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The Effects of Preterm Birth and Associated Neonatal Interventions on Cardiac Structure in Early Postnatal Life

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Bachelor of Science (Honours)

A thesis submitted for the degree of Doctor of Philosophy at
Monash University in 2020

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Abstract

Adults born preterm (<37 weeks' gestation) exhibit altered cardiac growth and are susceptible to impaired cardiac function. Findings from sheep studies have shown that moderate preterm birth, without postnatal respiratory support, is associated with maladaptive structural remodelling of the cardiac ventricles. It was hypothesised that this maladaptive remodelling would be exacerbated with increasing severity of preterm birth. Therefore, the overall aim of this thesis was to determine the effects of preterm birth (at timepoints equivalent to early childhood) on ventricular structure and biochemical composition in postnatally ventilated lambs born prematurely at the saccular stage of lung development.

Lambs delivered preterm at 128 days' gestation and administered required clinical treatments (including antenatal glucocorticoids, surfactant, postnatal mechanical ventilation and caffeine citrate) were compared to unventilated lambs born at term (150 days' gestation) at 2 and 5 months term-equivalent age (TEA). The structure and biochemical composition of the former-preterm and term lamb ventricular myocardium was comprehensively assessed.

Firstly, our histological and stereological findings showed that cardiomyocyte number and cross-sectional area, in both the left ventricle plus septum (LV+S) and right ventricle (RV) of the lamb hearts, were not affected by preterm birth or age. Interstitial collagen levels in the LV+S increased with age and were exacerbated by preterm birth. In the RV, interstitial collagen levels increased with age but were not affected by preterm birth. Levels of cardiomyocyte proliferation and apoptosis were negligible in all lambs.

The second experimental study of this thesis used Fourier-transform infrared micro-spectroscopy to determine whether protein, lipid, nucleic acid and carbohydrate content is altered in the LV+S myocardium of lambs born preterm. The LV+S of former-preterm lambs showed an increase in collagen, and the secondary structure of proteins was significantly shifted towards a β-sheet conformation, whereas the LV+S of term lambs showed an increase in proteins with α-helical structure. At 5 months of age, the former-preterm LV also had an increase in triglyceride and phospholipid content compared to the LV+S of lambs born at term.

Finally, we determined the feasibility of using a novel MRI technique called *ex-vivo* diffusion tensor imaging in archived fixed lamb hearts, a technique that characterises parameters of cardiac microstructure that are important for efficient ventricular contraction. Although only a limited number of scans in this study were

viable, our preliminary experimental findings support the view that the LV microarchitecture in early life is different following preterm birth.

In conclusion, using a clinically-relevant sheep model of preterm birth, this thesis has shown that preterm birth combined with modern perinatal treatments results in structural and biochemical alterations in the ventricular myocardium of lambs. While cardiomyocyte growth itself was not affected in either ventricles, alterations in cardiomyocyte orientation and the biochemical composition of the LV myocardium suggest that the preterm heart is altered in early life; this may render vulnerability to cardiac dysfunction and disease later in life. The pre-clinical findings from this thesis add to a growing recognition that preterm birth disrupts the growth and development of many organs, resulting in long-term adverse health consequences.

Declaration

This thesis is an original work of my research and 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.

Bianca Le

Date: 26/03/2020

Thesis including published works declaration

I 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.

This thesis includes one review paper published in a peer reviewed journal and three submitted publications. The core theme of the thesis is the effect of preterm birth and perinatal treatments on cardiac development and structure. The ideas, development and writing up of all the papers in the thesis were the responsibility of myself, the student, working within the Department of Anatomy and Developmental Biology, and my supervisors Professor Jane Black, Dr Megan Sutherland, and Professor Kurt Albertine. The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

I acknowledge the work of Professor Kurt Albertine, Mar Janna Dahl, Sydney Bowen, Toshio Aoki, and Katie Zuspan from the University of Utah for their work on the animal experiments outlined in Chapter 2.

In the case of Chapters 1, 3, 4, and 5, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Publication status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
1	Maladaptive structural remodelling of the heart following preterm birth	Published	I conducted a review of the literature and wrote the manuscript. Total contribution: 80%	1) Dr Megan Sutherland, edited manuscript 2) Prof Jane Black, edited manuscript Total co-authors' contribution: 20%	No
3	Preterm birth with modern neonatal interventions accelerates myocardial collagen deposition in the left ventricle of lambs at 2 and 5 months of age without affecting cardiomyocyte development	Submitted	Assisted in some animal work, performed all of the experimental laboratory work, performed all data analyses, and wrote the manuscript. Total contribution: 80%	1) Mar Janna Dahl, performed animal studies 2) Prof Kurt Albertine, contributed to the development of the project, provided scientific direction and the facilities for the animal studies, contributed to the undertaking of the animal studies, edited manuscript 3) Dr Megan Sutherland, contributed to oversight of the project, assisted with statistical analyses, edited manuscript 4) Prof Jane Black, provided scientific direction and	No

				oversight throughout the development and undertaking of the project, provided oversight of the data analyses, provided wet laboratory equipment and facilities, edited manuscript Total co-authors' contribution: 20%	
4	Biochemical alterations in the left ventricular myocardium following preterm birth and assisted ventilation in lambs: a Fourier-transform infrared micro-spectroscopic study	Submitted	Assisted in some animal work, performed all of the experimental laboratory work, performed all data analyses, and wrote the manuscript. Total contribution: 70%	1) Dr Kamila Kochan, assisted with principal component analysis, edited manuscript 2) Prof Bayden Wood, contributed to study design, provided spectroscopy equipment and resources, provided oversight of spectroscopy data analyses, edited manuscript 3) Mar Janna Dahl, performed animal studies 4) Prof Kurt Albertine, contributed to the development of the project, provided scientific direction and the facilities for the animal studies, contributed to the undertaking of the animal studies, edited manuscript 5) Dr Megan Sutherland, contributed to oversight of the project, edited manuscript 6) Prof Jane Black, provided scientific direction and oversight throughout the development and undertaking of the project, provided wet laboratory equipment and facilities, edited manuscript Total co-authors' contribution: 30%	No
5	Microarchitecture of the hearts in term and former-preterm lambs using diffusion tensor imaging	Submitted	Assisted in some animal work, performed all of the experimental work (except	1) Dr Pedro Ferreira, developed computational software for DTI analysis and edited manuscript 2) Samar Merchant: Performed DTI scanning of hearts	No

			the DTI scanning and developing computational software), performed all data analyses, and wrote the manuscript. Total contribution: 50%	3) Mar Janna Dahl, performed animal studies 4) Prof Kurt Albertine, contributed to the development of the project, provided scientific direction and the facilities for the animal studies, contributed to the undertaking of the animal studies, edited manuscript 5) Dr Megan Sutherland, contributed to oversight of the project, edited manuscript 7) Prof Jane Black, provided scientific direction and oversight throughout the development and undertaking of the project, provided wet laboratory equipment and facilities, edited manuscript 8) Dr Gang Zheng, contributed to the DTI file conversion Total co-authors' contribution: 50%	

Student name: Bianca Le

Student signature: **Date:** 26/03/2020

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Mary Jane Black

Main Supervisor signature: **Date:** 25 March 2020

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I dedicate this thesis to my wonderful family, without whom this PhD would not be possible. I am where I am – and who I am – today, thanks to the unconditional love, care, and generosity of my parents and sisters, Anh and Dai. To mum and dad, thank you for making the difficult journey from Vietnam to Australia all those years ago. Thank you for all the sacrifices you've made, and continue to make, to ensure your three daughters have successful, healthy, and fulfilling lives. I hope to one day repay in kind. Cảm ơn nhiều má và ba. To Ti and Vi, I could not have asked for two better sisters. Thank you for always keeping me grounded, for being my moral compass, and for being my most enthusiastic cheerleaders throughout my entire life. I can't imagine a life without your unwavering support. I have learnt so much from you two and hope to make you both very proud.

Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BPD	Bronchopulmonary dysplasia
D_1	Primary eigenvalue
D_2	Secondary eigenvalue
D_3	Tertiary eigenvalue
DTI	Diffusion tensor imaging
E1	Primary eigenvector
E1	Secondary eigenvector
E1	Tertiary eigenvector
E2A	Sheetlet angle
FTIR	Fourier-transform infrared
g	Gram
HPA	Hypothalamic–pituitary–adrenal
IMV	Invasive mechanical ventilation
iv	Intravenous
kg	Kilogram
LV	Left ventricle/Left ventricular
LV+S	Left ventricle plus septum
mEq	Milliequivalent
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
n	Number of samples

PBS	Phosphate buffered saline
PC1	First principal component
PC2	Second principal component
PCA	Principal component analysis
pCO_2	Partial pressure of carbon dioxide
pO_2	Partial pressure of oxygen
PPROM	Preterm premature rupture of membranes
RDS	Respiratory distress syndrome
ROS	Reactive oxygen species
RV	Right ventricle/Right ventricular
TEA	Term-equivalent age

Chapter 1

Introduction

1.1 Preterm birth

1.1.1 Definition

Preterm birth in humans, also known as premature birth, is defined as birth prior to 37 completed weeks of gestation [1]. This definition can be further sub divided into three classifications according to the severity of prematurity as shown in Figure 1: moderately preterm (birth between 32 and up to 37 weeks' gestation), very preterm (birth between 28 and up to 32 weeks' gestation), and extremely preterm (birth before 28 weeks' gestation) [2]. The minimum gestational age in which babies can survive out of the womb, termed the limit of viability, is 22 weeks' gestation [3].

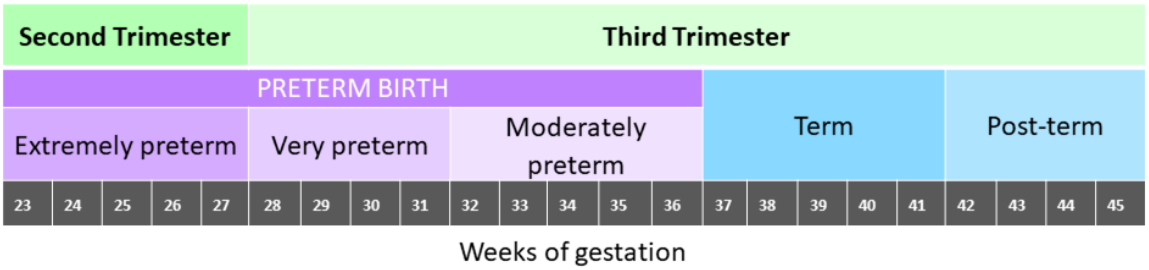

Figure 1. The sub-classifications of preterm birth, from Goldenberg, R. *et al*. **[2].**

Preterm birth can also be classified according to the clinical presentation, as either medically indicated or spontaneous preterm birth (Figure 2). Medically indicated preterm birth involves the induction of birth through either vaginal or caesarean delivery, prompted due to serious maternal illness and/or a deteriorating intrauterine environment [4]. The most common maternal health related reasons for medically inducing preterm birth are gestational hypertension, pre-eclampsia [5], and eclampsia [6]. Conversely, spontaneous preterm birth occurs without medical intervention. It is further sub divided into spontaneous onset of labour with intact membranes, and preterm premature rupture of membranes (PPROM). It is generally accepted that PPROM is caused by infection within the amniotic sac [7]. Subsequent oxidative stress and inflammation of the fetal membranes reduces the structural integrity of the supporting extracellular matrix, thereby weakening the membranes and resulting in

preterm delivery [8]. The aetiology of these underlying clinical classifications varies considerably across and within populations; however, it is estimated that 60-85% of all preterm births are spontaneous [9].

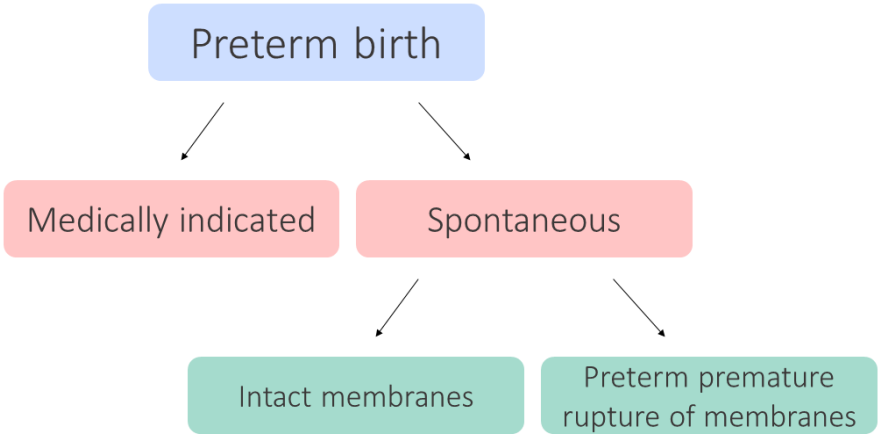

Figure 2. Preterm birth can be classified according to the clinical presentation.

1.1.2 Incidence

Preterm birth occurs in 9-11% of human pregnancies worldwide [1, 10, 11]. This translates to approximately 15 million preterm births annually [11]. The global distribution of preterm births is uneven, with certain regions and ethnic groups bearing a disproportionate amount of the burden. The highest absolute number of preterm births occur in Africa and Southern Asia, where 85% of total global preterm births are concentrated [1]. However, this number is associated with a substantially greater number of deliveries and high fertility rates occurring in these regions compared to other areas [12]. Other factors that may contribute to these high rates of preterm birth include intrauterine infection, and a lack of availability of drugs such as tocolytic agents [13]. Addressing these potentially preventable causes of preterm birth should be made a priority in developing regions of the world.

In developed countries such as Australia and North America, the rate of preterm birth has increased in the past two decades [14-16]. Rates of preterm birth in Australia rose from 6.8% in 1991 to 8.2% in 2009 [15, 16]. Similarly, rates of preterm birth in the USA increased from 10.6% to 12.2% over the same 18-year period [17]. This is partly due to the advances in fetal medicine and obstetric care available in

these developed countries, whereby high-risk infants that would otherwise die during pregnancy are now being delivered prematurely in a controlled, supervised environment. Along with the improvements in neonatal care that have led to a decline in perinatal mortality [10, 18], the incidence of preterm births in developed countries has also increased. Expectedly, the number of individuals with short and long-term morbidities associated with preterm birth have increased as a direct result of these advances in fetal medicine and obstetric care.

Although 8.7% of births in Australia are preterm [19], this absolute figure masks significant racial, regional, and social disparities relating to preterm birth. In this regard, Australian Aboriginal and Torres Strait Islander (Indigenous) women suffer disproportionately high rates of adverse pregnancy outcomes compared to other Australian women, with preterm births occurring in 14.2% of Indigenous mothers compared to 8.5% of non-Indigenous mothers [19]. Hence, analysis of the demographic breakup of the women presenting with preterm births can often indicate 'at risk' groups within a population. In the most recent data for Australia reported in 2019, the greatest proportion of preterm births across states and territories occurred in the Northern Territory (11%) [19]. This is likely to be associated with the higher proportion of teenage pregnancies and Indigenous mothers in the Northern Territory, both of which are high risk factors for preterm birth [19]. Australian babies born preterm are more common to mothers residing in very remote areas (13.5% of births) than to those residing in major cities (8.4% of births) [19]. These intra-national incidences are further described in Table 1 and Table 2.

Table 1. The percentage of preterm births occurring in Australia according to maternal factors and features of pregnancy.

		Preterm Births (%) in 2017
Maternal factors	Indigenous	14.2
	Non-Indigenous	8.5
	Very remote areas	13.5
	Major cities	8.4
	Maternal age <20	11.3
	Maternal age 20-39	8.5
	Maternal age >40	13.1
Sex of baby	Female	8.3
	Male	9.1
	Indeterminate/Not stated	78.8
Method of birth	Vaginal	6.9
	Caesarean	12.9
	Not stated	7.7

Data prepared from 'Australia's mothers and babies 2017—in brief', published by Australian Institute of Health and Welfare, *Perinatal Statistics* [19].

Table 2. The percentage of preterm births occurring in Australia in 2017 according to state or territory.

State/Territory	Preterm births (%) in 2017
Northern Territory	11
Tasmania	11
South Australia	9.6
Queensland	9.4
Western Australia	9.5
New South Wales	7.4
Australian Capital Territory	9.9
Victoria	8.9

Data prepared from 'Australia's mothers and babies 2017—in brief', published by Australian Institute of Health and Welfare, *Perinatal Statistics* [19].

1.1.3 Risk factors associated with preterm birth

There is currently a poor understanding of the underlying physiological mechanisms leading to premature delivery. Identifying the risk factors that contribute to preterm birth is essential to enable an accurate assessment of the prognosis of the neonate. It is also important to recognise at-risk mothers in order to target specialised services and administer the appropriate preventative measures and/or risk-specific interventions. Preterm deliveries occur most commonly due to spontaneous preterm births, rather from medical indication [2, 9]. Studying the causes of spontaneous preterm birth is complicated, as their aetiologies are often multi-factorial [20-23]. As mentioned above, the difference in preterm birth rates in developing versus developed countries may be accounted for by differences in the underlying cause(s).

Women residing in developing countries are generally subjected to malnutrition, gender inequality, limited access to health care facilities and educational programs, short birth intervals, and marriage/conception at a young age [24]. As a result, the risk factors contributing to preterm birth in these regions are affected by these sociocultural factors. Genitourinary infections, such as urinary tract infections [25] and asymptomatic bacteriuria [26], are the leading causes of preterm birth in developing countries [13, 27]. It has also been found that underweight women have an increased risk of preterm pregnancies in developing countries [24]. Maternal undernutrition is a serious problem in most countries in sub-Saharan Africa and South-Central/South-Eastern Asia, where more than 20% of women have a body mass index less than 18.5 [28]. Unsurprisingly, these regions have the highest percentage of preterm births worldwide (as mentioned above). Additionally, women (particularly primiparae) from developing countries may suffer from communicable diseases such as malaria [29, 30] and HIV [31, 32], which further exacerbate their already poor nutritional status [24]. Other maternal risk factors include multiple pregnancy [33, 34], pregnancy-induced hypertension [24, 27], incompetent cervix [27], history of preterm pregnancies [27, 33], and maternal age <20 years [35, 36]. Behavioural and social factors that are linked to a poorer socioeconomic status (such as cigarette smoking [37-39], strenuous physical

labor [27], and lack of antenatal care [34]) also contribute greatly to higher incidences of preterm birth in developing countries [40].

In contrast, women residing in developed countries have relatively better access to obstetric and gynaecological health facilities. An increase in the use of assisted reproductive technologies (ART), such as donor sperm and embryo transfer, in Australia and New Zealand over the past decade has been linked to higher rates of preterm birth [41, 42]. Preterm births occurred in 17.6% of those who received ART, compared to 6.8% in those who did not [15]. In high resource countries, women are delaying childbearing to pursue educational and career goals now more than ever [43, 44]. The percentage of mothers aged over 35 in Australia has gradually increased from 10.6% in 1991 to 23.7% in 2017 [15, 16, 19]. This demographic shift of increasing maternal age is of increasing clinical and public health concern because of its association with preterm births [44, 45], and other adverse maternal/fetal outcomes [22, 46, 47]. The rates of multiple births (twins, triplets, or higher order multiples) have also increased over the last decade [15], which may be partly explained by the combined factors of increasing maternal age and use of ART [42]. It is well known that a correlation exists between the number of fetuses per pregnancy and a higher incidence of preterm birth [48-50]. Approximately 57% of twins, 93% of triplets, and 96% of quadruplets are born preterm in the USA, compared to 10% of singletons [51]. Other major risk factors for preterm birth in women in developed countries are intrauterine infections and inflammation. The most common trigger of PPROM is chorioamnionitis, that is, infection of the chorion, amnion, and placenta. Histological chorioamnionitis is asymptomatic and occurs in approximately 40% of preterm births [52], and is particularly common in extremely and very preterm births. Pre-eclampsia [53], antepartum haemorrhage [54], previous preterm pregnancies/caesarean [47], obesity and gestational/pre-existing diabetes [55-57], and bacterial vaginosis [58, 59] are just some of the other maternal risk factors that are associated with preterm birth, predominantly in developed countries.

There are several studies that support a genetic predisposition to spontaneous preterm delivery. Mothers who were themselves born preterm are more likely to deliver preterm, with the relative risk increasing as the gestational age at which the mother was born decreases [60, 61]. Furthermore, the

risk of a woman giving birth preterm is almost doubled if her sister has given birth to a preterm infant [62]. Given that intrauterine infection is a common cause of preterm delivery, genes involved in the inflammatory response (such as tumor necrosis factor-α, interleukins and their respective receptors) are the most commonly studied candidates [63-66]. Similarly, polymorphism of genes involved with physical remodelling of the cervix and fetal membranes are found to be associated with PPROM, such as matrix metalloproteinases 1 and 9 (MMP1 and MMP9) [67, 68]. It is currently unclear, however, whether genetic susceptibility to preterm delivery occurs via the maternal genes in the mother or via the maternally-inherited genes in the fetus (or a combination of both) [69] , or how it may be influenced by the interplay of epigenetic and environmental factors. Interestingly, there is currently little evidence to suggest that paternal genetics contribute to preterm delivery risk [69].

1.1.4 Morbidity and mortality

Preterm birth is now the second most common cause of death (after pneumonia) in children under 5 years of age [70]. Although the rate of preterm birth in developed countries has steadily increased, the survival rates of preterm neonates have improved, particularly for those born extremely preterm [71, 72]. Moreover, moderately preterm neonates now have the same chances of survival as term neonates [73]. This is due to advances in clinical management, neonatal intensive care practices, and the use of antenatal steroids, paediatric ventilators, and surfactants in the past three decades [74-78]. Because of this reduction in mortality, the long-term outcomes of preterm birth into adolescence and early adulthood are now becoming clinically apparent [79-82].

Organ development is often still ongoing at the time of preterm birth; therefore, these immature organs are likely functionally and structurally compromised in postnatal life. Consequently, the preterm baby is highly susceptible to a spectrum of diseases. Morbidity is inversely proportional to gestational age at birth, where those born extremely preterm are most vulnerable to both short and long-term complications [83-85]. Many of the adverse outcomes of preterm birth are apparent within the first year of life, including respiratory distress syndrome (RDS), necrotising enterocolitis, apnoea of

prematurity, intraventricular haemorrhage, neonatal hypoglycaemia, neonatal sepsis, low birth weight, and patent ductus arteriosus (PDA) [86-90]. Subsequently, those who survive into young adulthood are predisposed to hypertension, insulin resistance, and metabolic and neurological aberrations, which could ultimately lead to the development of cognitive deficits, type II diabetes, and cardiovascular, renal, and respiratory diseases [42, 91-95]. Preterm birth is therefore not only associated with fetal, infant, and childhood morbidity and mortality but also with adverse outcomes persisting through to adulthood. The prevention and management of preterm birth should therefore be one of the highest priorities of obstetric research.

1.1.5 The male disadvantage in neonatal health

The male disadvantage was first described in 1971 by Naeye *et al*. [89] as the excessive risk of fetal and neonatal death in males compared to females. Males are more susceptible to preterm birth, particularly spontaneous preterm birth with intact membranes, than females [96]. This increased incidence of preterm birth in males is exacerbated with decreasing gestational age [96]. Furthermore, the prognosis for males born preterm is far worse compared to females born at the same gestational age [97-99]. Within the first few years of life, males born preterm have poorer outcomes associated with preterm birth-related morbidities are such as intraventricular haemorrhage [97], sepsis [97], respiratory distress syndrome [100] and neurodevelopmental impairment [101]. Preterm male neonates also require more respiratory and circulatory support within the first days of life [102]. Antenatal steroids are more frequently administered to mothers at risk of preterm delivery when they are carrying male infants, while the preterm males themselves receive postnatal doses of surfactant earlier, and are intubated more frequently, than preterm females [103]. Therefore, male sex should be considered as a risk factor for preterm birth.

