. P. 2010. Molecular Mediators of Angiogenesis. *Journal of Burn Care & Research: official publication of the American Burn Association,* 31**,** 158.

UTHAYA, S., THOMAS, E. L., HAMILTON, G., DORE, C. J., BELL, J. & MODI, N. 2005. Altered adiposity after extremely preterm birth. *Pediatr Res,* 57**,** 211-215.

VAN DER VEN, H., MONTAG, M. & VAN DER VEN, K. 2002. Performance of in vitro fertilization in Germany. *Zeitschrift für ärztliche Fortbildung und Qualität im Gesundheitswesen,* 96**,** 369-374.

VAN VONDEREN, J. J., ROEST, A. A., SIEW, M. L., BLOM, N. A., VAN LITH, J. M., WALTHER, F. J., HOOPER, S. B. & TE PAS, A. B. 2014. Noninvasive measurements of hemodynamic transition directly after birth. *Pediatric Research,* 75**,** 448-452.

VANGEN, S., STOLTENBERG, C., HOLAN, S., MOE, N., MAGNUS, P., HARRIS, J. R. & STRAY-PEDERSEN, B. 2003. Outcome of pregnancy among immigrant women with diabetes. *Diabetes Care,* 26**,** 327-332.

VARNER, M. W. & ESPLIN, M. S. 2005. Current understanding of genetic factors in preterm birth. *British Journal of Obstetrics and Gynaecology: an International Journal of Obstetrics and Gynaecology,* 112 Suppl 1**,** 28-31.

VERKLAN, M. T. & PADHYE, N. S. 2004. Spectral analysis of heart rate variability: an emerging tool for assessing stability during transition to extrauterine life. *Journal of Obstetric, Gynecologic, & Neonatal Nursing,* 33**,** 256-265.

VOHR, B. R. & ALLEN, M. 2005. Extreme prematurity--the continuing dilemma. *New England Journal of Medicine,* 352**,** 71-72.

VOHR, B. R., WRIGHT, L. L., POOLE, W. K. & MCDONALD, S. A. 2005. Neurodevelopmental outcomes of extremely low birth weight infants <32 weeks' gestation between 1993 and 1998. *Pediatrics,* 116**,** 635-643.

VOIGT, M., OLBERTZ D FAU - FUSCH, C., FUSCH C FAU - KRAFCZYK, D., KRAFCZYK D FAU - BRIESE, V., BRIESE V FAU - SCHNEIDER, K. T. M. & SCHNEIDER, K. T. 2008. The influence of previous pregnancy terminations, miscarriages and still-births on the incidence of babies with low birth weight and premature births as well as a somatic classification of newborns. *Zeitschrift für Geburtshilfe und Neonatologie*.

WADHWA, P. D., CULHANE, J. F., RAUH, V., BARVE, S. S., HOGAN, V., SANDMAN, C. A., HOBEL, C. J., CHICZ-DEMET, A., DUNKEL-SCHETTER, C., GARITE, T. J. & GLYNN, L. 2001. Stress,

infection and preterm birth: a biobehavioural perspective. *Paediatric and Perinatal Epidemiology,* 15**,** 17-29.

WAGENSEIL, J. E. & MECHAM, R. P. 2012. Elastin in large artery stiffness and hypertension. *Journal of Cardiovascular Translational Research,* 5**,** 264-273.

WAITES, K. B., SCHELONKA, R. L., XIAO, L., GRIGSBY, P. L. & NOVY, M. J. 2009. Congenital and opportunistic infections: Ureaplasma species and Mycoplasma hominis. *Seminars in Fetal and Neonatal Medicine,* 14**,** 190-199.

WAKAI, R. T. 2004. Assessment of fetal neurodevelopment via fetal magnetocardiography. *Experimental Neurology,* 190 Suppl 1**,** S65-71.

WALKER, A. M., CANNATA, J., DOWLING, M. H., RITCHIE, B. & MALONEY, J. E. 1978. Sympathetic and parasympathetic control of heart rate in unanaesthetized fetal and newborn lambs. *Biology of the Neonate,* 33**,** 135-143.

WALLER, D. K. & DAWSON, T. E. 2005. Relationship between maternal obesity and adverse pregnancy outcomes. *Nestlé Nutrition Institute Workshop Series: Pediatric Program,* 55**,** 197-207; discussion 207-211.

WALTHER, F. J., BENDERS, M. J. & LEIGHTON, J. O. 1993. Early changes in the neonatal circulatory transition. *Journal of Pediatrics,* 123**,** 625-632.

WATKINS, A. M., WEST, C. R. & COOKE, R. W. 1989. Blood pressure and cerebral haemorrhage and ischaemia in very low birthweight infants. *Early Human Development,* 19**,** 103-110.

WEISS, S. J. & NIEMANN, S. 2015. Effects of Antenatal Corticosteroids on Cortisol and Heart Rate Reactivity of Preterm Infants. *Biological Research For Nursing*.

WELLS, J. C. 2007. Sexual dimorphism of body composition. *Best Practice & Research Clinical Endocrinology & Metabolism,* 21**,** 415-430.

WELLS, S. M., LANGILLE, B. L., LEE, J. M. & ADAMSON, S. L. 1999. Determinants of mechanical properties in the developing ovine thoracic aorta. *American Journal of Physiology - Heart and Circulatory Physiology,* 277**,** H1385-H1391.

WELSH, A. & NICOLAIDES, K. 2002. Cervical screening for preterm delivery. *Current Opinion In Obstetrics & Gynecology,* 14**,** 195-202.