A delay in organ development in males may explain these sex differences in the outcomes of preterm birth. Many papers suggest that fetal pulmonary maturation is 1.2 to 2.5 weeks behind in males during the last two weeks of pregnancy [100, 104, 105]. Specifically, the formation of surfactant in the fetal

lung may be inhibited, or at least delayed, by the presence of androgens such as testosterone [104, 106]. Indeed, androgen receptors are present at higher concentrations in the lungs of male compared to female prepubescent rats [107].

This phenomenon, however, can only be partly explained by innate biological differences between males and females. An analysis of male and female mortality rates from 15 developed countries over a 253-year period showed that the sex gap in neonatal mortality changes over time and between countries [108]. The excess in male mortality increased from 10% to 30% from 1751 to 1970 but has steadily decreased to ~25% in 2004. This reduction is associated with declines in infectious diseases and improvements in obstetric and neonatal care in developed countries. However, the mechanisms in which environmental factors influence the biological vulnerability of males have not yet been explored.

1.2 Recent improvements in survival rates for preterm neonates

Preterm birth occurs at a time when lung development and maturation are incomplete. Respiratory disease mortality is high in preterm newborns without clinical interventions. Understanding the effects of preterm birth on lung function is therefore crucial in improving the survival rates for neonates born preterm. Since the early 1990s, there have been major advances in medical treatments aimed at improving lung maturation and function in preterm neonates [109]. Specifically, the use of antenatal glucocorticoids, assisted ventilation, and postnatal surfactant have become standard practice to maintain adequate respiratory function in preterm neonates. As a result, survival rates for preterm neonates (particularly for those born extremely preterm) have markedly improved. This unique population of preterm-born survivors are now entering adulthood and thus the long-term health outcomes of preterm birth are becoming clinically apparent.

1.2.1 Lung development in late gestation

Development of the lungs and the pulmonary circulation in the context of both term and preterm birth has been well described (Figure 3); however, the discreet timing of each developmental stage has not been unanimously defined.

Most preterm neonates are born in the saccular stage of lung development, which occurs after the canalicular stage (approximately 16 to 24 weeks' gestation) (Figure 3). In this stage, whole clusters of saccules form on the terminal bronchioles as the last generation of air spaces in the respiratory branches. The primary septum of the saccules develop a double layer of capillary vessels, gradually increasing the total pulmonary vascular surface area [110]. Differentiation of type II alveolar cells in the saccular stage initiates surfactant production, however the biochemical properties of the surfactant differs from surfactant produced by mature pneumocytes later in gestation [111]. Consequently, the saccules' capacity for gas exchange is limited. It is not until the final stage of lung development, alveolarisation, where these saccules subdivide into 'true' alveoli with a single layer of capillary vessels and increased surfactant production.

In contrast, term neonates are born at the alveolarisation stage of lung development, which begins at 36 weeks of gestation (Figure 3) [112]. This stage involves extensive angiogenesis in the respiratory zone of the lungs, and dramatic remodelling of the parenchyma. Specifically, true alveoli lined with type I and II alveolar cells are formed and matured, increasing the surface area of the lung 20-fold [113]. Pulmonary alveoli are thin-walled sacs roughly 150-200 nm in diameter, that are found at the terminal ends of the respiratory tree [114] at the site of gas exchange. The differentiation of alveolar epithelial cells and septation of the alveolar sacs that occur in these early stages are crucial for laying the foundations for alveolarisation. Many references state that alveolarisation occurs between gestational week 36 to 2 years postnatal age [115, 116], although others suggest alveolar formation continues into late childhood [117, 118]. It is, however, generally accepted that 85% of alveolarisation takes place after birth [119]. Timing aside, the process of alveolarisation seems to be similar across all vertebrates, and

human lung development can therefore be modelled in a variety of species. This developmental process is of clinical interest, as disruptions to alveolar development are associated with the pathogenesis of bronchopulmonary dysplasia (BPD) [120] (a common complication of preterm birth) and emphysema [121, 122].

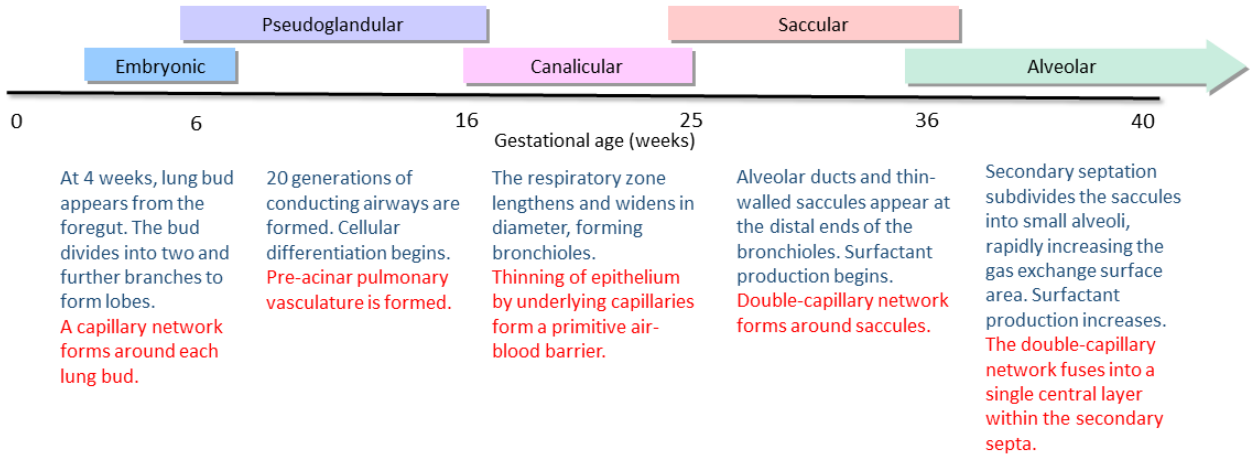

Figure 3. The five stages of respiratory airway [123] and pulmonary vascular [124] development.

Secondary septation occurs during the alveolar stage of development, where new alveolar septa are formed from within the terminal saccules of the lungs [125]. This septal formation corresponds with an increase in surfactant production within the alveoli [113]. The lengthening and thinning of the secondary septa result in the fusion of the double-capillary network into a single central layer within the septa, via intussusception [113, 126]. The lung then enters the growth phase of alveolarisation, where air space volume, rate of vessel formation, and gas-exchange surface area increase [125].

Unlike term newborns, preterm neonates are born within the saccular stage of lung development. Therefore, air breathing in the preterm neonate occurs with structurally and functionally immature lungs that are deprived of both sufficient surface area for gas-exchange and surfactant production. It is

apparent that without some level of alveolarisation occurring prenatally, postnatal respiratory function is significantly impaired.

1.2.2 The pulmonary transition at full-term birth

The adaptation of a fetus to the extrauterine environment requires the immediate transition from utilising maternal blood oxygen via the placenta and umbilical cord *in utero*, to independent air breathing via functioning lungs *ex utero*. The first breath of a newborn initiates a series of synchronised events in the respiratory and cardiovascular systems that are crucial for normal postnatal development. Deviation from this strictly coordinated running sheet of physiological events can adversely affect developmental programming, potentially leading to poor growth, altered body composition, metabolic dysfunction, and early maturation of certain organ systems in the neonate. It is reasonable to assume that preterm birth would be most detrimental to the organs that play a significant role in the haemodynamic transition at birth, including the lungs and heart. In order to understand the short and long-term effects of preterm birth on organ development, it is important to appreciate the physiology of the complex haemodynamic transition that normally occurs during term birth, and the structural remodelling that follows.

The placenta serves as the organ of gas exchange for fetuses *in utero*. The fetal lungs are filled with liquid, which allows them to be sufficiently expanded whilst undergoing development (branching morphogenesis). Exposure to the extrauterine environment at birth induces lung aeration, secretion of surfactant into alveoli, and an *ex utero* pattern of pulmonary circulation in the newborn, which are all crucial for *ex utero* breathing. It has been demonstrated that lung aeration coincides with inspiration in term newborns, with 3 ml/kg of lung fluid clearing over the first 5 breaths, with clearance continuing at a rate of 35 l/kg/h [127]. Increasing levels of adrenaline and arginine vasopressin in the fetal circulation, stimulated by active labour, also trigger a cascade of biochemical events that initiate the reabsorption of lung fluid at the time of birth [125]. An increase in fetal cortisol levels throughout late gestation and labour induces multiple physiological changes that facilitate normal neonatal

adaption. Cortisol levels in the neonate peak at ~200 µg/ml several hours after term delivery [128]. Cortisol not only facilitates fetal lung fluid clearance, but also stimulates surfactant secretion in the alveoli [129]; these two events must be coordinated in a timely manner during birth. Surfactant secretion is necessary to decrease surface tension in the lungs postnatally, thereby preventing alveolar collapse (atelectasis), particularly as the neonatal lungs no longer contain fluid to maintain their volume. During the transition, liquid is cleared more quickly from the airways than the lung tissue, forcing the chest to expand to accommodate for the increase in lung volume [130]. Lung aeration is widely considered to be the primary cause of the increase in pulmonary blood flow and, consequently, a decrease in pulmonary vascular resistance [131-133] at birth. The mechanism that links these two events, however, is largely unknown [134].

The fetal pulmonary circulation is physiologically hypertensive due to thick-walled pulmonary arteries [135, 136], low compliance [136] and high vasomotor tone induced by low oxygen saturation [137, 138]. Failure of the normal cardiopulmonary transition in the neonate results in persistent pulmonary hypertension, a clinical syndrome that is associated with respiratory failure [139]. Closure of the foramen ovale and ductus arteriosus at birth diverts oxygenated blood towards the pulmonary circulation, while clamping of the umbilical cord restores venous return to the heart. These inter-dependent biomechanical events shift the role of gas exchange from the placenta to the neonatal lungs within minutes of birth.

1.2.3 The pathological consequences of preterm birth on the pulmonary system

Development of the preterm lungs must continue postnatally in the extrauterine environment, whilst also providing gas exchange. At birth, the underdeveloped preterm lungs are abruptly subjected to mechanical breathing forces, changes in lung perfusion and blood volume, and the loss of nutritional and endocrine factors that were provided by the placenta [140]. Structural remodelling thus occurs in the preterm lungs as an adaptive mechanism to survive these premature functional changes.

Since most preterm neonates are born before alveolarisation has occurred, pulmonary outcomes following preterm birth often include enlarged and immature 'alveoli' (saccules), thick and fewer pulmonary capillaries that are further from the air surface, and surfactant deficiency. Structural outcomes of preterm birth also include increases in bronchial muscle and goblet cells, air-blood barrier thickness, fibrosis, and elastin [141, 142]. This remodelling of the respiratory anatomy is detrimental and leads to impaired gas exchange and increased risks of developing respiratory pathologies.

When the structural integrity of the respiratory system is compromised after preterm birth, the efficiency of gas exchange is diminished, which further harms the already vulnerable neonate. Firstly, the enlarged, simplified alveoli provide little gas exchange surface area, thereby reducing expiratory flow [143, 144] and resulting in an insufficient volume for efficient gas mixing [140]. Secondly, fewer capillaries in the thickened alveolar septa, increased muscularisation of pulmonary arterioles, and increased hydrostatic pulmonary oedema also compromise gas exchange by increasing pulmonary vascular resistance [145-148]. This can result in hypoxaemia, hypercapnia, and, most importantly, the pathogenesis of pulmonary hypertension after birth [147-149]. Thirdly, surfactant deficiency [150] and the increase in elastin [140] reduces lung compliance, placing further stress on the premature lungs. All of these respiratory outcomes of preterm birth ultimately lead to the development of respiratory distress syndrome (RDS; formerly known as hyaline membrane disease), which is a major cause of morbidity and mortality amongst preterm infants [151].

The primary factor that initiates RDS is pulmonary surfactant deficiency. At the time of preterm birth true alveoli have not yet formed, thinning of the pulmonary blood/air barrier has only just commenced, pulmonary vascularisation is incomplete, and, most importantly, type II epithelial cells (which produce surfactant) are immature [152]. Consequently, preterm neonates produce less pulmonary surfactant, resulting in increased alveolar surface tension and decreased compliance. A secondary factor that plays a role in the pathogenesis of RDS in preterm neonates is the inability to effectively clear fluid from the fetal lungs [153]. Infants with RDS have a higher lung fluid volume than normal, even 8 hours after birth [153]. Many animal and human studies suggest that lack of active transport of sodium in the epithelium

of an immature respiratory tract may compromise lung fluid clearance [154-157]. Indeed, widespread filling of airspaces with lung fluid is a major cause of reduced total lung capacity in premature neonates with RDS [158].

RDS survival rates have significantly increased since the introduction of assisted ventilatory support and antenatal corticosteroids. However, the increased use of prolonged mechanical ventilation with oxygen-rich gas has led to a rise in BPD, a chronic form of lung injury [159, 160]. BPD is characterised by prominent airway injury induced by mechanical ventilation and oxygen toxicity. The features of BPD include enlarged distal airspaces, decreased septation, thickened mesenchyme, vascular muscle hyperplasia, and alternating sites of over-inflation and fibrosis [161, 162].

Mechanical ventilation is known to cause BPD by promoting lung inflammation and injury in two ways: overstretching of the underdeveloped airways and oxygen toxicity. Firstly, overdistension of the lungs damages the alveolar-capillary barrier, increasing its permeability and allowing an influx of neutrophils and macrophages into the airways [163]. In addition, overstretch can cause structural damage to lung resident cells, particularly alveolar type II cells (responsible for surfactant production), resulting in apoptosis [164]. Secondly, mechanical ventilation exposes the preterm neonate to high concentrations of oxygen, resulting in inflammation mediated through reactive oxygen species (ROS) [163]. Preterm infants with RDS are particularly vulnerable to hyperoxia-induced lung injury, as their underdeveloped antioxidant system is unable to counterbalance ROS activity. This inflammatory response results in airway lesions, pulmonary oedema, fibrosis, saccular haemorrhage and atelectasis, all of which are features of BPD [165]. Consequently, infants with BPD have pulmonary dysfunction, substantial retardation of growth and development, increased susceptibility to infections, and decreased survival rates [166, 167].

The post-surfactant era in the 1990s saw an improvement in neonatal care and outcomes of preterm neonates, primarily due to the use of exogenous surfactant, pre and postnatal corticosteroids, and gentler, non-invasive ventilation strategies. This has led to increased survival rates; however, a different, milder form of BPD has evolved. This ‘new’ BPD is characterised primarily by an arrest in pulmonary

development, rather than injury caused by mechanical ventilation. The histopathology of this syndrome shows less fibrosis and airway injury compared to 'old' BPD, along with a distinct cessation in postnatal alveolar septation and vascular development [168]. Fewer and simplified alveoli, decreased acinar complexity, and dysmorphic capillaries with a lack of additional branching represents the interruption in the saccular stage of lung development [168]. New BPD is common in preterm infants, particularly those with extremely low birth weights [169]. Serial measurements of lung function in survivors of new BPD show poor pulmonary outcomes persisting into childhood, including increased lung reactance (a marker of respiratory dysfunction), wheezing, asthma, reduced exercise capacity, and increased use of daily inhaled steroid therapy compared with preterm children without BPD [170-175]. The extensive involvement of the peripheral airways suggests that preterm children diagnosed with BPD are potentially at risk of developing chronic obstructive pulmonary disease in adulthood. Furthermore, studies in children and adolescents show that preterm birth is associated with decreased lung capacity and overall respiratory function, regardless of a neonatal lung disease diagnosis or the use of ventilator support [143, 176-179].

1.2.4 Glucocorticoids

Glucocorticoids are a class of corticosteroids that play vital roles in immunity, glucose metabolism, body fluid homeostasis and fetal development. The glucocorticoid receptor is expressed in most cells in vertebrates, giving glucocorticoids great therapeutic potential. Synthetic glucocorticoids such as betamethasone and dexamethasone are most commonly used for the treatment of inflammatory and immune diseases, and act by modulating the transcription of many genes. Glucocorticoids are crucial for *in utero* organ maturation and differentiation, particularly during the transition from fetal to neonatal life. Most notably, glucocorticoids are essential for stimulating the production of pulmonary surfactant in the fetal lungs. Cortisol in the amniotic fluid peaks in late gestation and is highly synchronised with the timing of fetal lung maturation during the third trimester [180-182]. When preterm infants are delivered prior to the surge of glucocorticoids in late gestation, lung development

is severely compromised. The decreases in neonatal deaths and incidence of RDS is attributed to the introduction of antenatal administration of synthetic glucocorticoids to mothers at risk of preterm delivery.

In 1972, Liggins and Howie conducted a controlled trial of prenatal betamethasone therapy in mothers that either were at risk of premature delivery or had planned deliveries before 37 weeks' gestation [183]. The mothers received an intramuscular injection of either betamethasone or a control of cortisone acetate. If delivery had not already occurred, the mothers received a second injection 24 hours later. The incidence of RDS was markedly lower in the treated preterm infants than in controls, but only in those infants born earlier than 32 weeks' gestation (9.0% treated vs 25.8% control, $p = 0.003$) [183]. They also noted that the steroid only had an effect if the mothers received the injection within 24 hours before delivery. Since this pioneering study, many others have demonstrated the therapeutic benefits of prenatal glucocorticoids, such as betamethasone and dexamethasone, for reducing the risk of neonatal death, the development of RDS, and intraventricular haemorrhage [184, 185]. Furthermore, timely antenatal administration of glucocorticoids reduces the time infants spend on ventilatory support and in intensive care [184]. It is now known that glucocorticoid administration accelerates lung development and maturation in preterm neonates through several mechanisms, including stimulating surfactant production, reducing fibroblast proliferation, facilitating the maturation and differentiation of airway surface epithelial cells, increasing levels of antioxidant enzymes, and reducing the inflammatory response commonly elicited by mechanical ventilation [186, 187].

As expected, the efficacy of antenatal glucocorticoids in reducing respiratory disorders is heavily dependent on timing and dosage of administration. Currently, the standard protocol for women who are at risk of delivering preterm involves maternal administration of a single prenatal course of betamethasone or dexamethasone between 24-34 weeks' gestation, which correspond to the surge in cortisol levels that normally occurs in late gestation. Prenatal administration between 32-34 weeks' gestation does appear to decrease neonatal death, time spent on respiratory support, cerebroventricular haemorrhage, necrotising enterocolitis and risk of developing RDS [184, 188].

1.2.5 Exogenous surfactant therapy

When lung fluid is reabsorbed after birth, air enters the lungs forming an air-liquid interface. This creates surface tension, which reduces functional residual capacity and causes the lungs to recoil from the chest cavity [189]. However, the presence of pulmonary surfactant in developed lungs reduces surface tension, minimising recoil and preventing the alveoli from collapsing during expiration [190]. Pulmonary surfactant is a mixture of lipids and proteins coating the alveolar surface and is produced by alveolar type II cells.

Preterm babies are born at a time when their pulmonary surfactant system is still immature, and are therefore vulnerable to small airway collapse at end expiration [191]. This, combined with structurally immature alveoli, is the major cause of RDS in preterm infants [192]. Exogenous surfactant therapy for preterm infants was introduced in the early 90s and has since drastically reduced mortality and respiratory morbidity for this population [193].

1.2.6 Assisted ventilation

Many preterm neonates (particularly those born very or extremely preterm) not only require postnatal exogenous surfactant to survive, but also respiratory support to maintain patency of the airways. Respiratory support typically involves mechanical ventilators that use positive end-expiratory pressure to aerate the lung and increase functional residual capacity for efficient pulmonary gas exchange. Invasive mechanical ventilation (IMV; mechanical ventilation applied in infants intubated with an endotracheal tube) has been used to treat preterm neonates with RDS for nearly 40 years, and has extended the limit of viability of infants to those born extremely preterm [194]. Intermittent positive pressure mechanical ventilation is a common form of IMV and acts by providing cycles of inspiration and expiration, where the inspiratory period drives humidified, heated, oxygenated gas into the infant's lungs via an endotracheal tube at a set pressure, and the expiratory period maintains a positive end-expiratory pressure [195].

Unfortunately, BPD can occur following IMV. This has necessitated clinicians to turn to non-invasive alternatives to mechanical ventilation. Currently, several non-invasive forms of ventilation are being used in neonatal care units, albeit to a lesser extent [196]. While alternatives such as nasal continuous positive airway pressure have the potential to reduce the risk of developing BPD [197], use of these non-invasive therapies are not effective in extremely preterm infants [198]. Furthermore, 34-83% of these infants require subsequent intubation and mechanical ventilation [198].

Whilst the use of assisted postnatal ventilation has negative pulmonary consequences, survival rates for preterm neonates have markedly increased [74, 169]. As this cohort of preterm survivors are now entering adulthood, public health experts are becoming increasingly aware of the long-term outcomes of preterm birth, particularly those related to cardiovascular health. Recent evidence has shown that adults born preterm are more susceptible to developing hypertension, ischaemic heart disease, and heart failure, however the underlying mechanisms are unknown. Understanding the impact of preterm birth on the heart is crucial in addressing this issue.

1.3 Cardiac development during the haemodynamic transition

In order to understand the impact of preterm birth on the heart, it is important to understand cardiac development and the haemodynamic transition that takes place at birth. The following sections will describe our current knowledge of how preterm birth alters cardiac structure and function from early life to adulthood. Animal studies are now starting to provide insight into the mechanisms leading to these changes at a cellular level, crucial information that cannot be adequately addressed in human studies.

1.3.1 The haemodynamic transition

Perfusion of neonatal lungs at birth cannot occur without major physiological changes simultaneously taking place in the cardiovascular system at birth. The pulmonary circulation has direct effects on the distribution of fetal cardiac output; only 13-25% of combined ventricular output supplies the fetal lungs,

depending on gestational age. This is due to high resistance in the fetal pulmonary vessels [199]. The remainder of cardiac output is shunted directly to the fetal systemic system *via* the thoracic descending aorta (bypassing the pulmonary circulation), facilitated by the ductus arteriosus and right to left shunting through the foramen ovale. As a result, the fetal systemic circulation is, in contrast to the fetal pulmonary system, a high blood flow, low resistance circuit [200, 201]. While this physiological condition is advantageous for a developing fetus, it is not conducive to life after birth. Aeration of the lungs and clamping of the umbilical cord at birth prompts the onset of the structural and physiological cardiovascular alterations necessary for postnatal life.

The placenta is a major source of venous return and preload for the human fetal heart. At 40 weeks gestational age, the placenta supplies the fetus with approximately 265 mL/min of blood via the umbilical vein [202]. Physiological changes occurring in the cardiovascular system occur in response to the occlusion of the umbilical cord after birth. Within 30 seconds of umbilical cord occlusion after birth, venous return to the heart is reduced by approximately 50% as the low resistance placental circulation is removed from the systemic circuit, which ultimately causes an immediate increase in systemic vascular resistance [203]. Arterial pressures in the neonatal systemic circulation increases, becoming higher than that in the pulmonary circulation within minutes [204]. These alterations are closely associated with the change in direction of net blood flow through the ductus arteriosus, from right (pulmonary artery) to left (descending aorta) *in utero*, to the opposite (left to right) postnatally [203]. Left ventricular (LV) output increases from 150 mL/min/kg of fetal body weight to 400 mL/min/kg of body weight in the neonate, providing sufficient blood flow to vital organs [205, 206]. This increased cardiac output facilitates postnatal increases in energy-requiring functions such as thermogenesis, basal metabolism, and breathing. Plasma concentrations of cortisol, prostaglandin, and vasoactive hormones such as vasopressin, catecholamines, and angiotensin II are increased following delivery, which initiates these changes in blood pressure and cardiac output [207]. Overall, the transition of the

neonatal systemic circulation to a low blood flow, high resistance circuit occurs within ten minutes of occluding the umbilical cord [200, 201].

1.3.2 Closure of shunts

The major structural changes of the cardiovascular system that occur during the haemodynamic transition at birth include closure of three shunts, the ductus arteriosus, the foramen ovale and the ductus venosus. The ductus arteriosus and foramen ovale have significant clinical relevance to preterm birth, as patency of these shunts postnatally is a common complication amongst preterm infants [208, 209].

The ductus arteriosus is a blood vessel that connects the pulmonary artery to the proximal descending aorta in the fetus. This shunt allows most of the blood from the right ventricle (RV) to bypass the fetus's non-functioning lungs. Closure of the ductus arteriosus is necessary for separation of the pulmonary and systemic circulations after birth. Increased arterial partial pressure of oxygen [210], decreased circulating prostaglandin E_2 [211], and decreased pulmonary vascular resistance [201] initiate smooth muscle constriction of the vascular shunt, leading to functional closure of the ductus arteriosus within 10-15 hours after birth [212]. The loss of blood flow through the ductus arteriosus leads to hypoxia in the smooth muscle, causing complete anatomical closure of the shunt within 2-3 weeks after birth in infants born at term [213].

The foramen ovale shunts blood from the right atrium to the left atrium in the fetal heart. Increased pulmonary flow at birth increases left atrial pressure, causing the flap of the septum primum to press against the septum secundum, thus closing the foramen ovale [214]. Functional closure of the foramen ovale occurs shortly after birth, however anatomical closure may take 3 months to occur [215]. Coalescence of the two septa creates the definitive interatrial septum, preventing the mixture of oxygenated and deoxygenated blood. A visible depression remains in the interatrial septum after closure, known as the fossa ovalis.

1.3.3 Ventricular remodelling

The RV is the dominant ventricle of the heart *in utero*, in terms of both cardiac output and stroke volume [216, 217]. Ventricular volume and radius to wall thickness ratio is greater in the RV compared to the LV [218]. The RV provides 66% of cardiac output into the fetal systemic circulation [219]. As the pulmonary circulation is recruited at birth, the haemodynamic transition creates a fundamental redistribution of workload between the RV and LV. During the transition, LV stroke volume and cardiac output increase dramatically [216]. Also at birth, there is an increase in arterial blood pressure and heart rate [220]. The postnatal heart responds to the new postnatal hemodynamic demands by increasing LV compliance and wall thickness, whereas the reduction in pulmonary vascular resistance attenuates growth of the RV free wall. The LV is thus the dominant ventricle in the postnatal heart.

1.3.4 Cardiomyocyte maturation

Cardiomyocytes are the muscle cells of the heart (myocardium) that work synchronously to generate the contractions of the atria and ventricles. Prenatal growth of the myocardium requires cardiomyocyte proliferation, differentiation, migration, and apoptosis. Normal growth and maturation of cardiomyocytes during gestation is necessary to adequately prepare the heart for the haemodynamic transition that occurs at birth. The major determinant of cardiac size in early gestation is the proliferation of cardiomyocytes [221]. In the fetal heart, glucose and lactate are the major substrates for adenosine triphosphate (ATP) production in proliferating cardiomyocytes (Table 3) [222]. Nakano *et al.* have shown that glucose promotes cardiomyocyte proliferation and inhibits cardiac maturation in a dose-dependent manner, which strongly suggests that glycolytic metabolism contributes to cardiac growth by hyperplasia *in utero* [223].

During the S phase of the cell cycle, eukaryotic cells typically replicate their diploid genome generating two equal diploid sets of chromosomes. A nuclear envelope is formed around each set of chromosomes during telophase, creating two daughter nuclei. The nuclei are then distributed during cytokinesis into two daughter cells. Human fetal cardiomyocytes are predominantly diploid mononucleated [224],

meaning each cell has one nucleus containing two sets of chromosomes, however a small portion of cardiomyocytes are binucleated in the fetal mammalian heart. As expected, there is a high rate of cardiomyocyte proliferation in early gestation; however, this tapers off in late gestation and early neonatal life [225-228] (Figure 4). Later in gestation, most cardiomyocytes slowly begin to undergo maturation and become terminally differentiated in preparation for the increased work load and growth postnatally [229]. By birth, most cardiomyocytes exit the cell cycle, limiting the heart's potential to regenerate and restore function after significant injury. However, cardiomyocytes (or perhaps a subset of cardiomyocytes) retain the ability to proliferate postnatally up until adulthood [224, 230-232]. Cellular hypertrophy and deposition of the extracellular matrix are the major contributors to postnatal cardiac growth [233-235] (Figure 4).

The oxygen-rich postnatal environment may contribute to permanent cardiomyocyte cell-cycle arrest, however the mechanisms involved are still poorly defined and species-specific differences exist [236, 237]. This loss in proliferative capacity with age is associated with binucleation and polyploidisation of cardiomyocytes (Table 3) [238-240]. Roughly 26% of adult cardiomyocytes are binucleated [241], compared to 8-12% at the time of birth in humans [242]. Binucleation in cardiomyocytes occurs when DNA synthesis is followed by karyokinesis (division of the nucleus) but not cytokinesis (cellular division) [243]. Both dividing and binucleating cardiomyocytes form an actomyosin ring, however, binucleating cardiomyocytes are unable to completely disassemble their intracellular contractile apparatus [238, 239]. Partially intact myofibrils are found at the site of cleavage furrow formation, preventing the actin-myosin ring from constricting and dividing the cell, resulting in binucleation. This phenomenon of binucleation seen in the myocardium is in contrast with the process of cell fusion (fusion of two G1 phase mononucleated cells), which is typically seen in the formation of multinucleated skeletal myotubes [244].

In the human heart, a large proportion of cardiomyocytes can become polyploid over the life-course (57.5 ± 4.5% [224]), with the nuclei containing four or more gene copies (>4N DNA) [224, 245-247]. Polypoidisation occurs when there is DNA synthesis without either karyokinesis or cytokinesis. Little is

known about the mechanisms underlying cardiomyocyte polyploidisation. Polyploidy and multinuclearity may have some physiological advantages, including increased gene expression and therefore protein translation, thereby boosting tissue-specific functions. For example, it has been shown that polyploidisation and binucleation of alveolar luminal cells of lactating mammary glands allow for the increased expression of milk protein [248]. Polyploidisation within nuclei and multinucleation within cardiomyocytes is associated with the loss of proliferative capacity in early postnatal life. This is particularly problematic in the context of cardiac injury in adult hearts, as damaged myocardium is not reparable by cardiomyocyte proliferation. Analyses of cardiomyocytes in human hearts following infarction show that the cells in the border zone of the infarct re-enter the cell cycle and undergo DNA replication leading to polyploidy, but do not continue onto cell division [249, 250].

Besides cardiomyocyte number and nuclearity, the size and shape of cardiomyocytes also changes with age. Some key differences between fetal cardiomyocytes and the mature adult phenotype are summarised in Table 3. Morphologically, immature cardiomyocytes in human fetuses are cylindrical with tapered ends, have reduced sympathetic innervation, and have a lower proportion of contractile elements (approximately 30%, versus 60% in the adult) compared to mature cardiomyocytes in adults [251]. The Z discs are thick and irregular while the M band is absent in early myofibril development [110, 252]. Stiffness of the myocardium and decreased contraction and relaxation in the fetal heart are direct results of this immature cardiomyocyte structure [253].

After birth, the shape of human cardiomyocytes changes most rapidly in the LV. The volume of myocytes in the LV increases 30-40 fold between infancy to adolescence [253], and only 30% of the hypertrophied cardiomyocytes contain non-contractile elements [251]. Cardiomyocytes firstly mature into longer, rod-shaped cells with a larger cross-sectional area [254]. Following this, the sarcomeres lengthen and orientate along the direction of the long axis of the cell, increasing the space between the parallel Z-bands [255]. As the energy demands of the maturing cardiomyocytes increase, the density of mitochondria doubles [206] and the dominant cardiomyocyte fuel source transitions from carbohydrates to long-chain fatty acids (Table 3) [222]. The reduction in glucose metabolism has been

shown to coincide with the decrease in the proliferative ability of the cardiomyocyte [223, 256], while the increase in lipid metabolism produces a higher yield of energy production [222]. These major anatomical and physiological changes in cardiomyocytes after birth lead to an increased ability to produce contractile forces, particularly in the LV. Maturation of cardiomyocytes is therefore considered to be a vital process in the haemodynamic transition.

Table 3: Differences between the fetal and adult heart in humans.

Parameter	Fetal heart	Adult heart
Ventricular dominance	Right ventricle	Left ventricle
Contribution to cardiac growth	Mostly proliferating cardiomyocytes	Mostly hypertrophying cardiomyocytes
Metabolic substrate	Carbohydrate (glucose and lactate) oxidation [222]	Lipid (long-chain fatty acid) oxidation [222]
Nuclearity	Most cardiomyocytes are mononucleated (~68% [224])	Most cardiomyocytes remain mononucleated = ~63-74% [224, 242, 247]
Ploidy	diploid nuclei [247]	polyploid nuclei = ~58% [224]
Morphology	Cylindrical with tapered ends	Rod-shaped, branched [254]
	30% contractile elements [251]	60% contractile elements [251]

During gestation a 'critical window' exists for cardiac development [257], where perinatal insults such as nutrient restriction, hypoxia, and high levels of cortisol, angiotensin II and insulin growth factor-1 can have negative effects on cardiac growth (Figure 4) [257]. These developmental insults result in reduced proliferative capacity and/or increased apoptotic activity in the neonatal heart [257]. The resultant

reduction in the number of cardiomyocytes formed in the myocardium reduces the functional reserve of the heart. Consequently, any of these perinatal insults may ultimately result in increased susceptibility to hypertension, myocardial infarction, heart failure, arrhythmia, and ischaemic heart disease [258]. Reduced cardiomyocyte number can trigger a compensatory response where hypertrophy of cardiomyocytes increases above normal rates postnatally [259]. Importantly, preterm birth is another perinatal insult that has been shown to have adverse effects on cardiac growth.

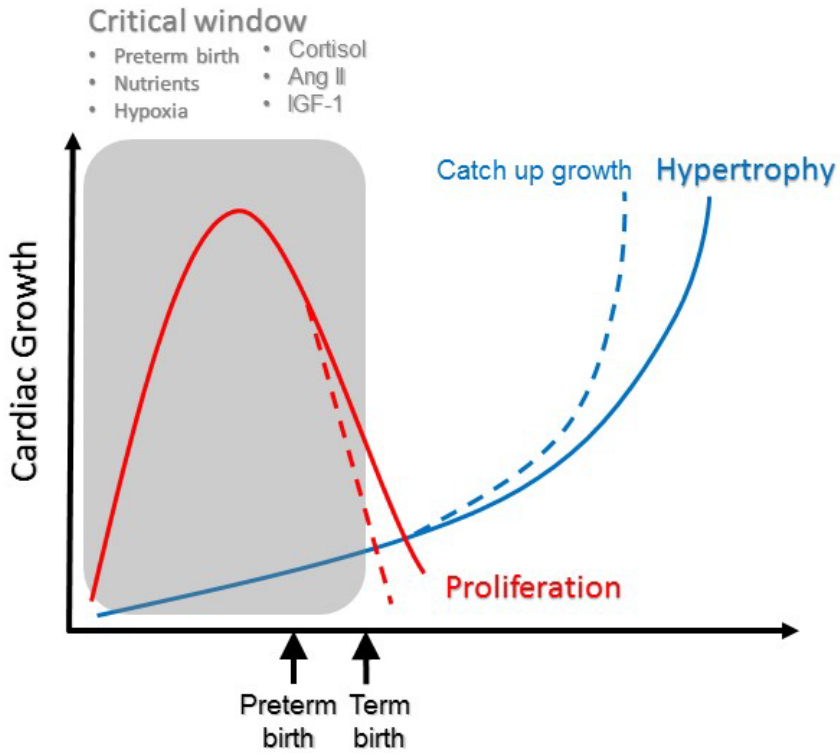

Figure 4. Normal cardiac growth in humans is attributable to cardiomyocyte proliferation prior to birth and cardiomyocyte hypertrophy after birth. A critical window of cardiac development exists where perinatal factors influence the cardiomyocyte population for the remainder of an individual's life. Solid lines represent proliferation (red) and hypertrophy (blue) in normal cardiac development. Broken lines represent decreased proliferation (red) and increased hypertrophy (blue) as a result of perinatal insults occurring during the critical window.

Adapted from Porrello ER, *et al*. 2008 *[257].*

1.3.5 Animal models for cardiomyocyte development

Commonly used animal models in cardiac research include zebrafish, rodents, and sheep. Animal models can be advantageous experimentally in studies of cardiac development, since immature and mature cardiomyocytes are easily identified depending on their nuclearity; immature cardiomyocytes are generally mononucleated (although this may not always be the case under stress conditions) and mature cardiomyocytes are generally binucleated [260]. In contrast, ~64% of adult human cardiomyocytes remain mononucleated, even though only ~0.0009% of cardiomyocytes are undifferentiated in adults [224].

The zebrafish is a common model used in developmental and regenerative biology, as their cardiomyocytes are all mononucleated and thus retain their proliferative capacity throughout their entire lifespan, unlike those found in mammals [261, 262]. Mammals, on the other hand, have cardiomyocytes that eventually lose their ability to proliferate. In rodent models, such as mice and rats, the cardiomyocytes are mononucleated and are still proliferating at birth. Within two weeks after birth, rodent cardiomyocytes become mature, differentiated and binucleated [260]. Cardiomyocyte maturation occurs earlier in sheep, during late gestation, as is seen in humans. Ovine cardiomyocyte binucleation begins at around 110 days' gestation (term, 150 days) and is completed just after birth, resulting in a limited capacity for regeneration in postnatal life [225, 263, 264]. Therefore, sheep are an excellent animal model to examine the effects of disease or perinatal insults (such as preterm birth) on the immature heart, as their cardiomyocyte development is similar to that of humans [265, 266].

1.4 The effect of preterm birth on the newborn heart

The adverse anatomical and physiological effects of preterm birth have been well-described for the lungs. The haemodynamic transition at birth, however, requires close interplay between the respiratory and cardiovascular systems meaning that cardiovascular development and function is also affected by premature delivery. Indeed, following preterm birth the heart is prematurely exposed to the

hemodynamic transition at birth; the premature exposure to the necessary postnatal increase in blood pressure and heart rate and the premature switch from placental oxygen dependency to air breathing at birth is damaging to the underdeveloped heart, causing immediate clinical complications in the neonate. As a consequence, preterm birth can cause structural remodelling of the heart at a cellular and gross anatomical level. A unique cardiac phenotype persists into adulthood, and cardiovascular sequelae are evident in the long-term.

1.4.1 Patent ductus arteriosus (PDA)

Closure of the ductus arteriosus is a crucial step in the haemodynamic transition. Failure of the ductus arteriosus to close within 72 hours of birth is defined as PDA [89]. PDA is common amongst preterm and low birth weight infants [208], and is associated with neonatal hypotension [267], BPD [268, 269], intraventricular haemorrhage [270], and increased ventilatory needs in intensive care [271, 272]. An increased fraction of inspired oxygen and mean airway pressure is required to overcome the decrease in lung compliance caused by PDA; however, this increased ventilatory assistance may contribute to the development of chronic lung disease [273]. Surgical ligation or pharmacological (administration of non-steroidal anti-inflammatory drugs) closure of the PDA in preterm infants reduces the need for prolonged ventilatory assistance [272].

1.4.2 Neonatal hypotension

Neonatal hypotension is common in preterm neonates during the first few weeks after birth, particularly for those born very and extremely preterm [274]. Hypotension in the first few days of life can be calculated as a mean blood pressure that is lower than the number of their gestational age in weeks plus 2 mmHg [275]. For example, a preterm neonate with a gestational age of 26 weeks would be considered hypotensive if their mean blood pressure was less than 28 mm Hg. Systemic hypotension is associated with low end-organ perfusion and tissue hypoxia, which can impede the postnatal

development of immature organs. Cardiac factors that may contribute to inadequate systemic perfusion include an immature heart with a patent foramen ovale and PDA [276].

The persistence of fetal shunt patency combined with decreasing pulmonary pressure causes blood to flow from the left to right atrium (in the case of patent foramen ovale), or from the aorta to the pulmonary artery (in the case of PDA) [276], thereby reducing the volume of oxygenated blood entering the systemic circulation. Treatment of PDA is therefore a priority in preterm infants with hypotension.

Non-cardiac factors also contribute to neonatal hypotension. Blood pressure is the product of cardiac output and systemic vascular resistance. Reduced LV cardiac output is not associated with low blood pressure in preterm infants requiring mechanical ventilation, suggesting that immature vascular control of arterial pressure leads to neonatal hypotension [277]. Indeed, neonatal hypotension is effectively treated by vasopressor medications and/or volume replacement, both of which increase systemic blood pressure [274]. Studies have shown that neonatal hypotension can be prevented in some cases through umbilical cord milking, delayed umbilical cord clamping, and administration of antenatal glucocorticoids [278, 279].

1.4.3 Cardiac remodelling

The effects of preterm birth on the structure and function of the human heart have been previously studied using various modes of echocardiography [280-284] and magnetic resonance imaging (MRI) [285, 286]. These imaging techniques are non-invasive and can be used for serial measurements over time in live individuals. Although echocardiography and MRI have low spatial resolution, they do allow analysis of the gross anatomy and physiology of the heart.

The first papers to describe structural remodelling of the heart as a result of preterm birth found that the interventricular septum remains flat in preterm neonates [287-289]. This phenotype is typical in fetal anatomy due to RV dominance, but is normally resolved soon after birth as a result of decreased pulmonary vascular resistance [218]. However, persistence of a flattened septum due to premature birth leads to an abnormal 'D' shape in the transverse cross-section of the LV postnatally [288] (Figure

5); this may be associated with high pulmonary vascular resistance that persists into infancy, however the exact causal relationship is still unknown. Some studies found that within 12-14 days after preterm birth, the LV eventually established a normal degree of circularity, and thus there was an improvement in LV function [287, 289]. One study, however, found that the shape of the ventricles was not completely normal in preterm infants, even after 51 days [288].

Studies additionally suggest that preterm birth results in ventricular hypertrophy in early life. A recent echocardiographic study in 83 very preterm infants found that although most of the preterm infants had normal LV geometry, just under half of them had dilatation of the LV, with or without LV hypertrophy [284]. Furthermore, a cardiac ultrasound study conducted in preterm infants showed that there is a disproportionate postnatal increase in RV and LV mass, relative to body weight. These differences were not present at birth, indicating abnormal growth of the preterm heart in the neonatal period [290]. In addition, a recent study showed premature infants born <29 weeks' gestation had increased LV sphericity, greater weight-indexed LV mass, and overall heart shape was more globular compared to healthy term newborns, suggesting a premature shift to LV dominance [291]; increased ventricular sphericity is one of the first observations of pathophysiological remodelling of the LV [292]. One autopsy study in infants (1 to 42 days postnatal age) found that preterm birth resulted in a marked reduction in the proliferation of cardiomyocytes relative to age-matched stillborn infant controls [293], where decreasing gestational age at birth was correlated with a decrease in the number of proliferating cardiomyocytes. This suggests that preterm birth reduces cardiac functional reserve. Preterm birth did not affect heart weight, myocardial capillarisation, interstitial collagen deposition or cardiomyocyte maturation, ploidy, or size. This study, however, did not control for any postnatal factors, such as mechanical ventilation and medication administration, which may have influenced whether postnatal development of the heart in individual infants can overcome the consequences of preterm birth.

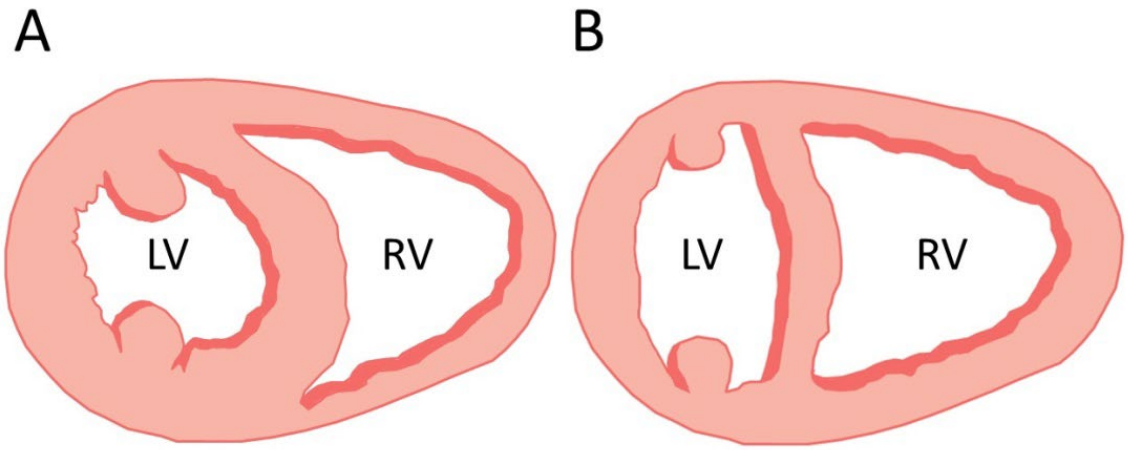

Figure 5. A diagrammatic representation of the transverse cross section of hearts from (A) an infant born at term and (B) an infant born preterm. Persistence of a flattened interventricular septum is seen in the preterm neonatal heart. *LV,* left ventricle; *RV,* right ventricle

As expected, cardiac physiology has also been shown to be abnormal in preterm infants compared to infants born at term. Echocardiography studies have shown that LV diastolic function is diminished in preterm infants during the first three months of life [294-298]. Specifically, maximal velocity of LV lengthening and diastolic filling time are lower in preterm infants compared to infants born at term. LV systolic function and myocardial contractility, however, are not affected in the the preterm newborn [281, 296, 297]. Only one study has shown a slight decrease in RV longitudinal systolic strain rate at 1 year corrected age in preterm infants compared to 1-year-old infants born at term [299]. In contrast, several studies have shown that LV and RV systolic function is impaired in adolescence and early adulthood [285, 286, 300]. This switch from impaired diastolic function in infancy to impaired systolic function later in life may be the result of long-term maladaptive structural remodelling of the ventricles, and could contribute to the development of heart failure and hypertension in adulthood.

Fortunately, early interventions may reduce the impact of preterm birth on long-term cardiac remodelling. A randomised controlled trial involving young adults (aged 23-28 years) born preterm found that being fed human breast milk during early postnatal life is beneficial to cardiac structure and

function. Preterm-born individuals fed exclusively human milk as infants had increased left and right ventricular end-diastolic volume index and stroke volume index compared with age-matched preterm-born individuals who were exclusively formula fed as infants. Although there were no differences in blood pressure between the two groups by this timepoint, the differences in cardiac structure in early adulthood may eventually become clinically significant later in life [301].

1.4.4 Long-term impact of preterm birth on the heart

In the past 15 years, several research groups have focused on establishing the medium- and long-term structural effects of preterm birth on the heart. Overall, an inverse correlation has been observed between gestational age at birth and the severity of adverse structural remodelling. A decrease in interventricular septal size, LV wall thickness, LV and RV systolic and diastolic internal diameters, and LV length was found in humans between 3 days and 40 years of age using echocardiography and MRI [283, 285, 286, 300]. A recent study looking at cardiac structure and function in 6-year-old children born extremely preterm found that LV length, width and aortic valve annulus diameter were 3-5% smaller compared to children born at term [302]. Additionally, LV mass was lower in the preterm participants than in the term control group [302].