WESTENBERG, L., VAN DER KLIS, K. A., CHAN, A., DEKKER, G. & KEANE, R. J. 2002. Aboriginal teenage pregnancies compared with non-Aboriginal in South Australia 1995-1999. *Australian & New Zealand Journal of Obstetrics & Gynaecology,* 42**,** 187-192.

WHITEHEAD, N., HILL, H. A., BROGAN, D. J. & BLACKMORE-PRINCE, C. 2002. Exploration of threshold analysis in the relation between stressful life events and preterm delivery. *American Journal of Epidemiology,* 155**,** 117-124.

WILCOX, A. J., SKJÆRVEN, R. & LIE, R. T. 2008. Familial Patterns of Preterm Delivery: Maternal and Fetal Contributions. *American Journal of Epidemiology,* 167**,** 474-479.

WINKVIST, A., MOGREN, I. & HOGBERG, U. 1998. Familial patterns in birth characteristics: impact on individual and population risks. *International Journal of Epidemiology,* 27**,** 248-254.

WITCOMBE, N. B., YIALLOUROU, S. R., WALKER, A. M. & HORNE, R. S. C. 2008. Blood Pressure and Heart Rate Patterns During Sleep Are Altered in Preterm-Born Infants: Implications for Sudden Infant Death Syndrome. *Pediatrics,* 122**,** e1242-e1248.

WOLINSKY, H. & GLAGOV, S. 1964. Structural Basis for the Static Mechanical Properties of the Aortic Media. *Circulation Research,* 14**,** 400-413.

WONG, J., VANDERFORD, P. A., FINEMAN, J. R. & SOIFER, S. J. 1994. Developmental effects of endothelin-1 on the pulmonary circulation in sheep. *Pediatric Research,* 36**,** 394-401.

WORLD HEALTH ORGANISATION. 2014a. *Every Newborn: An Action Plan to End Preventable Deaths* [Online]. Geneva: World Health Organisation. Available: http://www.who.int/maternal_child_adolescent/documents/newborn-action-plan/en/ [Accessed 20th March 2015].

WORLD HEALTH ORGANISATION. 2014b. *Preterm birth. Fact sheet No. 363.* [Online]. Geneva: World Health Organisation. Available: http://www.who.int/mediacentre/factsheets/fs363/en/ [Accessed 13th March 2015].

WYLLIE, J. 2006. Applied physiology of newborn resuscitation. *Current Paediatrics,* 16**,** 379-385.

XU, K., WANG, Y. A., LI, Z., LUI, K. & SULLIVAN, E. A. 2014. Risk factors associated with preterm birth among singletons following assisted reproductive technology in Australia 2007–2009 – a population-based retrospective study. *BioMed Central pregnancy and childbirth,* 14**,** 406.

YANCOPOULOS, G. D., DAVIS, S., GALE, N. W., RUDGE, J. S., WIEGAND, S. J. & HOLASH, J. 2000. Vascular-specific growth factors and blood vessel formation. *Nature,* 407**,** 242-248.

YANG, J., HARTMANN, K. E., SAVITZ, D. A., HERRING, A. H., DOLE, N., OLSHAN, A. F. & THORP, J. M., JR. 2004. Vaginal bleeding during pregnancy and preterm birth. *American Journal of Epidemiology,* 160**,** 118-125.

YI, B. A., WERNET, O. & CHIEN, K. R. 2010. Pregenerative medicine: developmental paradigms in the biology of cardiovascular regeneration. *Journal of Clinical Investigation,* 120**,** 20-28.

YU, V. Y. & DOYLE, L. W. 2004. Regionalized long-term follow-up. *Seminars in Neonatology,* 9**,** 135-144.

ZAFFRAN, S., KELLY, R. G., MEILHAC, S. M., BUCKINGHAM, M. E. & BROWN, N. A. 2004. Right ventricular myocardium derives from the anterior heart field. *Circulation research,* 95**,** 261-8.

ZEITLIN, J., ANCEL, P. Y., SAUREL-CUBIZOLLES, M. J. & PAPIERNIK, E. 2000. The relationship between intrauterine growth restriction and preterm delivery: an empirical approach using data from a European case-control study. *British Journal of Obstetrics and Gynaecology: an International Journal of Obstetrics and Gynaecology,* 107**,** 750-758.

ZEITLIN, J., SAUREL-CUBIZOLLES, M.-J., DE MOUZON, J., RIVERA, L., ANCEL, P.-Y., BLONDEL, B. & KAMINSKI, M. 2002a. Fetal sex and preterm birth: are males at greater risk? *Human Reproduction,* 17**,** 2762-2768.

ZEITLIN, J. A., SAUREL-CUBIZOLLES, M. J. & ANCEL, P. Y. 2002b. Marital status, cohabitation, and risk of preterm birth in Europe: where births outside marriage are common and uncommon. *Paediatric and Perinatal Epidemiology,* 16**,** 124-130.

ZHANG, J. & SAVITZ, D. A. 1992. Preterm birth subtypes among blacks and whites. *Epidemiology,* 3**,** 428-433.

ZHANG, Y. P., LIU, X. H., GAO, S. H., WANG, J. M., GU, Y. S., ZHANG, J. Y., ZHOU, X. & LI, Q. X. 2012. Risk factors for preterm birth in five Maternal and Child Health hospitals in Beijing. *PLoS One,* 7**,** e52780.

ZHU, B. P., ROLFS, R. T., NANGLE, B. E. & HORAN, J. M. 1999. Effect of the interval between pregnancies on perinatal outcomes. *New England Journal of Medicine,* 340**,** 589-594.

ZISK, J. L., GENEN, L. H., KIRKBY, S., WEBB, D., GREENSPAN, J. & DYSART, K. 2011. Do Premature Female Infants Really Do Better Than Their Male Counterparts? *American Journal of Perinatology,* 28**,** 241-246.