Importantly, Lewandowski *et al.* found a 20% increase in both LV and RV mass (indexed to body surface area) in former preterm adults at 20 to 39 years of age (LV: preterm = 66.5 ± 10.9, term = 55.4 ± 11.4 g/m^2; RV: preterm = 24.5 ± 3.5, term = 20.4 ± 3.4 g/m^2) [285, 286]. In contrast, a study in 18-year-olds by Kowalski *et al.* saw no effect of extremely preterm birth on LV wall thickness [300]. Most interestingly, however, they found that LV mass was reduced in adults born preterm (preterm = 75 ± 14, term = 81 ± 16 g/m^2, P = 0.02). Two major differences in the design of these studies may account for the disparity in their findings: firstly, different imaging techniques were used. Lewandowski *et al.* used MRI, which may be more accurate in estimating LV mass than the tissue Doppler echocardiography [303] utilised by Kowalski *et al.*. Secondly, the study by Lewandowski *et al.* included a mixed cohort of former moderately, very, and extremely preterm adults, with 32% of participants born small for gestational

age, whereas Kowalski *et al.* included only adults born extremely preterm in their study. It is known that people born small for gestational age often experience rapid postnatal growth and impaired organ development in childhood [304]. Therefore, both preterm birth and intrauterine growth restriction could ultimately affect LV mass and wall thickness.

Indeed, long-term alterations in cardiac anatomy are associated with impairments in cardiac function in adulthood. A recent clinical study showed that normotensive young adults born preterm had an impaired LV response to physiological stress when subjected to physical exercise, which suggested a reduced myocardial functional reserve [305]. Furthermore, results from a Swedish cohort study have found an association between preterm birth before 32 weeks of gestation and heart failure in childhood and young adulthood [306], a worrying finding given that the number of individuals surviving preterm birth, and therefore at risk of heart failure, is increasing. While these studies do not definitively demonstrate that preterm birth per se causes adult cardiovascular disease, there appears to be an association between preterm birth and risk factors of cardiovascular disease, such as cardiac remodelling and high blood pressure.

1.4.5 Vascular remodelling

Gestational age at birth is inversely proportional to the prevalence of multiple cardiovascular risk factors, particularly increased systolic and diastolic blood pressure in childhood, adolescence, and adulthood [91, 95, 307-309]. Survival rates and the lifespan of preterm individuals have increased over the past 20 years [74-78], so it is reasonable to assume that the prevalence of morbidities associated with hypertension and ageing, would also increase in this population. The relationship between preterm birth and the development of hypertension can be partially explained by the "developmental origins of health and disease" theory, also known as the Barker hypothesis, which proposes that cardiovascular morbidities in adulthood can result from an adverse intrauterine environment and/or perinatal factors (such as premature birth) [310]. In order to understand the mechanisms underlying

the pathology of hypertension in adults born preterm, we must appreciate the structural remodelling of the vascular system that occurs early in life.

Compliance and elastance of the aorta are vital for haemodynamic regulation, particularly after birth. Elastin synthesis begins early in gestation, but is at its highest rate in the perinatal period [311]. An increase in elastin content allows the large vessels to pre-adapt to the increase in blood pressure that occurs during the haemodynamic transition after birth, and vessel maturation in this period is crucial for ensuring long-term vascular health. Elastin synthesis ceases a few weeks after birth, and then slowly breaks down later in life, causing a natural increase in arterial stiffness in old age [312]. Early disruption of elastin synthesis during the critical window in fetal life results in pathological arterial stiffness, which is never fully recovered [313]. Tauzin *et al.* found that abdominal aortic wall distensibility and whole-body arterial compliance was reduced in very low birth weight preterm infants compared to those born at term [314]. Another observational study found that preterm children aged between 5-7 years have reduced distensibility and increased stiffness of the descending abdominal aorta [315]. Only one experimental study in a lamb model has addressed the effect of preterm birth on vascular histology; in that study, no abnormal collagen deposition or fibrosis was detected in the aorta [316].

One clinical study found that the end-diastolic diameters of the aorta and carotid arteries were narrower in very preterm infants at 3 months corrected age compared to those born at term (22% and 14%, respectively), with and without adjusting for body weight [317]. This decrease in lumen size was accompanied by high systolic blood pressure. Other studies found that this aortic narrowing persists into adolescence [37, 318] and artery stiffening persists into adulthood [319], suggesting a long-lasting phenotype in people born preterm. Additionally, clinical interventions commonly used to treat preterm neonates, such as antenatal glucocorticoids and intravenous lipid use, have been shown to also contribute to aortic stiffness in adulthood [320, 321]. Another study found evidence of capillary rarefaction (a major determinant of increased vascular resistance) in young adults born preterm [322]. It is well known that an association exists between intima-media thickening of major arteries and cardiovascular diseases such as hypertension, stroke, and coronary heart disease [323-325]. Thus,

preterm birth increases the risks of developing cardiovascular morbidities through structural remodelling of the vascular system.

1.5 Clinical interventions that may adversely affect cardiopulmonary development in those born preterm

The administration of antenatal corticosteroids and the use of postnatal surfactant treatment and paediatric ventilation have become standard practice to facilitate lung maturation in the preterm neonate. However, although it is well-established that these treatments are effective in the prevention of RDS, many studies have begun to reveal both the short and long-term sequelae that arise after antenatal glucocorticoid treatment and use of ventilatory support.

1.5.1 The effects of antenatal glucocorticoids on the heart

The advantages of antenatal administration of glucocorticoids to mothers at risk of delivering preterm, for accelerating fetal lung development, are well established. As seen in the fetal lungs, structural and functional maturation of the fetal heart is dependent on glucocorticoid signalling. However, sensitivity to glucocorticoids varies between tissues [326], and *in utero* overexposure of synthetic glucocorticoids for the purpose of accelerating lung development may increase the risk of cardiovascular and metabolic disorders in adult life [327].

Studies in rats found that maternal administration of excess dexamethasone during late gestation results in reduced birth weight and hypertension in offspring later in life [328, 329]. However, there is currently much controversy surrounding the effects of antenatal glucocorticoids on cardiac anatomy and histology. A plethora of rodent and sheep studies present conflicting results due to differences in study design, including the timing, dose and type of glucocorticoid administered. Most agree, however, that exogenous glucocorticoid exposure leads to transient cardiac hypertrophy (despite a general slowing of fetal growth) [330]; some studies claim this is solely the effect of increased cardiomyocyte

hypertrophy [266, 331-333], whilst others provide evidence of increased cardiomyocyte proliferation [334, 335]. Interestingly, one clinical study showed that *in utero* glucocorticoid exposure did not influence LV structure in young adults born preterm; however, aortic stiffness did increase in this cohort [320].

Moreover, the effects of antenatal glucocorticoids on cardiac remodelling may not be due to direct glucocorticoid signalling in the heart, but via the subsequent changes in blood pressure and suppression of the hypothalamic–pituitary–adrenal (HPA) axis [336], further confounding the origins of glucocorticoid-induced cardiac diseases. Considering the widespread use of glucocorticoid therapy in mothers at risk of delivering preterm, it is necessary to have a greater understanding of the effects of synthetic glucocorticoids on the cardiovascular system of preterm newborns.

1.5.2 The effects of ventilation on the heart

The effects of spontaneous breathing and mechanical ventilation on cardiac function are poorly understood. Even though the heart and lungs are very closely connected, interventions aimed at improving one may negatively affect the other. For example, intermittent positive pressure ventilation forces air into the immature lungs of preterm infants during inspiration, thereby increasing intrathoracic pressure. Other intrathoracic structures are also affected by this change in pressure, notably the heart and the great arteries and veins. As a result, right arterial pressure increases and LV afterload decreases, and venous return and cardiac output are reduced [337-340]. This LV dysfunction is associated with clinical hypotension and a leftward displacement of the interventricular septum [340]. Furthermore, intrathoracic pressure alone may be sufficient to cause mechanical stress and trigger autophagy of cardiomyocytes [341].

Interestingly, a recent paper suggested that diaphragm pacing via phrenic nerve stimulation is a safer alternative for producing alveolar ventilation without reducing cardiac output [342]. Although clinical approaches to neonatal ventilation have improved, the effects of assisted ventilation on cardiomyocytes, whether it be direct or indirect, has not yet been explored.

1.5.3 Animal models provide unique insight into the effects of preterm birth on the heart

Most studies that have assessed structural remodelling in the preterm heart have used non-invasive imaging techniques to determine cardiac morphometry and function in humans. However, many of the findings from these studies are contradicting, and lack appropriate controls. Factors such as the cause of preterm birth, cause of death, genetics, clinical interventions, lifestyle, and environment may influence cardiac development and growth in human infants; establishing an appropriate animal model of preterm birth allows us to control for these factors. Histological analyses in animal models remains the gold-standard method for analysing structural remodelling in cardiac tissue at a cellular level, although this requires sampling from tissue at necropsy.

Sheep are commonly used to model preterm birth, mainly due to their similarities to humans in pregnancy characteristics (size and weight of newborn singleton or twin babies, a long gestation length) and the timing of organ development and maturation. Specifically, the lungs of sheep and humans both reach the alveolar stage of development by term gestation (sheep: 150 days; humans: 40 weeks), making sheep an excellent species for neonatal respiration research [145]. Furthermore, newborn lambs are similar in size to human infants; therefore, preterm lambs can be treated with the same paediatric ventilations and catheters that are used clinically.

Furthermore, sheep are an excellent and well-established animal model to examine the effects of preterm birth on the immature heart, as their trajectory of cardiovascular and cardiomyocyte development is similar to that of humans [265, 266]. In contrast, in another commonly-used animal model, rodents, cardiomyocytes continue to proliferate and maintain regenerative capacity after term birth; thus, rodents are partially protected from perinatal events which may disrupt or impair cardiac development [343, 344].

To date, only two experimental studies have utilised histology to analyse cardiomyocytes in sheep born preterm. Bensley *et al.* [345] showed that collagen deposition in both the RV and LV was greater in moderately preterm, non-ventilated lambs than that found in term lambs (6.6-fold and 4.8-fold

increases, respectively) at 9 weeks post-term-equivalent age (equivalent to a 2 year-old child). They also found that former-preterm lambs had a higher ratio of mononucleated to binucleated cardiomyocytes and a higher number of tetraploid (4N) mononucleated cardiomyocytes in both ventricles, compared to the term-born lambs; these cellular characteristics are indicative of accelerated postnatal maturation [345].

Cardiomyocyte volumes were also found to be greater in both the RV and LV in the preterm-born lambs compared to lambs born at term; usually when cardiomyocyte hypertrophy is apparent, it is indicative of increased growth in compensation for low cardiomyocyte number or a decrease in cardiac function [345]. This study found no differences in cardiomyocyte number nor heart weight between the preterm and term groups, contrary to their hypothesis. Instead, they found an increase in cardiac fibrosis, a marker of myocardial stiffness and impaired ventricular function. Another study in adult sheep born preterm showed decreases in RV wall thickness and cardiomyocyte number, yet no significant change in levels of interstitial fibrosis or RV wall volume [346]. Both studies used moderately preterm sheep born at the saccular stage of lung development (equivalent to 35 weeks' gestation in humans), and thus they did not require assisted ventilation at birth. Currently, there are no published studies exploring the effects of preterm birth on the heart in sheep requiring assisted postnatal ventilation. This forms the focus of this thesis.

1.6 Summary and conclusion

Preterm birth occurs in 9-11% of deliveries worldwide [1, 10, 11]. The survival rates of preterm neonates have improved, particularly for those born very and extremely preterm [71, 72], due to modern advances in clinical management, neonatal intensive care practices, and the use of antenatal steroids, paediatric ventilators, and surfactants in the past three decades [74-78]. This modern cohort of survivors are now entering adulthood. As a result, the long-term outcomes of preterm birth are becoming clinically apparent [79-82].

Recent epidemiological and clinical studies have shown that preterm birth results in long-term cardiac remodelling, which is associated with impaired cardiac function and an increased susceptibility to cardiovascular disease. There are currently no published studies that have characterised the effect of very preterm birth and accompanying modern neonatal interventions (mechanical ventilation, antenatal glucocorticoids, administration of caffeine citrate, and exogenous surfactant therapy) on the heart. This thesis aims to address this gap in current knowledge, using a combination of gold standard techniques (histology and stereology) and novel imaging techniques (diffusion tensor imaging and Fourier-transform infrared micro-spectroscopy). Furthermore, the former-preterm lamb model used in the studies of this thesis is well-established and involves the use of the modern neonatal interventions mentioned above. The findings of this thesis provide a better understanding of the underlying mechanism of cardiac remodelling in the context of preterm birth and modern neonatal interventions.

1.6.1 Hypothesis and aims

The focus of this thesis is to examine the impact of preterm birth, in conjunction with modern perinatal interventions, on cardiac structure and myocardial growth in lambs. It was hypothesised that preterm birth, and the related factors involved in the perinatal care of preterm neonates (such as assisted postnatal ventilation), would result in an altered cardiac growth trajectory and maladaptive cardiac remodelling. The specific hypothesis of this thesis is that preterm birth (equivalent to 28 weeks' gestation in humans in terms of lung development) would adversely affect cardiac morphology, cardiomyocyte development, myocardial microstructure, levels of fibrosis, and the biochemical composition of the LV in lambs at two and five months term-equivalent age (TEA) (Figure 6).

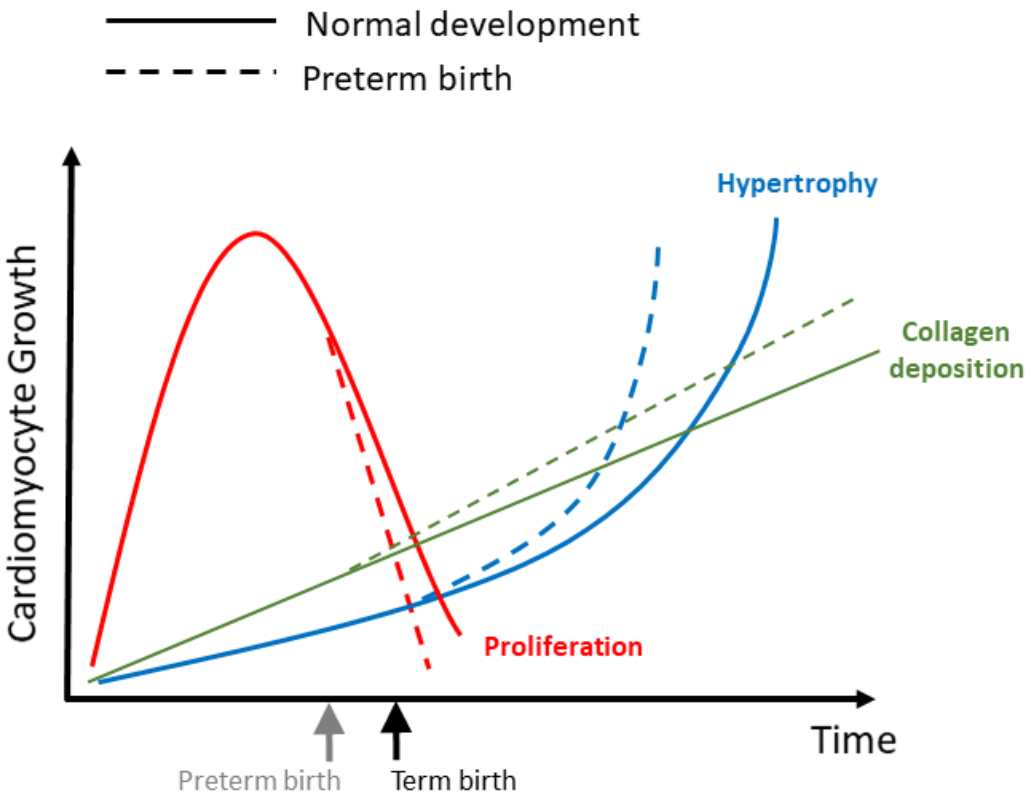

Figure 6. The specific hypothesis of this thesis is that preterm birth combined with modern neonatal interventions would adversely affect cardiomyocyte development, whereby there is a decrease in cardiomyocyte proliferation (green), and a compensated increase in cardiomyocyte hypertrophy (pink) and myocardial collagen deposition (blue).

To address these hypotheses, we used an established sheep model of preterm birth requiring assisted ventilation (described in **Chapter 2**). The aims of this thesis were:

Chapter 3: To determine the effects of preterm birth on the left and right ventricular myocardium in lambs born at two and five-months TEA using histology and stereology.

Chapter 4: To determine the effects of preterm birth on the biochemical composition of the left ventricular myocardium in lambs at two and five-months TEA using Fourier-transform infrared spectroscopy.

Chapter 5: To determine the effects of preterm birth on myocardial microstructure in the lamb heart at five months TEA using diffusion tensor imaging.

It was predicted that the adverse impacts of preterm birth on cardiac structure would be greater than previously reported in preterm lambs that were born moderately preterm and did not require respiratory support after birth.

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Current Opinion in Physiology

Maladaptive structural remodelling of the heart following preterm birth

Bianca Le, Megan R Sutherland and M Jane Black

Preterm birth (delivery prior to 37 completed weeks of gestation) is the leading cause of perinatal deaths worldwide. Preterm infants are born when their hearts are structurally and functionally immature; as a result, maladaptive cardiac remodelling occurs in the neonatal period which may lead to cardiac dysfunction later in life. Hypotension is a common co-morbidity of preterm birth in the neonatal period; however, hypertension often manifests in adulthood. Adults born preterm exhibit altered heart growth, which may in part be linked to their elevation in blood pressure. Clinical interventions used to facilitate lung function in preterm infants, including antenatal glucocorticoids and mechanical ventilation, may also impact cardiac growth early in life, with lifelong implications for cardiac structure and function.

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Introduction

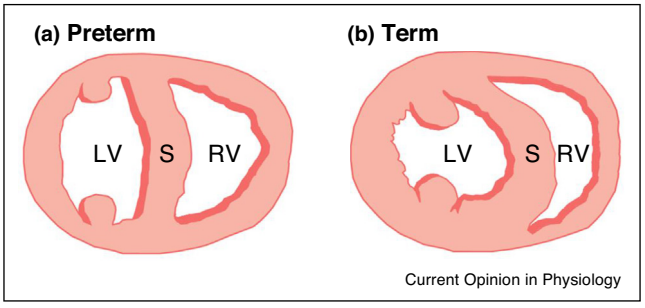
Preterm birth (delivery prior to 37 completed weeks of gestation) is the leading cause of perinatal deaths worldwide [1]. The survival rates of preterm neonates in developed countries have markedly improved over recent decades (particularly for those born extremely preterm), due to advances in neonatal care aimed at facilitating lung maturation and function. Along with the reduction in mortality rates, the adverse clinical consequences of preterm birth that emerge later in life are becoming increasingly apparent.

Hypotension is a common challenge faced by preterm neonates, and is clinically managed by volume replacement and/or vasopressor medications [2]. Later in life, however, it is of increasing concern that both children and adults born preterm are highly susceptible to developing hypertension [3], with a strong inverse correlation between gestational age at birth and blood pressure [4,5]. The later development of high blood pressure can be partly explained by the 'developmental origins of health and disease' hypothesis, which proposes that perinatal insults, such as preterm birth, result in compensatory mechanisms (including structural remodelling, physiological alterations and epigenetic changes), that are necessary for survival in the short term, but can program a later vulnerability to disease. In the case of the cardiovascular system, while the early postnatal adaptations are crucial for the neonate to increase systemic pressure for adequate organ perfusion, this short-term compensation can lead to impaired function later in life [6], thus increasing the risk of cardiovascular pathologies, such as heart failure [7••] and hypertension [4,5,8•]. This is true for all severities of preterm birth; even small reductions in gestational length are sufficient to induce long-lasting anatomical alterations [9]. Studying the underlying physiological mechanisms of preterm birth that result in cardiovascular sequelae is difficult, since the aetiologies of both preterm birth and cardiovascular disease are often multifactorial. This review will describe how maladaptive remodelling of the heart in preterm patients can influence cardiovascular physiology throughout life. The impact on the immature heart of clinical interventions (antenatal steroids and postnatal ventilatory support) used to improve postnatal respiratory function, and thus the survival of preterm infants, will also be discussed.

Structural remodelling of the heart following preterm birth

Before term birth, the total peripheral resistance of the foetal systemic circulation is low and hence systemic arterial pressure is relatively low [10]. Within minutes of umbilical cord occlusion at term birth, the peripheral resistance of the systemic circulation increases, primarily due to the loss of the placental vascular bed [11]. Thus, systemic arterial pressure is normally increased after term birth. In preterm infants, particularly those born very or extremely preterm, the immature heart cannot maintain cardiac output in the face of the sudden increase in systemic vascular resistance at birth, and these infants are consequently vulnerable to pathophysiological hypotension [2]. Compensatory structural remodelling of the heart and blood vessels may occur as a short-term adaptation to neonatal hypotension in infants born very or extremely preterm, but in turn could lead to the progressive development of high blood pressure later in life [4,5].

Figure 1



Comparative structure of the heart in term and preterm infants. A diagrammatic representation of the transverse cross section of hearts from **(a)** an infant born preterm and **(b)** an infant born at term. Persistence of a flattened interventricular septum (S) is seen in the preterm neonatal heart [18]. LV, left ventricle, RV, right ventricle.

Indeed, preterm birth has been shown to be associated with the induction of hypertension in adulthood, with blood pressure inversely related to age at birth [4,5]. Individuals born preterm also demonstrate an elevated blood pressure stress response [8•]. Narrowing of conduit arteries in children born preterm is well-described [12,13,14•], and studies have also shown altered postnatal cardiac growth [15••,16,17,18]. A recent study found that preterm neonates had reduced heart mass to body weight ratio at birth compared to infants born at term [15••]. However, ventricular mass increased disproportionately in relation to body size within the first 3 months of life in the preterm infants, which was associated with cardiac dysfunction [15••]. Other studies have found that the interventricular septum remains flat for 9.5 days after preterm birth, resulting in a distorted 'D' shape in the transverse cross-section of the left ventricle (LV) (Figure 1a) [16–18]. While this phenotype is typical in the foetal heart where the right ventricle (RV) is dominant, the reduction in pulmonary vascular resistance (and thus a decrease in RV afterload) normally occurring at birth causes the intraventricular septum to bow into the RV, creating a circular LV by approximately 5 days after term birth (Figure 1b) [19]. However, the persistence of high pulmonary vascular resistance in preterm neonates is likely the reason why their septum remains flat after birth. This anatomical variation is associated with a reduction in the fractional shortening of the LV [18], and thus reduced LV diastolic filling [16]. Some studies found that within 12–14 days after preterm birth, the LV establishes a normal degree of circularity, and therefore an improvement in LV function [16,17]. One study, however, found that the shape of the ventricles was still abnormal in preterm infants after 51 days [18].

Recently, it has become apparent that altered cardiac growth following preterm birth continues into childhood and later life. One study found that the interventricular septum was thicker and left ventricular end-diastolic diameter was reduced in preterm-born children at 5 years of age, compared to age-adjusted population reference values [20]. This altered cardiac morphology, combined with increases in systolic blood pressure, are indicative of increased cardiac afterload in preterm individuals [20]. Increased cardiac afterload and elevated blood pressure may reflect reduced distensibility in the large arteries [21], and importantly in this regard, abdominal aortic wall distensibility and whole-body arterial compliance has been shown to be reduced in very low birth weight preterm infants examined at 7 weeks of age [22].

Cardiac and vascular remodelling can also occur following moderate to late preterm birth (between 32 and 36 weeks' gestation) even though hypotension is not an issue in babies born at this gestational age. Indeed, it may be the premature exposure of the immature heart and vasculature to the increase in blood pressure that normally occurs at birth that leads to the cardiac and vascular remodelling following moderate or late preterm birth [23]. At the cellular level, we have shown through experimental studies that preterm birth leads to an increase in collagen deposition and alterations in cardiomyocyte maturation and size within the myocardium, early in life [9,23]. Bensley *et al.* [9] found that cardiomyocyte volume was markedly increased in both the LV and RV of 11 week old lambs born preterm, as compared to term-born lambs, despite no differences in absolute or relative heart weight, wall thickness, or luminal areas of the RV or LV. The accelerated cardiomyocyte hypertrophy was accompanied by findings of altered cardiomyocyte nuclearity and increased cardiomyocyte ploidy in the preterm myocardium [9]; polyploidy is common in hearts subjected to pathological hypertrophy, and is linked to impaired cardiac function [24]. Furthermore, preterm-born lambs were shown to have narrowing of the ascending aorta and altered cellular composition of the aortic and pulmonary artery walls, with some animals exhibiting significant aortic intimal injury [25].

Anatomical alterations in the hearts of people born preterm have also been reported in adulthood. Lewandowski *et al.* [6,26] used magnetic resonance imaging (MRI) to analyse cardiac structure in 25-year-old adults. Cavity size was significantly reduced in the LV and, to a greater extent, the RV of adults born preterm compared to those born at term, which was associated with decreased stroke volume and ejection fraction [6,26]. Preterm-born adults also had significantly greater ventricular mass, with ventricular mass inversely associated with gestational age at birth (-0.98 g/m^2 LV mass and -0.67 g/m^2 RV mass per additional week of gestation) [6,26]. Importantly, LV mass in the young adults born preterm was disproportionately increased relative to blood pressure [6]. It may be speculated that the LV hypertrophy observed in preterm-born adults occurs due to the increased cardiac afterload

and increased peripheral vascular resistance reported in preterm infants and children [20,22].

Notably, in contrast to the MRI findings of Lewandowski *et al.* [6] a recent echocardiography study conducted in 18 year-old adults born preterm, by Kowalski *et al.* [27••], showed that although preterm-born adults had a reduced left ventricular volume compared to those born at term, there was no significant difference in LV wall thickness. Furthermore, they found that LV mass was significantly reduced in adults born preterm compared to those born at term (preterm = 75 ± 14 g/m^2, term = 81 ± 16 g/m^2, $P = 0.02$).

There are a number of differences in these studies that may account for the disparity in findings. Firstly, different imaging techniques were used; Lewandowski *et al.* [6,26] used MRI, which may be more accurate in estimating tissue mass than echocardiography [28] used by Kowalski *et al.* [27••]. Secondly, Lewandowski *et al.* [6,26] included a mixed cohort of adults born moderately (31%), very (55%), and extremely (14%) preterm, with 31% of participants also intrauterine growth restricted (IUGR), whereas Kowalski *et al.* [27••] only included adults born extremely preterm (<28 weeks gestation) or weighing <1000 g at birth, the vast majority of whom were non-IUGR. Certainly, it is well described in animal models that IUGR, independent of preterm birth, leads to altered cardiac growth [29] and to a reduced complement of cardiomyocytes at birth [30,31]. Clinical studies have also shown that IUGR leads to alterations in vascular and cardiac morphology, and cardiac dysfunction in neonates [29]. Given the limited proliferative capacity of cardiomyocytes after birth, it is likely that IUGR as a co-morbidity of preterm birth would further impact postnatal cardiac growth, particularly in the setting of accelerated catch-up growth which often occurs in IUGR infants [32]. Therefore, the combined effect of preterm birth and IUGR could have a greater influence on ventricular mass, and ultimately, cardiac function, than preterm birth alone.

It is well-known that males born preterm are more susceptible to developing respiratory distress syndrome (RDS) than their female counterparts [33], likely due to delayed maturation of the pulmonary surfactant system [34]. To our knowledge, the limited number of experimental studies that have explored sex as a factor in cardiovascular physiology at the time of preterm birth have not reported any significant differences [35–37]; there is a lack of evidence regarding whether there are sex differences in the prevalence of long-term cardiovascular morbidities. In one study, female adults born preterm were shown to have a significantly greater RV mass to end-diastolic volume ratio compared to males (female: 0.33 ± 0.059 g/ml, male: 0.30 ± 0.060 g/ml) [26], however this was not associated with any differences in RV function. This area of research warrants further investigation, particularly given the known sex differences in the prevalence of cardiovascular disease, with males at greater risk than females prior to middle-age [38,39].

Interventions directed at improving survival following preterm birth have the potential to adversely impact cardiac development

Clinical approaches to preterm birth are primarily targeted towards improving respiratory function postnatally. Specifically, the use of antenatal glucocorticoids, postnatal surfactant, and assisted ventilation have become standard practice to maintain adequate respiratory function in preterm neonates and this has markedly decreased neonatal mortality and morbidity [40–42]. It has recently become apparent, however, that antenatal steroids and postnatal ventilation have the potential to adversely impact the immature heart, either independently of preterm birth, or additive to the respiratory outcomes of preterm birth.

Antenatal glucocorticoids

Glucocorticoids are a class of corticosteroids that play vital roles in the development and maturation of foetal organs. Cortisol, the major human glucocorticoid, is notable for stimulating pulmonary surfactant production in the foetal lungs. The developing foetus is unable to produce cortisol, and thus receives its supply from the maternal circulation [43,44]. Cortisol levels in the amniotic fluid peak in late gestation, and this is highly synchronised with the timing of lung maturation during the third trimester [45,46]. However, if birth occurs prior to the surge of cortisol in late gestation, lung development of the preterm neonate is severely impaired.

It is well-known that the prenatal administration of synthetic glucocorticoids to mothers at risk of delivering preterm accelerates foetal lung development, markedly improving short term survival [47]. Much like foetal lungs, structural and functional maturation of the foetal heart is highly dependent on glucocorticoid signalling [48]. However, glucocorticoid sensitivity varies between organs [49], and it is unknown whether the dosage of antenatal glucocorticoids necessary for foetal lung maturation is suitable for the developing heart.

The effects of antenatal glucocorticoids on cardiac development in preterm infants is not yet fully elucidated. Certainly, there are a number of studies that report a link between antenatal corticosteroids and the onset of hypertension and aortic stiffness later in life [50,51], and this can, in turn, lead to cardiac remodelling in the adult heart. Although there are some conflicting reports in sheep studies relating to the direct effects of steroid exposure on the growth of cardiomyocytes in early life, the majority of studies support the view that glucocorticoids accelerate cardiomyocyte maturation [37,52]; evidence of decreased cardiomyocyte proliferation and increased cardiomyocyte

hypertrophy and/or binucleation after glucocorticoid exposure (all of which are markers of mature cardiomyocytes) support this concept [37,53–56]. Premature cell cycle arrest following accelerated maturation may be particularly detrimental for the developing heart, as it may lead to a reduced cardiomyocyte endowment and thus, reduced cardiac functional reserve, rendering the heart susceptible to cardiovascular injury and dysfunction later in life.

Considering the widespread use of glucocorticoid therapy for mothers at risk of delivering preterm, it is necessary to develop a better understanding of the long-term effects on the developing cardiovascular system. Indeed, given that maternal antenatal glucocorticoids have only been used clinically in the past 40 years, the full extent of the long-term cardiovascular consequences may not have yet emerged.

Neonatal ventilation

Aeration of the neonatal lungs is one of the first obstacles of preterm life. Intermittent mandatory ventilation (IMV) is an invasive form of ventilation requiring endotracheal intubation, and it provides cycles of positive pressure during inspiration and expiration to prevent airway collapse [57]. IMV use has extended the limit of viability of preterm infants and significantly improved survival rates, particularly in neonates with RDS [58]. This form of ventilation may, however, cause volutrauma and atelectrauma due to over-inflation of the developing lungs, which can lead to chronic lung disease.

Although cardiac and pulmonary physiologies are closely interconnected, interventions aimed at improving one, may negatively impact the other. IMV facilitates gas exchange by forcing air into the lungs during inspiration, thereby increasing intrathoracic pressure. Other intrathoracic structures are consequently affected, notably the heart and great vessels. Firstly, increased intrathoracic pressure causes right atrial pressure to increase, resulting in decreased venous return and cardiac output, with subsequently increased pulmonary vascular resistance and right ventricular afterload [59]. These outcomes contribute to increased workload in the right ventricle, causing maladaptive hypertrophy of the RV [60]. Indeed, Lewandowski *et al.* [26] found that increased duration of postnatal IMV following preterm birth was directly correlated with increased RV mass in adulthood. Additionally, increased intrathoracic pressure can also result in decreased LV afterload, venous return and cardiac output [61–63]. This LV dysfunction is associated with hypotension, and a leftward displacement of the interventricular septum occurs in adults requiring respiratory mechanical ventilation [63]; however, as yet no studies have examined whether postnatal ventilation affects the structure of the developing LV in preterm infants.

Ventilator-induced lung injury and its associated sequelae necessitates a reduction in the time that preterm infants spend on IMV. While gentler alternatives, such as nasal continuous positive airway pressure (nCPAP), have the potential to reduce the risk of lung disease [64], they are not effective in extremely preterm infants who cannot yet produce spontaneous breaths [65]. Furthermore, 46% of infants on nCPAP ultimately require subsequent intubation and IMV [65]. Although clinical approaches to neonatal ventilation have improved, the effects of assisted ventilation on the developing preterm heart warrant further investigation. Indeed, studies in sheep have shown that ventilation after birth reduces glomerular capillary growth in both preterm and term born lambs [66], thus suggesting that there are haemodynamic changes that may then influence cardiovascular growth.

Conclusion

With the first generation of medically treated preterm patients now approaching mid-adulthood, we are beginning to see the long-term cardiovascular consequences of being born early. There is substantial evidence to suggest that the immature preterm heart undergoes structural remodelling as a maladaptive response to the functional demands associated with postnatal life, and that these abnormal structural changes in cardiac growth persist into adulthood. It is important to also take into account that current clinical interventions used to treat preterm neonates may further exacerbate cardiac remodelling following preterm birth. Further research is required to fully understand the potential long-term pathophysiological consequences of these treatments.

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Conflict of interest statement

None declared.

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Chapter 2

Animal model and methods

2.1 Introduction

Preterm birth is an emerging risk factor for hypertension and heart failure in humans [1, 2]. Studies using MRI have found that long-term cardiac dysfunction in humans born preterm is linked to structural remodelling of the ventricles [3, 4]. However, little is known about the cellular processes driving this maladaptive change in ventricular anatomy. This is particularly difficult to study in humans, as autopsy tissue is required for analysis. It is also difficult to control for factors such as the cause of preterm birth, cause of death, lifestyle and environment, all of which may influence cardiac development and growth. Therefore, establishing an appropriate animal model of preterm birth is currently the most feasible solution to understanding the pathogenic mechanisms that underlie ventricular remodelling.

Few large animal models of preterm birth are available, as animal studies that aim to recapitulate the settings of neonatal intensive care units are laborious, costly, time-consuming and require highly-experienced animal technicians [5]. Sheep are commonly used to model preterm birth, mainly due to their similarities to humans in gestational length and foetal lung anatomy. Specifically, the lungs of sheep and humans both reach the alveolar stage of development by term gestation (sheep: 150 days; humans: 40 weeks), making sheep an excellent species for neonatal respiration research [5]. Furthermore, newborn lambs are similar in size to human infants; therefore, preterm lambs can be treated with the same paediatric ventilations that are used clinically.

Cardiac development, anatomy and physiology in sheep and humans are also comparable. Sheep have a four-chambered heart that is left ventricle dominant, much like humans, but have a slightly lower heart to body weight ratio (adult sheep: 3-3.13 g/kg [6, 7]; adult human: 5 g/kg [8]). Cardiomyocyte development and maturation has also been well-studied in sheep [9, 10]. It is generally accepted that human and sheep cardiomyocytes have extremely low proliferative capacity soon after term birth [10, 11], unlike rodent cardiomyocytes which continue to proliferate and maintain regenerative capacity after term birth [12, 13]. Using Ki-67 immunohistochemistry staining, Jonker *et al.* [10] found that only

~1% of cardiomyocytes in both the left and right ventricles of sheep were active in the cell cycle by the first week of postnatal life. Cardiomyocyte hypertrophy then becomes the main determinant of cardiac growth after term birth [10]. Sheep cardiomyocytes also have the unique characteristic of being mononucleated and capable of proliferating when immature, then becoming binucleated once they have terminally differentiated [9]. Thus, the nuclearity of cardiomyocytes in sheep serves as a useful marker for cell maturation.

This chapter describes a sheep model of preterm birth that was used throughout this PhD project. This project is part of an ongoing sheep study at the Albertine laboratory at the University of Utah, Salt Lake City, U.S.A. Lambs used in this study were born between 2008 and 2017 at the Albertine sheep laboratory. The protocols of this study adhered to APS/NIS guidelines for the humane use of animals for research and were prospectively approved by the IACUC at the University of Utah Health Sciences Center. The following methods apply to all animals used in the subsequent chapters of this thesis.

2.2 Methods

All caesarean deliveries were performed by Ms Mar Janna Dahl and Mr Li Dong, with the assistance of Ms Sydney Bowen (senior laboratory manager), Mr Toshio Aoki, and Ms Kaitlin Zuspan (laboratory managers). In 2017, I spent 4 months in the Albertine laboratory to assist with the prenatal care of the ewes, and the delivery and postnatal care of term and preterm lambs. Details of this former-preterm lamb model have previously been published [14].

Figure 1 summarises the timeline, preterm lamb delivery, and postnatal care protocols for the former-preterm lamb model.

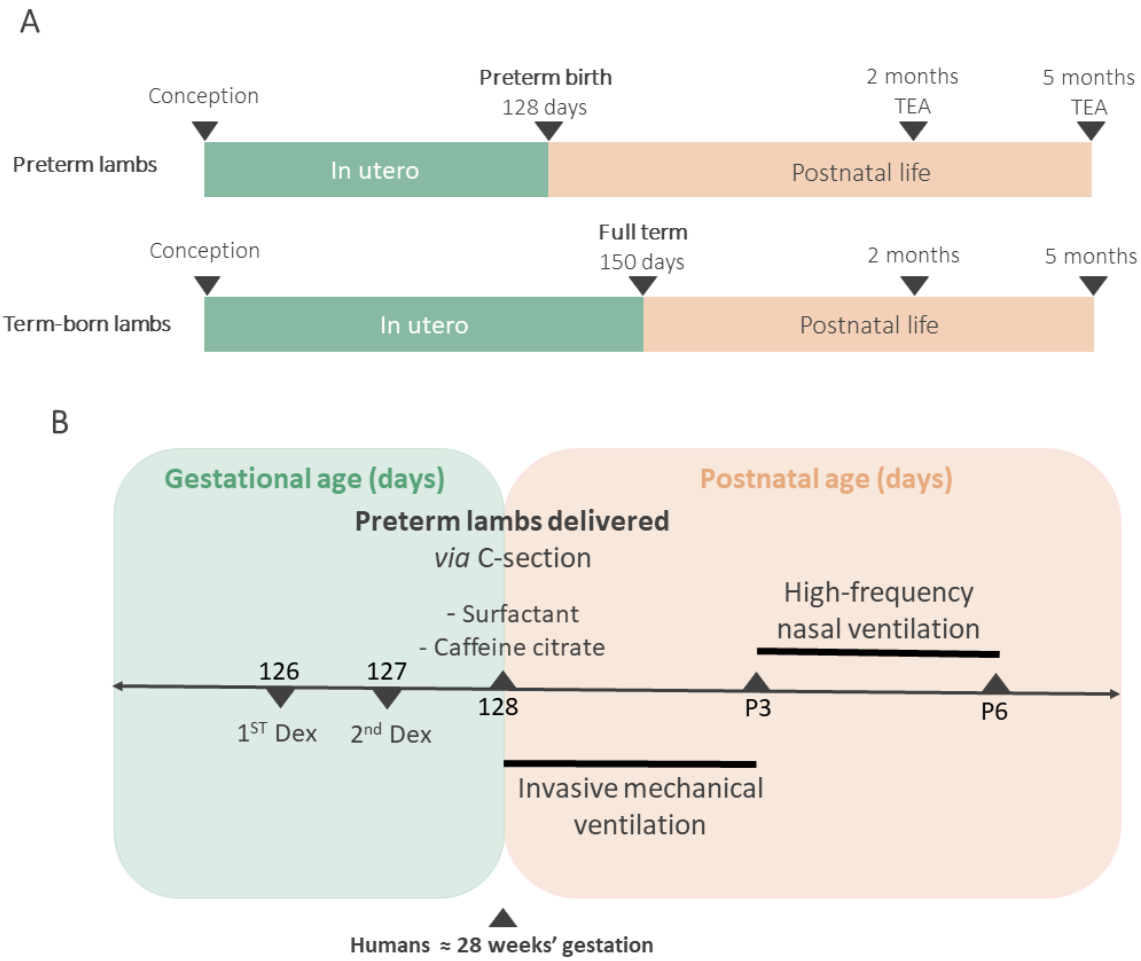

Figure 1. A) Timeline for the life span of preterm lambs and lambs born at term. Lambs from each group were randomly assigned to be culled at 2 months term-equivalent age (TEA) or at 5 months TEA. **B)** Timeline for the preterm lamb delivery and postnatal care protocol. Ewes received two courses of dexamethasone 48 hours and 24 hours before delivery. Preterm lambs were delivered on day 128 of gestation via a caeserean section (C-section). The preterm lambs received exogenous surfactant and caffeine citrate soon after birth. They were then placed on invasive mechanical ventilation for 3 days, then placed on high-frequency nasal ventilation for 3 more days.

2.2.1 Prenatal care of ewes

Rambouillet-Columbia cross ewes were date-mated with Rambouillet rams (Deweyville, Utah). Pregnant ewes were transported to the animal housing facility at the University of Utah (Salt Lake City, Utah, U.S.A.) approximately 2 weeks before delivery. The ewes were fed alfalfa hay and water *ad libitum* and were monitored daily to ensure they remained healthy and alert. The ewes chosen for preterm delivery received an intramuscular injection of dexamethasone phosphate (6 mg; Vedco, Inc., St. Joseph, MO, U.S.A.) 48 hours and 24 hours before operative delivery.

2.2.2 Preterm lamb delivery protocol

The ewes were randomly assigned to deliver preterm via caesarean section at 128 days' gestation (0.85 gestation; n = 13) or at term via natural vaginal delivery following spontaneous labour at 150 days' gestation (n= 15). The ewes are fasted from food, but not water, 16 hours before delivery to reduce the risk of bloating and regurgitation during surgery. On the day of delivery, the ewe received an intramuscular injection of ketamine hydrochloride (10-20 mg/kg; Vedco Inc., St. Joseph, MO, U.S.A.) in the rump. Once sedated, the ewe was transferred onto a scale and her weight was recorded. Inhalation anaesthesia with isoflurane was given (2.5% isoflurane; Piramal Healthcare, Bethlehem, PA, U.S.A.) via an anaesthesia mask placed over the ewe's mouth and nose. Meanwhile, the abdomen and neck fleece

were shaved and scrubbed with betadine (10% povidone iodine; Purdue Products L.P., Stamford, CT, U.S.A.).

The ewe was then transferred to the operating table and her legs were tied down onto the table to avoid kicking. A laryngoscope was used to intubate the ewe with a cuffed endotracheal tube (9.0 – 10.0 mm; AIRCARE® Smiths Medical, Ashford, United Kingdom). This allowed general anaesthesia to be maintained by use of a positive pressure ventilator (Narkomed 2B Anesthesia System; North American Dräger, Telford, PA, U.S.A.). A pulse oximeter was attached to the cheek to measure oxygen saturation during the course of the surgery. A grounding pad (Covidien™ Valleylab™ Non-REM Polyhesive™ Patient Return Electrode; Medtronics, Minneapolis, MN, U.S.A.) was placed on the chest, lateral to the sternum, to permit electrosurgery. A blood pressure cuff was placed on the front, right limb of the ewe. The shaved abdominal and neck areas were scrubbed with betadine again to ensure that the areas were sterile.

Surgery was performed under strict aseptic conditions; facemasks, surgical caps and sterile surgical gowns and gloves were worn. All surgical equipment and instruments were sterilised by autoclave at 133° C. The ewe was covered in adhesive sterile drapes (Basic sterile pack, 3M™ Steri-Drape™ 9000; 3M Healthcare, St Paul, MN, U.S.A.) and sterile surgical linen drapes, such that only the abdomen and neck were exposed.

2.2.3 Monitoring the ewe prior to and during delivery

Catheters (14 gauge) were placed in the carotid artery and jugular vein of the ewe. Heparinised saline was administered via the jugular vein for fluid replacement. Ringer's lactate solution containing 5% dextrose was given at a rate of 1 L/hour. Four ml of penicillin G potassium (Pfizerpen®; Pfizer, New York, U.S.A.) was also administered through the intravenous line. The caesarean delivery commenced, as described below. Simultaneous to the caesarean delivery, a catheter filled with heparinised saline was placed in the carotid artery for arterial blood gas analysis. One ml of heparinised blood was drawn from

the arterial line using a heparinised syringe every 30 minutes during the delivery and analysed on the blood gas machine (SIEMENS RAPIDPoint® 500; Munich, Germany); pH, partial pressures of carbon dioxide and oxygen, sodium bicarbonate, glucose and calcium levels were recorded. Heart rate, mean arterial blood pressure and oxygen saturation were recorded every 15 minutes until the ewe was killed. The target ranges for these measurements are noted in Table 1. Ventilator settings were adjusted if blood gas and vital measurements were outside their target range.

Table 1. Target physiological ranges for ventilated ewes under general anaesthesia during caesarean deliveries.

Parameter	Target range
pH	7.25 – 7.45
pCO_2	45 – 60 mmHg
pO_2	60-80 mmHg
Sodium bicarbonate	25 – 40
Glucose	60 – 100
Calcium	1.13 – 1.32
Heart rate	80 – 120 beats per minute
Mean arterial pressure	50 – 80 mmHg
Oxygen saturation	95 – 100%

Partial pressure of carbon dioxide (pCO_2), partial pressure of oxygen (pO_2).

2.2.4 Caesarean delivery

Once an intravenous line was placed in the jugular vein, the ewe underwent a laparotomy under general anaesthesia, and the foetus was exposed via midline hysterotomy (Figure 2). The foetus's common carotid artery and saphenous vein were catheterised (0.040") and an arterial blood sample (0.3 ml) was

taken to monitor its blood gases prior to delivery. The foetus was then intubated with a cuffed endotracheal tube (3.5-4.0 French) using a paediatric laryngoscope. All foetal catheters were anchored using 3-0 silk.

Ten ml of lung liquid was aspirated from the foetal lungs and replaced with calfactant (6 ml, Infasurf; New York, U.SA.), a non-pyrogenic pulmonary surfactant used to reduce surface tension within the lungs. The foetus was dried from amniotic fluid with warm, sterile towels to prevent the spread of infection. The foetus was then removed from the uterus and its umbilical cord was ligated and cut. 10 ml/kg (body weight of lamb) of blood was taken from the umbilical artery into a heparinised tube to be transfused into the preterm lamb once resuscitated. Finally, the ewe was euthanised by an overdose of concentrated pentobarbital (30 ml, Beuthanasia®-D Special; Merck Animal Health, New Jersey, U.S.A.) administered through the venous line.

Figure 2. A preterm lamb is delivered *via* Caesarean section

2.2.5 Invasive mechanical ventilation of preterm lambs

The lamb was manually resuscitated using a Neopuff™ T-piece resuscitator (Fisher & Paykel; Auckland, New Zealand) whilst being weighed on an infant scale. All preterm lambs were placed in a veterinary sling mounted on a radiantly heated bed to restrict mobility during ventilation. As soon as the lamb was positioned (Figure 3), the lamb was placed on invasive mechanical ventilation (IMV) (Babylog® VN500; Dräger, Lübeck, Germany). The ventilator was attached to an air compressor and air humidifier (Figure 3). The initial ventilator settings are noted in Table 2.

The fraction of inspired oxygen (FiO_2) was gradually lowered to 21% over 3 – 6 days to wean the lamb off IMV. Preterm lambs were mechanically ventilated for 3 to 6 days, depending on how well the lamb could adjust to the decreasing FiO_2. The lambs were then extubated and placed on high-frequency nasal ventilation. The target physiological ranges for preterm lambs on IMV are noted in Table 3. IMV is a form of ventilation requiring endotracheal intubation that provides cycles of positive pressure during inspiration and expiration to prevent airway collapse [15]. IMV use has extended the limit of viability of preterm infants and significantly improved survival rates, particularly in neonates with respiratory distress syndrome [16]. This form of ventilation may, however, cause volutrauma and atelectrauma due to over-inflation of the developing lungs, which can lead to a chronic lung disease known as bronchopulmonary dysplasia [17].

Figure 3. A preterm lamb on synchronised invasive mechanical ventilation. The lamb was placed in a paediatric sling on a radiantly heated bed immediately after resuscitation. The lamb was intubated with an endotracheal tube attached to the Dräger VN500 ventilator to the left. A vital signs monitor is placed on the right, displaying heart rate and oxygen saturation measured from a pulse oximeter (not in view), and displaying blood pressure measured from an arterial line pressure transducer.

Table 2. Initial ventilator settings for preterm lambs on invasive mechanical ventilation.

Setting	Value
Fraction of inspired oxygen	50%
Respiratory rate	60 breaths/minute
Inspiration time	0.34 seconds
Peak inspiratory pressure	23 cmH_2O
Positive end-expiratory pressure	7 cmH_2O
Flow	50% oxygen at 8 L/min
Expiratory tidal volume	5 – 7 ml/kg

Table 3. Target physiological ranges for preterm lambs on invasive mechanical ventilation.

Parameter	Target range
pH	7.25 – 7.45
pCO_2	45 – 60 mmHg
pO_2	60-80 mmHg
Sodium bicarbonate	20 – 40 mmol/L
Base excess	-2 – +2 mmol/L
Sodium	135 – 148 mmol/L
Potassium	3.5 – 5 mmol/L
Calcium	1 – 1.32 mmol/L
Chloride	98 – 106 mmol/L
Glucose	60 – 100 mg/Dl
Hematocrit	35 – 45%
Total serum protein	3.5 – 5 g/dL
O_2 saturation	89 – 95%
Temperature	38 – 39.5°C
Heart rate	100 – 200 beats per minute
Mean arterial pressure	60 – 80 mmHg
Oxygen saturation	95 – 100%

Partial pressure of carbon dioxide (pCO_2), partial pressure of oxygen (pO_2).

2.2.6 High-frequency nasal ventilation of preterm lambs

HFNV was performed using the same ventilator used for IMV. Once the preterm lamb was extubated, an uncuffed oral/nasal cannula (3.0 French; 13 cm length) was inserted ~5 cm into a nostril, and was

secured onto the lamb's head using umbilical tape (Figure 4). Lidocaine (1% solution; Hospira, Inc., U.S.A.) was injected subcutaneously within the nostril to minimise pain and discomfort. Initial ventilator settings for HFNV are described in Table 4. Preterm lambs were placed on HFNV for ~3 days, then received supplementary blow-by oxygen through a cone until fully stable. Preterm lambs were then placed in the same housing as the term lambs.

High-frequency nasal ventilation (HFNV) is a mode of non-invasive ventilation that provides high-frequency breathing cycles of sub-physiological tidal volumes around a relatively high but constant mean airway pressure. Tidal volumes used in HFNV must be lower than the anatomical dead space volume. High-frequency ventilation facilitates spontaneous breathing whilst avoiding extreme pressures which could cause the airways to overextend (barotrauma) and/or collapse (atelectrauma) [18]. HFNV also incorporates cycles of single 'sigh' breaths. A sigh breath is a sustained ventilator-induced breath with a tidal volume greater than the pre-set tidal volume. This temporary hyperinflation of the lungs is used to prevent heterogeneous alveolar collapse typically seen during modes of ventilation using low tidal volumes, thus improving gas exchange [19].

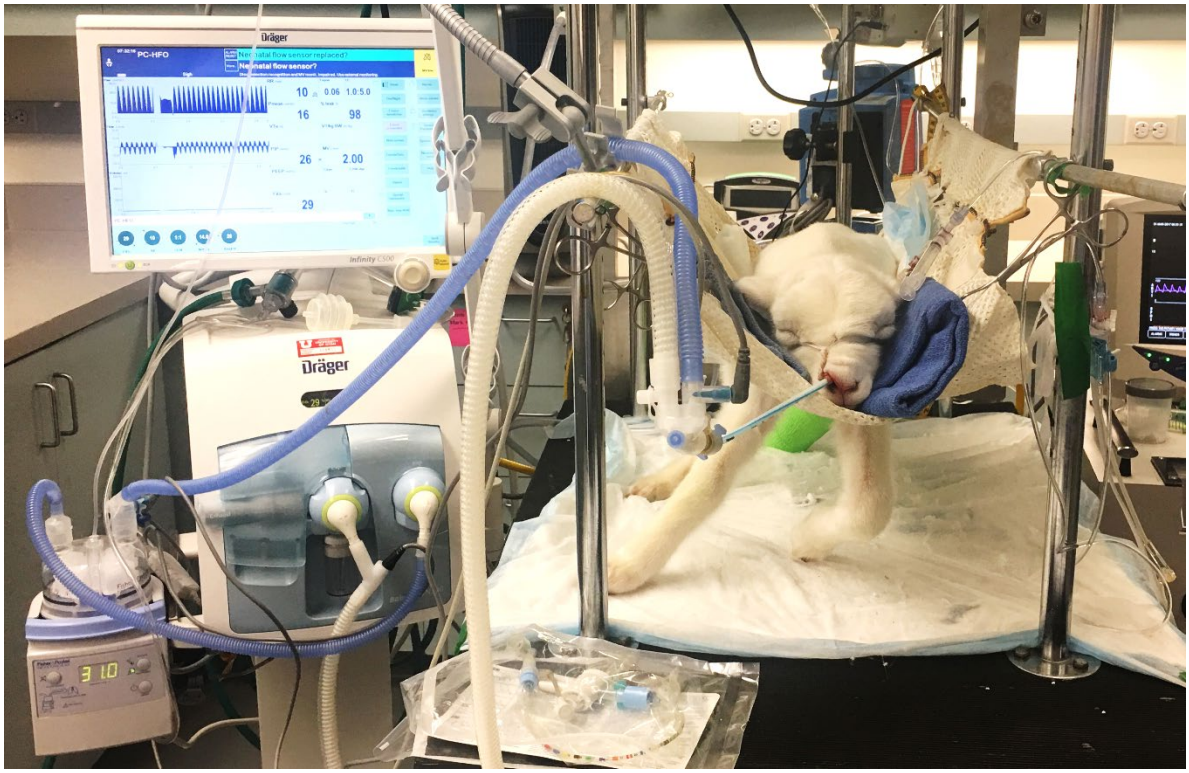

Figure 4. A preterm lamb on high-frequency nasal ventilation. A nasal cannula attached to the Dräger VN500 ventilator (left) was placed in the nostril of the preterm lamb.

Table 4. Initial settings for high-frequency nasal ventilation of preterm lambs.

Parameter	Ventilator setting
Inspiratory to expiratory ratio	1:1
Frequency	8 Hz
Amplitude	22 Hz
Mean airway pressure	10.0 mmHg
Pressure of sigh breath	23 mmHg
Length of sigh breath	0.8 – 1 seconds
Respiratory rate of sigh breath	20 breaths per minute

2.2.7 Management of preterm lambs

Intravenous fluids and drugs

Within the first 30 minutes of life, the preterm lamb received intravenous saline (0.9%) and dextrose (10%). Blood milked from the umbilical artery was also delivered intravenously to the lamb, at a rate of 5 ml/kg for two hours. In sequential order, pentobarbital (1 mg/kg), buprenorphine (5mg/kg), caffeine citrate (15 mg/kg), potassium penicillin (100,000 units/kg) and amikacin (18 mg/kg) were administered intravenously. The preterm lambs continued to receive the same dose of amikacin every 36 hours for the first 3 days of life. This dosage decreased to 15 mg/kg, administered once every 24 hours, until the lamb was removed from respiratory support. The preterm lamb continued to receive buprenorphine every 6 hours (2.5 mg/kg) until the lamb was extubated, and potassium penicillin and caffeine citrate (dosage was reduced to 5 mg/kg) every 24 hours until the lamb was off respiratory support. Dopamine (6mg/kg) was given at a rate of 5 mcg/kg/min if blood pressure was below 32 mmHg.

Monitoring vital signs

An arterial line pressure transducer and pulse oximeter placed on the tail were used to measure heart rate, mean blood pressure, systolic and diastolic blood pressure and oxygen saturation every 15 minutes until the lamb was removed from respiratory support. Heparinised arterial blood was taken every 15 minutes to monitor blood gases. Ventilation settings were adjusted if blood gases were not within their target ranges. Once the lamb was stable, blood gases were only taken once an hour. Rectal temperature was taken hourly, and plasma concentrations of total serum protein, white blood cells and haematocrit were measured every 12 hours to check for signs of infection. Daily measurements of body weight and abdominal girth were recorded to monitor growth and to check for signs of infection. Once the preterm lambs were placed in sheep housing, daily measurements of body weight, abdominal girth and rectal temperature continued to be recorded.

Feeding

Beginning at hour 3 of life, preterm lambs were fed colostrum (3 ml/kg every 3 hours; Bovine IgG; Land O Lakes®, Minnesota, U.S.A.) via an orogastric feeding tube for the first 48 hours of life. The volume of colostrum gradually increased by 3-5 ml increments, with the total intravenous fluid intake reduced proportionately. The preterm lambs were then fed milk (Sav A Lam milk replacer; Sav A Calf®, Wisconsin, U.S.A.) through the feeding tube. Once extubated, a sterilised nipple was gradually introduced during feedings, and once tolerated, feeding quantity increased whilst feeding frequency was reduced until the lamb was bottle-fed 300 ml every 12 hours for the remainder of its life. The preterm lambs also had access to alfalfa pellets, hay and water *ad libitum*.

2.2.8 Management of term lambs

Term lambs were left with their mothers to suckle colostrum for the first 24 hours of life. The term lambs were then removed from the ewe and placed in sheep housing with other term and preterm lambs. Term lambs were bottle-fed 50 ml of milk every 3 hours, then gradually increased in quantity and decreased in frequency to match the feeding pattern of the preterm lambs. By the first month of life, the term lambs were bottle-fed 300 ml of milk every 12 hours, and this was continued for the remainder of life. The term lambs also had access to alfalfa pellets, hay and water *ad libitum*. Daily measurements of body weight, abdominal girth and rectal temperature were also recorded in the term lambs to monitor growth and to check for signs of infection.

2.2.9 Euthanasia protocol and organ collection

Term lambs were randomly assigned to be euthanised at 2 months or 5 months of life. Preterm lambs were randomly assigned to be euthanised at 3 months (or 2 months term-equivalent age (TEA]) or at 6 months (5 months TEA) (Figure 1A). The number of singletons, twins, and triplets (only one animal in a triplet was included) in each animal group is shown in Table 5. With a sample size of at least 8 animals per group, based on previous cardiac stereological and fibrosis studies, we had approximately an 80%

chance of detecting a 15% difference between the groups at $\alpha = 0.05$. The survival rate for this animal model, where successful experiments were defined as reaching the 2- and 5- month endpoint, was 50% [14]. All lambs selected for the studies in this thesis survived to the end-points.

Table 5. The number of lambs included in this study that were born preterm (128 days' gestation) or at term (150 days' gestation) and were culled at 2 or 5 months term-equivalent age (TEA). The columns present the number of lambs that were singletons, twins, and triplets. The number in the parenthesis is the number of females within each sub-category.

	Singletons	Twins	Triplets	Total
Preterm 2 months TEA	3 (2)	5 (2)	0	**8 (4)**
Preterm 5 months TEA	1 (0)	7 (4)	0	**8 (4)**
Term 2 months TEA	2 (1)	8 (4)	1 (1)	**11 (6)**
Term 5 months TEA	3 (3)	5 (3)	0	**8 (6)**
Total	**9 (6)**	**25 (13)**	**1 (1)**	**35 (20)**

The lambs were fasted 12 hours before the surgery. Inhalation anaesthesia with isoflurane was given via an anaesthesia mask placed over the lamb's mouth and nose. Once placed on the surgical table, the lamb was intubated and connected to a positive pressure ventilator to maintain lung inflation. A final arterial blood sample was taken to measure blood gases. Three ml of heparin was administered intravenously, followed by concentrated pentobarbital (10 ml: 2-month-old lambs; 20 ml: 5-month-old lambs) and 7 ml of potassium chloride. The chest cavity was opened and the trachea was ligated at end-inspiration to minimise atelectasis. The heart was excised at the base of the great vessels, then weighed. Once the heart was removed, the coronary vessels were perfused with cold phosphate buffered saline, with 20 mEq of potassium chloride added. A 5mm x 5 mm tissue biopsy was taken from the left and

right ventricular free walls, and snap frozen in liquid nitrogen. The remaining whole heart was stored in 10% buffered formalin at 4°C.

2.2.10 The common clinical scenario for preterm deliveries in Australia

Both betamethasone and dexamethasone are glucocorticoids that are used as antenatal glucocorticoids in clinical practice in Australia, with betamethasone most commonly administered to women who are at risk of delivering preterm [20]. In developed countries such as Australia, both glucocorticoids provide similar neonatal outcomes, a similar likelihood of survival free of neurosensory disability in early childhood, and a similar risk of maternal infectious morbidity [21, 22]. According to the New Zealand and Australian Clinical Practice Guidelines by the Liggins Institute, a repeated course of glucocorticoids is recommended when the gestational age of the baby is 32 weeks and 6 days or less and the preterm birth is planned or expected within the next seven days [20]. As such, a repeated course of dexamethasone (12 mg total exposure) was administered to the preterm lambs in this study.

The delivery methods chosen for our lamb model of preterm birth and the term-born controls reflect the common clinical scenario in Australia. In Australia, roughly 80% of preterm babies are admitted to a NICU or special care nursery, compared to babies delivered at term (13%) [23]. The main delivery method for preterm babies is via caeserean section (54%), followed by vaginal delivery without instruments (34%), then vaginal delivery with forceps or vacuum (7%) [24]. In contrast, the main delivery method for babies born at term is via vaginal delivery without instruments (53%), followed by caesarean section (34%), then vaginal delivery with forceps or vacuum (13%) [24]. Similarly, the preterm lambs included in our studies were delivered via a caesarean section, while the term-born controls were delivered spontaneously.

2.2.11 Conclusion

This chapter describes the sheep model of preterm birth used in the subsequent chapters in this thesis. Given the increasing importance of using antenatal steroids, exogenous surfactant and non-invasive

forms of ventilation in preterm infants, this animal model reflects the common clinical setting of preterm human infants. Furthermore, the hearts collected from this study have allowed us to acutely explore the composition and structure of the preterm myocardium. All lambs described in subsequent chapters were treated according to this method.

2.3 Summary of animal model

This chapter describes the sheep model of preterm birth used in the following chapters of this thesis. Lambs were delivered spontaneously at term (n= 19), or via caesarean section at 128 days' gestation (n = 16) at the University of Utah. The preterm lambs were intubated and placed on invasive mechanical ventilation for 3 - 6 days, then weaned off respiratory support using a high-frequency nasal ventilation for a further 3 days. They were also given dexamethasone (via the maternal circulation) and pulmonary surfactant, which reflects the typical procedure of a scheduled caesarean delivery for medically indicated preterm birth in humans. The term lambs did not receive any form of respiratory support. The preterm lambs lived for either 2 months TEA (equivalent to a 2 year old infant) or 5 months TEA (equivalent to a 6 year old child) and were age-matched to the term lambs.

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Chapter 3

Preterm birth with modern neonatal interventions accelerates myocardial collagen deposition in the left ventricle of lambs at 2 and 5 months of age without affecting cardiomyocyte development

Preterm birth with modern neonatal interventions accelerates myocardial collagen deposition in the left ventricle of lambs at 2 and 5 months of age without affecting cardiomyocyte development

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Abstract

Background: Adults born preterm (<37 weeks' gestation) exhibit altered cardiac growth and are susceptible to impaired cardiac function. Sheep studies have shown that moderate preterm birth without postnatal respiratory support results in maladaptive structural remodelling of the cardiac ventricles. The aim of this study was to examine (at timepoints equivalent to early childhood) the effects of preterm birth on cardiac ventricular structure in lambs born at a greater severity of preterm birth and ventilated postnatally.

Methods and Results: Former-preterm lambs delivered at 128 days' gestation, and mechanically ventilated for a week after birth, were compared to unventilated lambs born at term (150 days gestation) at 2 months (term: n=10, former-preterm: n=8) and 5 months (term: n=9, former-preterm: n=8) term-equivalent age. The right ventricle (RV) and left ventricle plus septum (LV+S) were analysed, using immunohistochemistry, histology, and stereology. Heart weight was not affected by preterm birth. Cardiomyocyte number and cross-sectional area were not affected by preterm birth or age. Interstitial collagen levels in the LV+S increased with age ($p=0.0015$) and were exacerbated by preterm birth ($p=0.0006$; 2 months term: 0.57 ± 0.07 %, former-preterm: 1.44 ± 0.18 %; 5 months term: 1.37 ± 0.25 %, former-preterm: 2.15 ± 0.31 %). In the RV, interstitial collagen levels also increased with age ($p=0.012$) but were not significantly affected by preterm birth. Levels of cardiomyocyte proliferation and apoptosis were negligible in all lambs.

Conclusions: This study is the first to explore the effect of preterm birth combined with modern neonatal interventions on the ventricular myocardium in lambs. There were no adverse impacts of preterm birth and/or mechanical ventilation on cardiomyocyte growth in early postnatal life. Of concern, however, there

was increased collagen deposition in the preterm hearts, which has the potential to induce cardiac dysfunction, especially if it becomes further exaggerated with ageing.

Key words:

Preterm birth, mechanical ventilation, heart development, myocardial remodelling, collagen, stereology

Abbreviations:

Left ventricle plus septum (LV+S), right ventricle (RV), term-equivalent age (TEA)

1. Introduction

Preterm birth (defined as delivery prior to 37 weeks of gestation) necessitates the cardiopulmonary haemodynamic transition to occur at a time when many of the cardiomyocytes are undifferentiated and immature (with the level of cardiomyocyte maturity directly proportional to gestational age at birth). Therefore, the immature myocardium of former-preterm infants is often unable to cope with the marked increase in systemic arterial blood pressure and subsequent acute increase in cardiac afterload that occurs soon after preterm birth [1]. As a result, pathophysiological instability of systemic blood pressure and pulmonary hypertension are challenges commonly faced by infants born preterm [2, 3].

Several clinical studies suggest that early remodelling of both cardiac ventricles following preterm birth occurs as a physiological adaptation to a premature increase in cardiac preload and afterload on the immature myocardium [4-7]. In addition, anatomical and functional differences in the heart following preterm birth, from early infancy through to mid-adulthood, have recently been reported. For example, an increase in left ventricle and septum (LV+S) and right ventricle (RV) mass relative to body size has been shown in former-preterm infants at 3 months of age, when compared to infants born at term [8]. Similarly, magnetic resonance imaging studies in young adults born preterm report an increase in ventricular mass and wall thickness, reduced ventricular length and internal cavity diameter, and a displaced cardiac apex when compared to young adults born at term [9]. However, not all studies report induction of cardiac hypertrophy in adulthood following preterm birth [10], thus implying variability in the long-term cardiac structural and/or functional consequences.

Individuals born preterm are also at increased risk of developing arterial and pulmonary hypertension later in life, with reports of high systolic blood pressure in childhood [6, 11-13], adolescence [10, 14] and in early [9, 15-19] and mid-adulthood [20, 21]. Decreasing gestational age at birth is associated with higher systolic blood pressure and greater systolic dysfunction. Both hypertension and ventricular hypertrophy (particularly in the LV) are major risk factors for cardiovascular disease. It is likely that the antecedents to hypertension and cardiovascular disease in people born preterm arise in early postnatal life, due to maladaptive compensatory remodelling of the immature heart and blood vessels in response to premature exposure to postnatal haemodynamics. These structural changes are likely to facilitate survival in the short-term but may ultimately increase the vulnerability to long-term cardiovascular disease [22-24].

In support of this idea, a study conducted in a sheep model of preterm birth (where the gestational timing of cardiomyocyte maturation closely resembles that in humans [25-28]) showed increases in interstitial fibrosis and abnormalities in cardiomyocyte growth (increase in cardiomyocyte volume, nuclearity and ploidy) in the hearts of lambs that were born moderately preterm (at 135 days' gestation) without requiring assisted postnatal ventilation, examined at 9 weeks post term-equivalent age (TEA) [29]. It is expected that this maladaptive structural remodelling of the preterm myocardium would be further exacerbated with decreasing gestational age at birth; this is addressed in the current study.

In contrast, no differences in myocardial fibrosis or in cardiomyocyte size, nuclearity or ploidy were detected in an autopsy study of preterm neonates that died at an early time point between 1 and 42 days after birth [30]. Of concern, however, there was a marked decrease in cardiomyocyte proliferation in the preterm neonates when compared to age-matched stillborn infants. This has the potential to adversely

impact lifelong cardiomyocyte functional reserve. Given the low proliferative capacity of cardiomyocytes postnatally [25], it is conceivable that abnormal cardiomyocyte and fibrotic growth (as observed in the former-preterm sheep study) may subsequently manifest in the human heart in early childhood.

Over the past three decades there have been major advances in the clinical approaches aimed at increasing the survival rates of preterm neonates, particularly those born very and extremely preterm. The use of exogenous surfactant, gentler forms of assisted ventilation and antenatal glucocorticoids in preterm neonates have markedly improved respiratory capacity, organ oxygenation and lung development, respectively [31]. However, these treatments are administered during a particularly vulnerable time when organ maturation is still ongoing, which may inadvertently affect the development of other vital organs, including the heart [32, 33]. Hence, as more clinically-treated preterm individuals are now entering adulthood, it is imperative to determine the impact of preterm birth (including severity of preterm birth) and associated neonatal clinical care on the cellular structure and growth of cardiomyocytes in the immature heart in early life. This will allow for focussed therapeutic strategies to be developed to mitigate the abnormal postnatal cardiac development following preterm birth and subsequently reduce the risk of cardiovascular disease.

The aim of this preclinical study was to assess, at 2 and 5 months of age (equivalent to approximately 2- and 6-years-of age in humans, respectively), the effects of preterm birth on cardiac structure in lambs born prematurely at a timepoint when lung development was at the saccular stage (equivalent to ≈28 weeks' gestation in humans); these lambs required assisted ventilation after birth as well as continuous 24 hour intensive care monitoring. Our sheep model of preterm birth closely reflects the typical management of a

scheduled caesarean delivery for medically indicated preterm birth in humans, including the administration of antenatal steroids, exogenous surfactant and caffeine citrate, and the use of neonatal ventilators [34]. We chose to specifically look at the impact on both cardiac ventricles separately in this study, given their distinct haemodynamic roles. We hypothesised that preterm birth would result in abnormal cardiomyocyte growth and structural remodelling of the ventricular myocardium at 2 months TEA and that this would be further exacerbated at 5 months TEA. We expected that the adverse impacts of preterm birth would be greater than those observed previously in a sheep model of moderately preterm birth, given the increased severity of preterm birth in the present study.

2. Methods

2.1. Ethical approval

The sheep studies adhered to the American Physiology Society and National Institutes of Health guidelines for humane use of animals for research and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah Health Sciences Center.

2.2. Sheep model and preterm delivery

Twenty-eight date-mated Rambouillet x Columbia ewes were randomly assigned to deliver preterm via caesarean section at 128 days' gestation (n = 19) or to deliver spontaneously at term (≈ 150 days' gestation) (n= 16). Healthy singleton and twin lambs were used in this study.

Details of the perinatal procedure have been previously reported [35]. Briefly, the ewes chosen for preterm delivery received intramuscular injections of dexamethasone phosphate (6 mg; Vedco, Inc., St. Joseph, MO,

U.S.A.) 48 hours and again at 24 hours before caesarean delivery. At 128 days' gestation, the ewes received an intramuscular injection of ketamine hydrochloride (10-20 mg/kg; Vedco, Inc., St. Joseph, MO, U.S.A.), followed by inhalation anaesthesia with isoflurane (2.5% isoflurane; Piramal Healthcare, Bethlehem, PA, U.S.A.). The fetuses were then exposed via midline hysterotomy and intubated with a cuffed endotracheal tube (3.0 – 4.0 French). Ten ml of lung liquid was aspirated and replaced with calfactant (6 ml, Infasurf; ONY Biotech, Amherst, New York, U.S.A.), a non-pyrogenic pulmonary surfactant used to reduce surface tension within the lungs.

2.3 Lamb ventilation and nutrition

Once the umbilical cord was ligated and cut, the former-preterm lambs were manually resuscitated, using a Neopuff™ T-piece resuscitator (Fisher & Paykel; Auckland, New Zealand). The former-preterm lambs (n = 8 female, n = 8 male) were then weighed and placed on a heated bed.

All former-preterm lambs were treated intravenously, within 30 min of delivery, with a loading dose of caffeine citrate to stimulate ventilator drive (15 mg/kg, given over 90 min; Sagent Pharmaceuticals, Illinois, USA). Parenteral dextrose was infused to maintain plasma glucose between 60 – 90 mg/dl.

The former-preterm lambs were connected to an invasive mechanical ventilator (Babylog® VN500; Dräger, Lübeck, Germany) configured to provide synchronized, intermittent, mandatory ventilation that was pressure controlled with warm and humidified oxygen. Initial ventilator settings were respiratory rate of 60 breaths/min, inspiratory time of 0.32 s, peak inspiratory pressure of 21 cmH_2O, and positive end-expiratory pressure of 8 cmH_2O. Tidal volume was kept at 5-7 mL/Kg. Arterial blood gases were taken every 15 min for the first 90 min of postnatal life. The fraction of inspired oxygen was gradually lowered to attain a target

oxygen saturation of 88-94% by pulse oximetry (model SurgiVet V92001BP/Temp; Smith Medical ASD, Minnesota, USA) to wean the lamb off mechanical ventilation.

The former-preterm lambs were mechanically ventilated for ~6 days, then transitioned onto non-invasive respiratory support, using nasal high-frequency oscillation. Once the former-preterm lambs were extubated, an uncuffed oral/nasal cannula (3.0 French; 13 cm length) was inserted ~5 cm into a nostril. Lidocaine (1% solution; Hospira, Inc., Illinois, USA) was applied within the nostril to minimise pain and discomfort. The former-preterm lambs were placed on noninvasive respiratory support for ~3 days, then had the nasal tube removed. The former-preterm lambs received supplementary blow-by oxygen through a cone until fully stable. Term lambs (n = 13 female, n = 6 male) did not receive any form of respiratory support.

Orogastric feeding of ewe's colostrum was started at ~3h of postnatal life (3 ml) for former-preterm lambs, and the volume was gradually increased as tolerated, with a target over the first week of postnatal life of ~60 $kcal \cdot kg^{-1} \cdot day^{-1}$. Term lambs stayed with their ewe for ~24 h to let them take colostrum. Then, lambs born preterm (n = 16) and at term (n = 19) were bottle-fed milk (Sav A Lam milk replacer; Sav A Calf®, Wisconsin, U.S.A.), and were given alfalfa pellets and water *ad libitum*.

2.3. Organ collection

At 2 months TEA (2 months following birth of term lambs, and 2 months following the day that term-equivalent age was reached for the former-preterm lambs), 8 former-preterm lambs (female n = 4, male n = 4) and 10 term lambs (female n = 7, male n = 3) were randomly assigned for euthanasia. At 5 months TEA, the remaining 8 former-preterm (female n = 4, male n = 4) and 9 term (female n = 6, male n = 3) lambs

were also euthanised. At the end of each 2- or 5-month study period, the lambs were weighed then received an intramuscular injection of ketamine (10-20 mg/kg) followed by inhalation anaesthesia with 1.0-2.5% isoflurane with oxygen. Lambs were intubated and ventilated with a tidal volume of 5-7 ml/kg and given heparin (1,000 U iv). The lambs were then administered Beuthanasia solution (0.25 ml/kg; Intervet, New Jersey, USA) followed by potassium chloride (10 mEq; Hospira, Illinois, USA) intravenously.

At necropsy, the chest cavity was opened and the heart was excised at the base of the great vessels, then weighed. The excised heart was immersed in cold saline solution containing 20 mEq potassium chloride. The coronary vessels were perfused via their respective coronary ostia with cold phosphate buffered saline (PBS) mixed with 20 mEq of potassium chloride, then with 10% buffered formalin. The heart was stored in 10% buffered formalin at 4°C.

2.4. Heart sampling

The left ventricle and septum (LV + S) were sampled together, separate from the right ventricle (RV). The smooth fractionator approach, as previously described in detail [36], was used to select 9 pieces of LV tissue (including the interventricular septum) and 9 pieces of RV tissue per lamb for glycolmethacrylate embedding; a further 8 pieces per ventricle per lamb were selected for paraffin embedding.

The researcher performing the following image analyses was blinded to the group allocations.

2.5. Interstitial collagen

Paraffin-embedded tissue was sectioned at 5 µm, post-fixed in Bouin's fixative and stained with 1% Sirius red in saturated picric acid (picrosirius red). Areas of tissue were randomly sampled (8 fields of view per 8

pieces of tissue per ventricle per lamb), and the percentage of interstitial collagen within the myocardium was assessed using Image-Pro Plus (Version 6.2 for Windows, Media Cybernetics, Bethesda, USA). Blood vessels and perivascular collagen were excluded from the analysis.

2.6. Cardiomyocyte proliferation

Paraffin-embedded tissue sections at 5 µm were immersed in a Tris-EDTA buffer (10mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) and microwaved for antigen retrieval. Endogenous peroxidase staining was prevented with a wash of hydrogen peroxide and non-specific background staining was prevented using CAS block (Invitrogen, USA). The sections were incubated overnight with a primary Ki-67 antibody (1:100; M724001-2, MIB-1 Clone, Dako, USA), which detects the nuclei of proliferating cells, and subsequently incubated with an HRP-conjugated goat anti-mouse secondary antibody (1:200; Invitrogen). A Dako EnVision + Dual Link HRP/DAB+ immunohistochemistry kit (Dako, USA) was used to visualise the proliferating nuclei. The sections were then counterstained with haematoxylin. Paraffin-embedded tissue from a fetal sheep heart at 100 days' gestation was used as a positive control, and sections excluding the primary antibody were used as negative controls.

The immuno-labelled cardiac tissue sections were randomly sampled (8 fields of view per 8 pieces of tissue per ventricle per lamb) using Image-Pro Plus software, and the average percentage of Ki-67 positive cardiomyocytes per field of view was quantified for each ventricle.

2.7. Cardiomyocyte apoptosis

A TUNEL (terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling) assay (S7110, ApopTag® Fluorescein In Situ Apoptosis Detection Kit; Millipore, Billerica, USA) was used to

determine the proportion of apoptotic cardiomyocytes within the left and right ventricles. Briefly, paraffin-embedded tissue, sectioned at 5 µm, was treated with proteinase K (21627; IHC Select®, Merck, Germany), incubated with a terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37°C and then subsequently incubated with FITC-labelled anti-digoxigenin. Sections were counterstained with DAPI (4,6-diamidino-2-phenylindole hydrochloride; Invitrogen) to stain cell nuclei. Sections were mounted using ProLong Gold (Invitrogen, USA). Rat breast tumour sections were used as a positive control.

2.8. Cardiomyocyte number

Cardiomyocyte number within the RV and LV+S was stereologically estimated using an optical disector-fractionator approach [37, 38]. To do this, the glycolmethacrylate-embedded tissue blocks were serially sectioned at 20 µm thickness with every 20th section collected onto glass slides. Sections were stained with Harris's Haematoxylin (Amber Scientific, Queensland, Australia) in a 1000 W microwave oven at 50% power. The sections were then viewed with a light microscope (Olympus BX4, Japan) fitted with a 100x objective oil immersion lens, a motorised stage, and a z-axis sensor. Using C.A.S.T software (Computer Aided Stereological Toolbox; Olympus, Denmark), the tissue sections were systematically sampled, using a fixed x- and y-axis step length of 1500 µm, beginning at a random starting point. An unbiased counting frame (544.5 μm^2) was superimposed over each field of view and the number of cardiomyocyte nuclei within the middle 10 µm of each section was counted. The total number of cardiomyocyte nuclei counted per ventricle, using the optical disector approach, was then multiplied by the inverse of all tissue sampling fractions to estimate the total number of cardiomyocyte nuclei in the LV+S and RV. The total number of cardiomyocytes

within each ventricle was subsequently calculated by halving the total number of cardiomyocyte nuclei to adjust for binucleation.

2.9. Cardiomyocyte cross-sectional area

Cardiomyocyte cross-sectional area was quantified using confocal microscopy. Paraffin-embedded sections of the LV+S and RV (8 per ventricle) were cut at 40 µm thickness. The sections were incubated with DAPI (1:5000; Invitrogen, USA) and Wheat Germ Agglutinin-Alexa Fluor 488 (1:20; W11261, Invitrogen, USA) in phosphate buffered saline overnight and subsequently mounted using ProLong Gold (Invitrogen, USA). Image acquisition, in random fields of view, was conducted using a Nikon C1 confocal microscope equipped with a 40x objective oil immersion lens (Nikon, Japan). The cross-sectional area of 250 randomly selected cardiomyocytes was measured, using Image J software (v6.2, National Institutes of Health; Maryland, USA). Only cardiomyocytes with a nucleus in the plane of view and centrally located within the cell were measured. The average cardiomyocyte cross-sectional area per LV+S and RV was then determined.

2.10. Statistical analysis

Statistical analyses were conducted using GraphPad Prism version 7.01 for Windows (GraphPad Software, La Jolla California, USA). Birth weights of the preterm and term lambs were analysed, using a two-way ANOVA, with the factors preterm birth (P), sex (S), and their interaction (P*S). Final body weight and heart weights were analysed using a three-way ANOVA, with the factors preterm birth (P), sex (S), postnatal age (A), and their associated interactions. Differences between groups (preterm and term) and postnatal ages (2 and 5 months TEA) for all remaining data, where no significant sex effects were apparent, were analysed using a two-way ANOVA, with the factors preterm birth (P), postnatal age (A), and their interaction (P*A).

All data are presented as the mean ± standard error of the mean. P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Body and heart weights

Birth weight was significantly lower in the former-preterm lambs compared to the term lambs (preterm: 3.20 ± 0.19 kg, term: 4.74 ± 0.39 kg; p = 0.003). Sex did not have a significant effect on birth weight (p = 0.90), with no significant interaction effect between preterm birth and sex (p = 0.54). The average birth weight of female former-preterm lambs was 3.08 ± 0.18, compared to 4.85 ± 0.47 in female term lambs. The average birth weight of male former-preterm lambs was 3.31 ± 0.35, compared to 4.50 ± 0.75 in male term lambs.

Final body weight was significantly lower in former-preterm lambs compared to term lambs across the two time points, and the female lambs were lighter than male lambs overall (Table 1). As expected, final body weight in lambs at 5 months TEA was significantly greater compared to lambs at 2 months TEA (Table 1). There were no significant interaction effects between preterm birth and postnatal age (p_{P*A} = 0.30) or sex (p_{P*S} = 0.89) on body weight.

Absolute heart weight and relative heart weight (heart weight to body weight ratio) did not differ between preterm and term lambs (Table 1). Female lambs had significantly lighter absolute heart weight compared to male lambs overall, but no sex differences were detected in relative heart weight. Across all lambs, absolute heart weight significantly increased with increasing postnatal age, whereas relative heart weight

significantly decreased. There were no significant interaction effects associated with absolute (p_{P*A} = 0.94; P_{P*S} = 0.84) or relative (p_{P*A} = 0.72; P_{P*S} = 0.48) heart weights.

Table 1. Body and heart weights of lambs born at term or preterm at 2 and 5 months term equivalent age.

	Term				Former-preterm				p-values		
	2 months		5 months		2 months		5 months		p_P	p_S	p_A
Sex	F	M	F	M	F	M	F	M			
N	7	3	6	3	4	4	4	4			
Body weight (kg)	23.3±1.9	29.5±4.8	48.7±3.8	56.4±6.2	19.1±1.4	23.5±2.4	37.2±5.6	45.2±5.9	0.01	0.04	<0.0001
Absolute heart weight (g)	105.56±9.93	142.63±14.63	211.42±13.27	254.70±27.87	95.71±6.47	121.27±18.61	196.41±12.41	241.73±12.12	0.20	0.0031	<0.0001
Heart weight to Body weight ratio (g/kg)	4.93±0.42	4.93±0.39	4.42±0.32	4.53±0.21	5.02±0.16	5.33±0.29	4.26±0.12	4.80±0.34	0.56	0.35	0.043

Data are shown as mean ± standard error of the mean. Data analysed by three-way ANOVA, with factors preterm birth (P), sex (S), and postnatal age (A).

3.2. Cardiomyocyte proliferation and apoptosis

Levels of Ki-67 positive nuclei were extremely low (< 0.001%) in the LV and RV of both former-preterm and term lambs at 2- and 5-months TEA. Similarly, TUNEL assays revealed negligible levels of apoptotic cardiomyocytes in the LV+ S and RV of both former-preterm and term lambs at 2- and 5-months TEA (data not shown).

3.3. Cardiomyocyte Growth

Total cardiomyocyte number in the LV+S and RV did not differ between former-preterm and term lambs, nor between lambs at 2- and 5-months TEA (Figure 1). Overall, the cross-sectional area of cardiomyocytes in the LV+S and RV was significantly greater in lambs at 5 months TEA compared to lambs at 2 months TEA (Figure 2). There were no significant differences in cardiomyocyte cross-sectional area between lambs born preterm compared to lambs born at term in either ventricle (Figure 2).

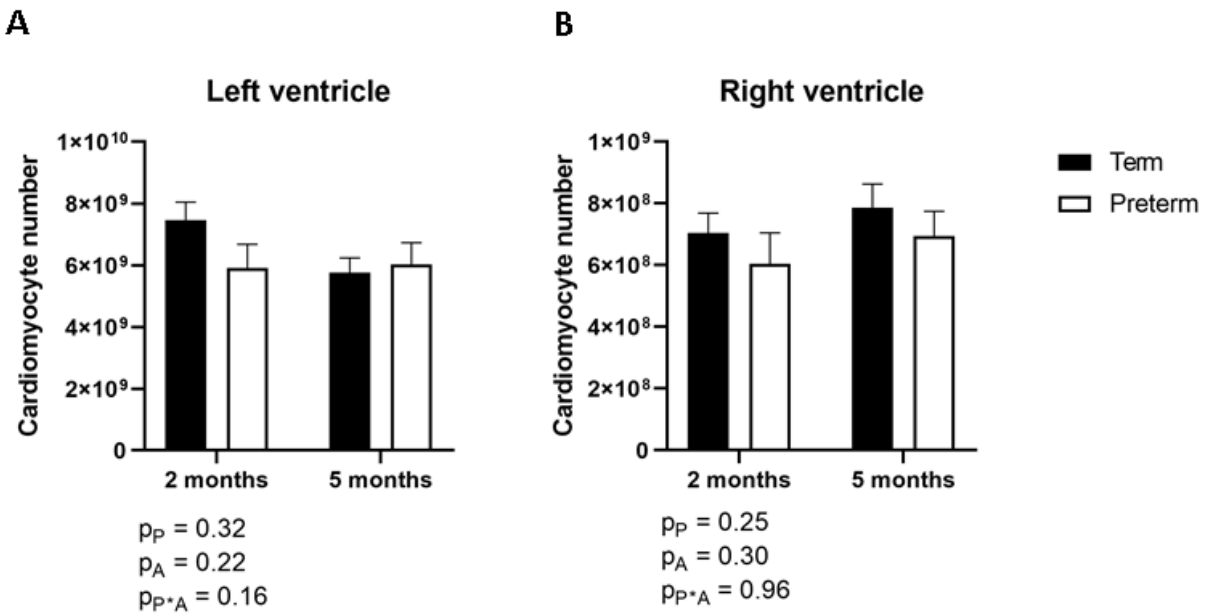

Figure 1. The total number of cardiomyocytes in the left ventricle plus septum (A) and right ventricle (B) of lambs born at term (150 days' gestation; black) or preterm (128 days' gestation; white) at 2 and 5 months term equivalent age. Analysis by two-way ANOVA, with the factors preterm birth ($_P$), postnatal age ($_A$), and their interaction ($_{P*A}$). Data are shown as mean ± standard error of the mean.

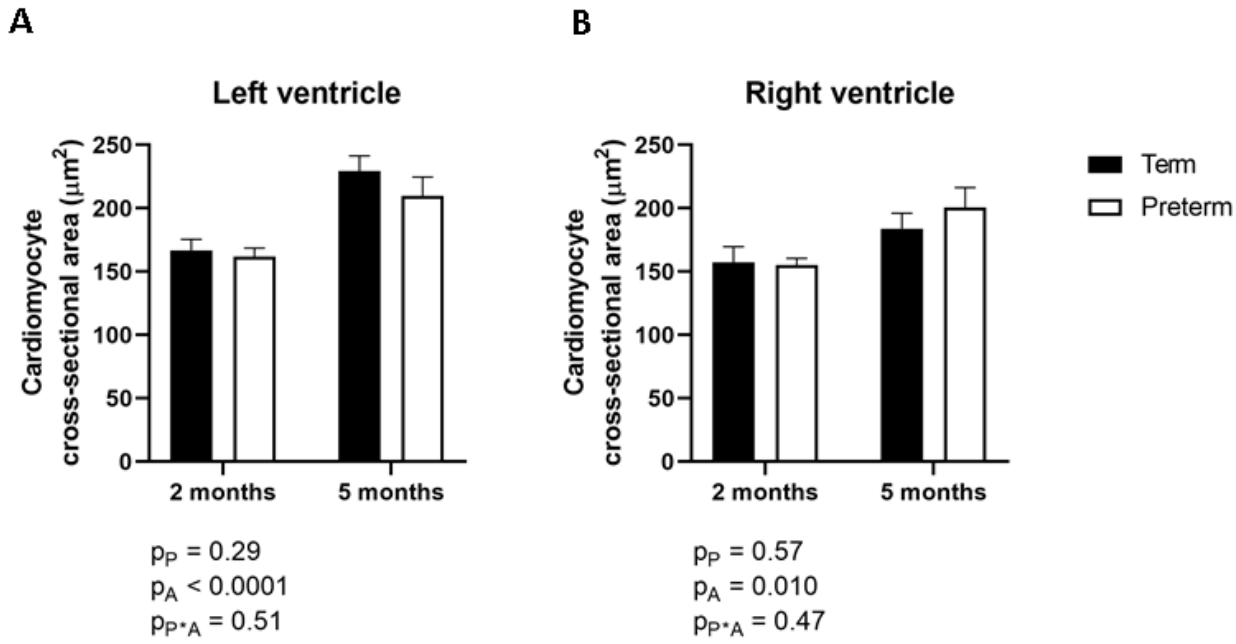

Figure 2. Cross-sectional area of cardiomyocytes in the left ventricle plus septum (A) and right ventricle (B) of lambs born at term (150 days' gestation; black) or preterm (128 days' gestation; white) at 2 or 5 months term equivalent age**.** Analysis by two-way ANOVA, with the factors preterm birth ($_P$), postnatal age ($_A$), and their interaction($_{P*A}$). Data are shown as mean ± standard error of the mean.

3.4. Myocardial interstitial collagen

The percentage of interstitial collagen significantly increased in the LV+S of lambs between 2 and 5 months postnatal age and was exacerbated by preterm birth (2 months: term = 0.57 ± 0.07 %, former-preterm = 1.44 ± 0.18 %; 5 months: term = 1.37 ± 0.25 %, former-preterm = 2.15 ± 0.31 %) (Figure 3A). Representative images of LV+S collagen staining are shown in Figure 3C-F. In the RV, however, interstitial collagen levels

increased with age but were not significantly affected by preterm birth (2 months: term = 2.99 ± 0.27 %, former-preterm = 3.061 ± 0.64 %; 5 months: term = 3.88 ± 0.31 %, former-preterm = 4.18 ± 0.31 %) (Figure 3B).

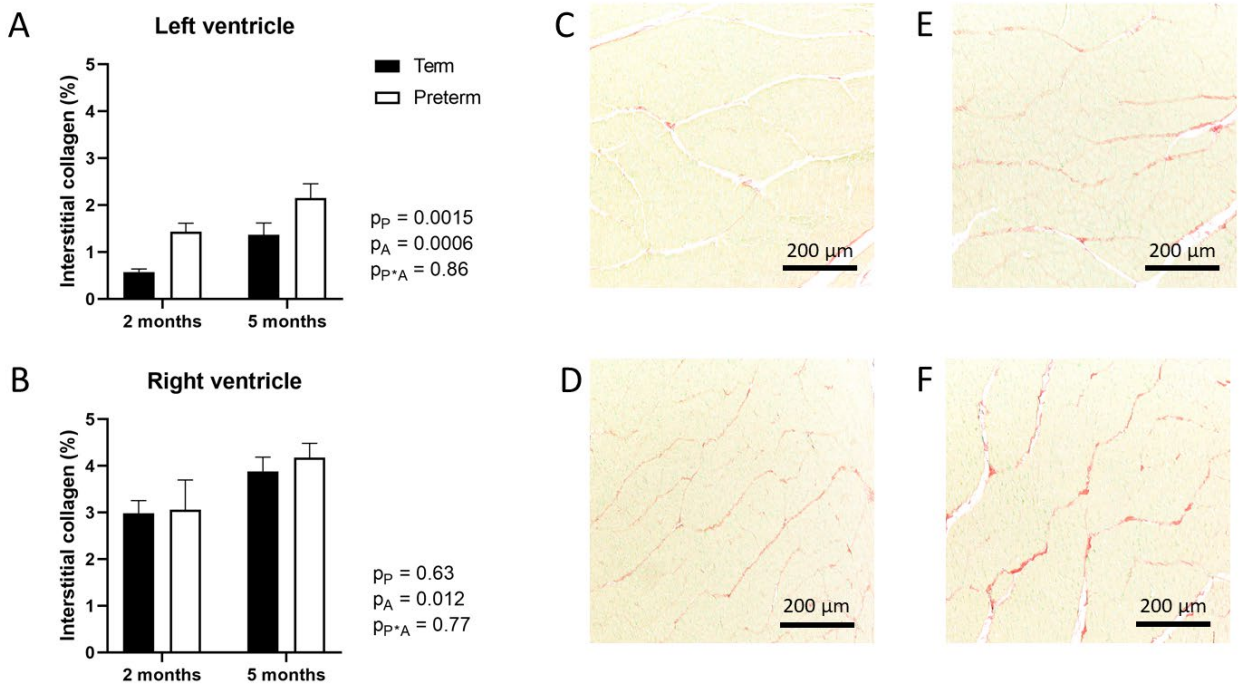

Figure 3. The percentage of interstitial collagen in the left ventricle plus septum (A) and right ventricle (B) of lambs born at term (150 days' gestation) or preterm (128 days' gestation) at 2 or 5 months term equivalent age. Analysis by two-way ANOVA, with the factors preterm birth ($_P$), postnatal age ($_A$), and their interaction($_{P*A}$). Data are shown as mean ± standard error of the mean. Representative photomicrographs of left ventricular myocardium stained with picrosirius red (collagen shown in red, and myocardium in

yellow) from term-born lambs at 2 (C) and 5 (E) months, and former-preterm lambs at 2 (D) and 5 (F) months term equivalent age. Scale bars = 200 µm.

4. Discussion

Although the lambs in the present study were delivered considerably earlier than in previous studies of moderate preterm birth in unventilated lambs [29], we found a less severe adverse cardiac phenotype. We showed that preterm birth (and its associated clinical management), at the saccular stage of lung development, does not affect cardiomyocyte growth at 2- and 5-months TEA; of concern, however, there was greater myocardial collagen deposition within the extracellular compartment of the LV+S. This is likely a consequence of the premature haemodynamic transition occurring at the time of preterm birth, with a compensatory increase in collagen content providing the underdeveloped LV+S myocardium with structural stability. If this accelerated collagen deposition continues into adolescence and/or adulthood, the LV+S myocardium could become susceptible to cardiac fibrosis – an irreversible pathway that may render the LV with functional impairment later in life.

4.1. Preterm birth reduced body weight but did not affect heart growth

In this study, former-preterm lambs were lighter than term lambs at birth and remained lighter across 2- and 5-months TEA, findings consistent with those reported in children born preterm [39, 40]. Despite the lower body weights of the former-preterm lambs, we found that preterm birth combined with postnatal ventilation had no apparent effect on heart growth in lambs over the study period, a finding also seen in previous studies of moderately preterm and term born lambs assessed at 9 weeks TEA [29]. Similarly, an

echocardiographic study showed that although body mass index and body surface area were reduced in 6 year old children born extremely preterm compared to age-matched term controls, LV mass was unaffected after adjusting for body surface area [40]. However, LV length, width, and aortic valve annulus diameter were 3-5% smaller in the preterm group; this was associated with impaired LV systolic function [40]. This suggests that measurements of LV geometry, rather than LV mass alone, are important anatomical indicators of cardiac function.

4.2. Cardiomyocyte growth was not impacted by preterm birth

It is now well established that early life adverse events can negatively impact the growth of cardiomyocytes [41, 42]. To date, there are few studies that have explored the effects of preterm birth (either early or later in life) on the number and size of cardiomyocytes. Contrary to our initial hypothesis, we found no apparent differences in cardiomyocyte size (as assessed by measurements of cross-sectional area) between former-preterm and term born lambs, at 2 or 5 months TEA. These findings were partly unexpected, given that previous studies in moderately preterm born lambs at a similar postnatal timepoint found a marked increase in the size of mononucleated and binucleated cardiomyocytes in both the right and left ventricles. Although it was proposed that the increased severity of preterm birth in the present sheep study (birth at 128 days of gestation) would have a greater detrimental impact on cardiomyocyte growth than previously reported in former-preterm lambs (born moderately preterm at 133 days' gestation), this was not the case. In this regard, the postnatal clinical course of infants following preterm birth likely has an important influence on cardiomyocyte growth. The lambs examined in previous studies were born moderately preterm and did not require early postnatal respiratory support with oxygen-rich gas [43] or neonatal

intensive care and monitoring. It may be the case that the postnatal clinical interventions (such as mechanical ventilation with oxygen-rich gas, caffeine citrate, and exogenous surfactant therapy) used in the present study to maintain haemodynamic stability of the former-preterm lambs may have had a protective effect on cardiomyocyte growth. Studies exploring the long-term effects of each of these clinical interventions are required to determine whether this is the case. In support of the current findings, no change in cardiomyocyte volume has previously been reported in human preterm neonates requiring mechanical ventilation after birth, compared to age-matched stillborn infants during gestation [30]. However, no clinical studies to date have examined the effects of preterm birth on cardiomyocyte size in childhood or later in life.

The total number of cardiomyocytes within the LV+S and RV directly impacts the functional capabilities and myocardial reserve of the respective ventricles. In human infants (and in sheep), there is a strong inverse correlation between gestational age and cardiomyocyte proliferation rate [34, 44, 45]. At the time of preterm delivery, cardiomyocyte proliferation in lambs is still ongoing, but then decreases markedly around full-term age [34]. Our study suggests that preterm birth combined with early postnatal mechanical ventilation in lambs does not adversely affect the trajectory of cardiomyocyte proliferation at an age equivalence of childhood, with no difference observed between groups in cardiomyocyte number or cell proliferation rate (almost negligible at this time point). Bensley *et al.* have also shown that cardiomyocyte number within the LV+S and RV did not differ between lambs born preterm or at term, when examined at 9 weeks TEA [1]. In contrast to these findings, a study in adult sheep born preterm (that received a higher clinical dosage of antenatal steroids and were not mechanically ventilated) showed a significant decrease in cardiomyocyte number within the RV compared to term-born sheep [43]; the left ventricle was not

examined in that study. In addition, an autopsy study in deceased preterm neonates (at 1-42 days postnatal age) found that preterm birth greatly reduced the rate of cardiac cell proliferation [30]. Differences in gestational age, postnatal age, and clinical care between studies likely accounts for the inconsistent findings. Given the importance of cardiomyocyte number on lifetime cardiovascular health, further controlled studies to examine the isolated effects of each of these factors on cardiomyocyte proliferation and growth are certainly required.

4.3. Increased myocardial collagen deposition following preterm birth

Excessive myocardial collagen is associated with impaired conductivity and contractility of the ventricles and is a hallmark feature of many cardiac diseases such as heart failure and cardiac hypertrophy [46]. A major finding of this study is that myocardial interstitial collagen deposition within the LV+S was exacerbated by preterm birth (with early postnatal assisted ventilation) in lambs assessed at the human-equivalent ages of 2 and 6 years; increased LV+S collagen deposition is a finding consistent with other preterm lamb studies [29]. Preterm birth, however, did not increase interstitial collagen content in the RV, in contrast to the previous report in lambs born moderately preterm and examined at a similar postnatal age [43].

In the case of the preterm heart, the premature increase in systemic arterial blood pressure that necessarily occurs (either spontaneously or through clinical intervention) likely leads to the observed increases in collagen content within the LV as a structural adaptation to maintain LV structural stability. Over time, this may render individuals born preterm susceptible to impaired cardiac function. Further studies in this animal model at later time points would help to elucidate this. Indeed, in support of this idea, young adults born

preterm have been shown to have impaired LV systolic function [9] and an impaired LV response to physiological stress when subjected to physical exercise [47] compared to adults born at term; however, myocardial collagen content has not yet been assessed in adults born preterm to confirm this relationship.

4.4. Conclusion

As more survivors of preterm birth are now entering adulthood, the long-term detrimental health impacts continue to emerge. Adverse myocardial remodelling, and thus an increased susceptibility to the development of cardiac disease, is a biological phenomenon recently revealed in individuals born preterm and is becoming an increasingly important issue in public health. This study is the first to analyse the impact of preterm birth, at a time when the lungs were still very immature (and thus requiring neonatal interventions including respiratory ventilation), on cardiomyocyte growth and myocardial structure in juvenile life. Whilst cardiomyocyte growth was not affected, an increase in myocardial interstitial collagen was found in the left ventricle of lambs born preterm with assisted ventilation. These findings add to the growing body of evidence that preterm birth results in early maladaptive structural remodelling of the heart, likely contributing to the functional deficits of the preterm heart in adulthood. Unexpectedly, the adverse impact of preterm birth on the ventricular myocardium was less than that previously reported in a sheep model of preterm birth where the lambs were delivered moderately preterm and did not require respiratory support after birth. The reasons for this are currently unknown and this is an important area for future research. Since the relationship between the severity of preterm birth and later cardiac remodelling could not be confirmed, there is the possibility that antenatal and postnatal factors associated with preterm birth are involved.

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Conflicts of Interest

None to disclose.

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Chapter 4

Biochemical alterations in the left ventricular myocardium following preterm birth and assisted ventilation in lambs: a Fourier-transform infrared micro-spectroscopic study

Biochemical alterations in the left ventricular myocardium following preterm birth and assisted ventilation in lambs: a Fourier-transform infrared micro-spectroscopic study

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Abstract

Background: Preterm infants are born when their hearts are structurally and functionally immature; therefore, maladaptive cardiac remodelling can occur in the neonatal period that may lead to later cardiac dysfunction. We hypothesised that preterm birth would be associated with biochemical changes within the left ventricular (LV) myocardium later in life.

Methods: Lambs delivered preterm at 128 days' gestation and mechanically ventilated after birth were compared to unventilated lambs born at term (150 days' gestation), at 2 and 5 months term-equivalent age (TEA). The LV was analysed using Fourier-transform infrared micro-spectroscopy combined with principal component analysis across the fingerprint region (1800-1000 cm^{-1}).

Results: Overall, the LV of lambs born preterm showed an increase in collagen and the secondary structure of proteins was significantly shifted towards a β-sheet confirmation. In contrast, the LV of term-born lambs showed an increase in proteins with an α-helical structure. At 5 months TEA, the LV of former-preterm lambs also exhibited an increase in triglyceride and phospholipid content when compared to term-born lambs. In the LV of term-born lambs, proteins with amide II α-helix structure and collagen increased with postnatal age. Contrastingly, the protein profile of the LV in lambs born preterm did not change to the same extent between 2 and 5 months TEA.

Conclusions: Our analysis provides evidence of altered biochemical composition of the LV myocardium following preterm birth.

General significance: These changes in the biochemical composition of the myocardium provide insight into the potential underlying mechanisms of preterm birth-associated cardiovascular disease later in life.

Key words:

Preterm birth, Fourier transform infrared spectroscopy, mechanical ventilation, heart development, myocardial remodelling, protein structure

Abbreviations:

Left ventricle (LV), term-equivalent age (TEA), Fourier-transform infrared (FTIR), principal component analysis (PCA), primary principal component (PC1), secondary principal component (PC2).

1. Introduction

Major advances in neonatal care practices since the early 1990s have greatly improved the survival rates of neonates born preterm (prior to 37 weeks of gestation) [1]. Many of these clinically-treated individuals born preterm are now entering adulthood, thereby exposing the long-term health impacts of both preterm birth and the neonatal treatments that helped this cohort survive after birth. Recent epidemiological studies have shown that adults born preterm are more susceptible to developing heart failure, as well as hypertension (a major risk factor for cardiovascular disease) [2-6]. In addition, young adults born preterm often exhibit impaired left ventricular (LV) function and altered LV morphology [7]. Sheep studies also report increased myocardial collagen deposition and altered cardiomyocyte growth in the LV following preterm birth in early life.

Gross morphological changes in organ structure are preceded, or accompanied by, cellular and/or biochemical changes within tissues, particularly in disease contexts [8]. Importantly, in this regard, Fourier-transform infrared (FTIR) spectroscopy is an emerging technology which can be used to gain an understanding of the biochemical composition of organs and tissues. It is a label-free analytical tool that provides biochemical fingerprints of cells and tissues in a fast and reliable way and can be used to gain an understanding of the underlying causes of structural alterations within organs and tissues under pathological conditions. FTIR spectroscopy exploits the fact that when a molecule is excited with mid-infrared light (4000-400 cm^{-1}), every chemical bond within that molecule absorbs a unique amount of infrared energy, shifting the bond from its ground state to an excited vibrational state [8]. By measuring the absorption of infrared light in biological samples (fresh, dried, or formalin fixed, with minimal preparation required), the characteristic wavenumber values and intensities of absorbance bands of macromolecules, including carbohydrates, lipids, proteoglycans, collagens, nucleic acids, and proteins, are detected at high spatial resolution [10]. This is beneficial for studies of diseases or conditions that result in uncharacterised and/or widespread changes in the biochemical profile of tissues, rather than genetic disorders that affect a particular subset of known proteins.

While infrared micro-spectroscopy is not a technique that enables the identification of individual macromolecules or their subunits, it is a method that can detect overall phenotypic changes in the spectral 'fingerprint', or biochemical profile, of tissues in the presence of a disease, environmental stress or genetic modifications. Characterising this biochemical difference can provide the first steps in understanding a biological or pathological process. FTIR spectroscopy has previously been used to reveal biochemical changes within the myocardium in various disease contexts, such as myocardial infarction [12-15], cardiomyopathy [16-19], chronic kidney disease [11], diabetes [20], and intrauterine growth restriction [21].

This study used FTIR spectroscopy to examine the effect of preterm birth on the biochemical composition of the LV myocardium early in life in a clinically-relevant sheep model. In this study, we chose to focus on the LV given it is the dominant pumping chamber of the heart in postnatal life. Furthermore, we have previously shown evidence of alterations in the extracellular matrix of the LV myocardium in former-preterm lambs at 2 and 5 months TEA (unpublished). Our preclinical sheep model offers unique opportunities to replicate contemporary neonatal intensive care in preterm lambs born at the saccular stage of lung development (equivalent to approximately 28 weeks' gestation in humans), and requiring administration of antenatal glucocorticoids, exogenous surfactant treatment, and ventilatory support in the same manner as human neonates born very or extremely preterm [22]. The specific aim of this study was to determine whether protein, lipid, nucleic acid and carbohydrate content is altered in the LV myocardium in sheep that were born preterm and examined at 2- and 5-months of age (equivalent to early childhood, ~2 and 6 years of age, in humans).

2. Methods

2.1. Ethical approval

The lamb studies adhered to the American Physiology Society and National Institutes of Health guidelines for humane use of animals for research and were prospectively approved by the Institutional Animal Care and Use Committee at the University of Utah Health Sciences Center.

2.2. Animal model and preterm delivery

Date-mated Rambouillet x Columbia ewes were randomly assigned for caesarean-section delivery of preterm lambs at ~128 days' gestation, or for spontaneous term delivery (~150 days' gestation). Details of the perinatal procedures have been previously reported in detail by our group [29]. Briefly, the ewes chosen for preterm delivery received an intramuscular injection of dexamethasone phosphate (6 mg; Vedco, Inc., St. Joseph, MO, USA) 48 and 24 hours before operative delivery. At ~128 days' gestation, the preterm fetuses were exposed via midline hysterotomy and administered calfactant (6 ml, Infasurf; generously provided by ONY Biotech, Inc, Amherst, NY, USA). Once the umbilical cord was milked, clamped, and cut, the preterm lambs were resuscitated with a t-piece resuscitator (NeoPuff; Fisher & Paykel, Auckland, New Zealand). Lambs were weighed, then mechanically ventilated (Dräger ventilator; model VN500, Lubeck, Germany). All preterm lambs were treated with a loading dose of caffeine citrate (15 mg/Kg, given over 90 min, Sagent Pharmaceuticals, Schaumburg, IL; iv) within 30 minutes of delivery, followed by maintenance treatment (5 mg/Kg) every 24 hours for 6 days. Preterm lambs were transitioned to non-invasive respiratory support 72 hours after birth for 3 days. Term lambs did not receive any form of respiratory support.

At 2 months after term-equivalent age (TEA), 8 former-preterm lambs female n=4, male n=3) and 10 lambs born at term (female = 4, male n=4) were weighed, then euthanised via administration of Beuthanasia (0.25mL/Kg, Intervet Inc., Madison, NJ) followed by potassium chloride (10 mEq; Hospira, Illinois, USA) intravenously. At 5 months after TEA, the remaining former-preterm lambs (female n=4, male n=4) and lambs born at term (female n=6, male n=2) were also weighed, then euthanised via

administration of Beuthanasia. The full terminal tissue collection procedure is reported by our group [29]. At necropsy, the hearts were excised, arrested in diastole via coronary perfusion with potassium chloride (10 mEq; Hospira, Illinois, USA), then immersion fixed in 10% buffered formalin at 4°C {Dahl, 2018 #159}.

2.3. Heart sampling and processing

The smooth fractionator approach, as previously described in detail [24], was used to select 3 representative samples of the LV free wall and adjoining interventricular septum. The LV tissue samples were initially washed in 30% sucrose (Sigma-Aldrich; St Louis, USA) in phosphate buffered saline overnight and then embedded in O.C.T (Tissue-Tek, Sakura Finetek; California, USA) and snap frozen.

The tissue was cryo-sectioned at a thickness of 5 µm and mounted onto low-e reflective-coated infrared microscope slides (MirrIR slides, Kevley Technologies; Ohio, USA) coated with poly-l-lysine (Sigma P-8920; St Louis, USA), then gently washed with distilled water overnight.

2.4. FTIR spectroscopy image acquisition

Spectra were acquired with an Agilent Cary 620 interferometer and infrared microscope (Agilent Technologies; California, USA) equipped with a liquid nitrogen cooled 128 x 128 pixel FPA and 4x calcium fluoride lens. FTIR spectral data images were recorded in the range 3600 – 1000 cm^{-1} at a spectral resolution of 8 cm^{-1}, a spatial resolution of 20.6 µm per pixel, and an integration sampling interval of 4. Sixty-four scans per sample were collected; background scans were also acquired directly prior to measurement from a clean region on the microscope slide. One hyperspectral image was taken for each piece of tissue, resulting in three images (over 40,000 spectra) per lamb.

2.5. Data processing and analysis

Spectral data were initially pre-selected, using the proprietary OPUS software package (Bruker Optics; USA) to exclude spectra with an amide I band (~1650 cm^{-1}) that had an absorbance lower than 0.4 or

greater than 0.8. The included spectra were further pre-processed using MATLAB 9.1 (2017b, MathWorks; USA) and the PLS_Toolbox package (Eigenvector Research Inc.; USA). Pre-processing included the calculation of second derivatives (Savitzky-Golay, 11 smoothing points), and normalisation via standard normal variate (SNV). Subsequently, the processed spectral data from each lamb were averaged by a factor of 100. The averaged data were SNV-normalised and then mean-centred.

Principal component analysis (PCA) was used in the spectral range from 1800 to 1030 cm^{-1}. This unsupervised method was applied to search for features of interest (spectral bands), eliminating subjectivity from the analysis. PCA converts the original dataset into a new set containing a reduced number of linear variables called principal components (PCs). The primary PC (PC1) accounts for the most variation among the dataset; each succeeding PC is orthogonal to the preceding PC and contains the highest amount of variance within its data matrix [25]. The scores plot calculates PC1 against the secondary principal component (PC2).

The scores plot from each PCA was interpreted in conjunction with the corresponding loadings plot. The loadings plots display the variables (in this case, wavenumber values), which contribute to most of the clustering patterns observed in the principal components of the scores plot. Due to processing the data into second derivatives (where maxima are converted to minima, and *vice versa*), samples that show positive score values in the PC1 scores plot are correlated to negatively loaded bands in the PC1 loadings plot. Likewise, samples showing negative scores in the scores plot are correlated with positively loaded bands in the PC1 loadings plot.

Band assignments from previous spectroscopic studies were used to define and interpret the data acquired from collected spectra in this current study. Generally, the studies included in Table 1 use samples of biochemical species (such as pure collagen extracted from rat skin [26, 27]) to determine the unique spectral profile and thus, the distinct absorption bands that characterise those functional groups. Assignments of vibrational bands discussed in this paper are given in Table 1.

Table 1. Absorption band assignments for FTIR tissue spectra

Band position (cm^{-1})	Assignment
1743-1747	*ν*(C=O) of ester carbonyl groups of phospholipids and triglycerides [28, 29]
1720	C = O stretching vibrations of nucleic acids [15]
1708	ν(C=O) of nucleoside side of nucleic acids [13, 29]
1670-1685	Amide I (anti β-sheet/random coils) [10, 29]
1662	Triple helix structure in collagen [15, 29]
1643-1658	C=O stretching vibration of Amide I (α-helix) [11-13, 21]
1620-1627	Amide I (β-sheet) [30]
1542-1562	Amide II (protein N–H bending, C–N stretching), α-helical structure [10, 20, 29]
1535-1539	Amide II, β-sheet structure [29]
1516	Tyrosine [30, 31]
1465	CH_2 scissoring: lipids [20]
1450	Collagen (CH2/CH3 deformation) [26, 27]
1395-1405	Symmetric CH_3 bending of the methyl groups of proteins [29]
1384	Structural proteins like collagen $\delta(OH) + \delta(CH_3)$ [29]
1312 and 1238	Collagen (triple helical structure) 1238: Stretching PO_2 asymmetric nucleic acid / amide III [10, 29]
1304	Myofibres [13]
1242	$\nu_{as}(PO_2^-)$ stretching of phospholipids, nucleic acids [13]
1160-1171	Tyrosine (collagen type I) [10, 29]
1080	$\nu_s(PO_2^-)$ symmetric stretching: phospholipids, nucleic acids) [20]

3. Results and Discussion

With the improved survival rates of preterm infants over recent decades, the long-term health outcomes are now becoming increasingly apparent. In this regard, survivors of preterm birth are more susceptible to impaired cardiac function and are, therefore, more vulnerable to cardiovascular disease later in life. However, the underlying mechanisms are still unknown. A crucial part of elucidating the developmental trajectory of cardiac disease in surviving adults is understanding the biochemical processes within the myocardium following preterm birth. Our study used FTIR micro-spectroscopy to show that preterm birth combined with perinatal clinical treatments alters the biochemical profile of the LV myocardium in lambs at 2 and 5 months TEA. Specifically, the LV of lambs born preterm had significantly increased collagen deposition and the secondary structure of proteins was shifted towards a β-sheet confirmation. In comparison, the secondary structure of proteins in the LV of term-born lambs exhibited a predominately α-helical structure. Furthermore, we showed at 5 months TEA that the LV of lambs following preterm birth had an increased triglyceride and phospholipid content compared to the LV of term-born lambs. These findings are summarised in Table 2.

We were also able to detect differences in the biochemical profile of the LV myocardium between 2 and 5 months TEA. Proteins with amide II α-helix structure and collagen content in the LV myocardium increased with increasing postnatal age in term lambs. In contrast, the protein profile of the myocardium in lambs born preterm did not change to the same extent between 2 and 5 months TEA, providing evidence of altered postnatal development of the LV myocardium following preterm birth. These findings are summarised in Table 3.

Table 2. Summary of upregulated biochemical components of the left ventricular myocardium in lambs born at term and lambs born preterm.

	Biochemical components increased in lambs born at term (compared to lambs born preterm)	**Biochemical components increased in lambs born preterm** (compared to lambs born at term)
A. **Two months TEA** *(Fig. 2)*	• amide I α-helix • amide II α-helix	• Amide I anti β-sheet • Amide I β-sheet • Collagen • Triglycerides and phospholipids • Tyrosine
B. **Five months TEA** *(Fig. 3)*	• amide I α-helix • amide II α-helix	• amide I anti β-sheet • amide I β-sheet • Collagen • Triglycerides and phospholipids

Principal component analyses detected which chemical species were increased in lambs born at term versus lambs born preterm and assessed at 2 (A) and 5 (B) months term-equivalent age (TEA).

Table 3. Summary of upregulated biochemical components of the left ventricular myocardium in lambs between timepoints 2 and 5 months term-equivalent age (TEA).

	Biochemical components increased at two months TEA (compared to the timepoint at 5 months TEA)	**Biochemical components increased at five months TEA** (compared to the timepoint at 2 months TEA)
A. **Term lambs** *(Fig. 4)*	• Amide I α-helix • Amide II β-sheet	• Amide II α-helix • Collagen
B. **Preterm lambs** *(Fig. 5)*	• Amide I α-helix • Amide I β-sheet • Amide II β-sheet	• Amide I anti β-sheet • Amide II α-helix • Collagen • Triglycerides and phospholipids

Principal component analyses detected which chemical species were increased at 2 months TEA versus 5 months TEA, and vice versa, in lambs born at term (A) and preterm (B).

3.1. Comparison of group-averaged second derivative spectra

Clear differences in the overall spectral profile of term and former-preterm lambs were detected at both 2 and 5 months TEA. Figure 1 shows the group-averaged second derivatives of FTIR spectra for each animal group in the spectral region 1800 – 1000 cm^{-1}. The use of 2^{nd} derivatives enabled us to resolve broad, overlapping bands into individual ones, thus increasing the accuracy of analysis. Most of the differences in the second derivative bands between preterm and term groups were observed in the spectral range 1740 – 1400 cm^{-1}; this range is specific to the secondary structure of proteins. In particular, a much greater intensity is seen in the ~1650 cm^{-1} band (amide I, corresponding to α-helix structure) in the term-born lambs, compared to the lambs born preterm. Within the 1740 – 1400 cm^{-1} spectral range, there also appears to be a small difference in the spectral profile between the 2 and 5 month age groups.

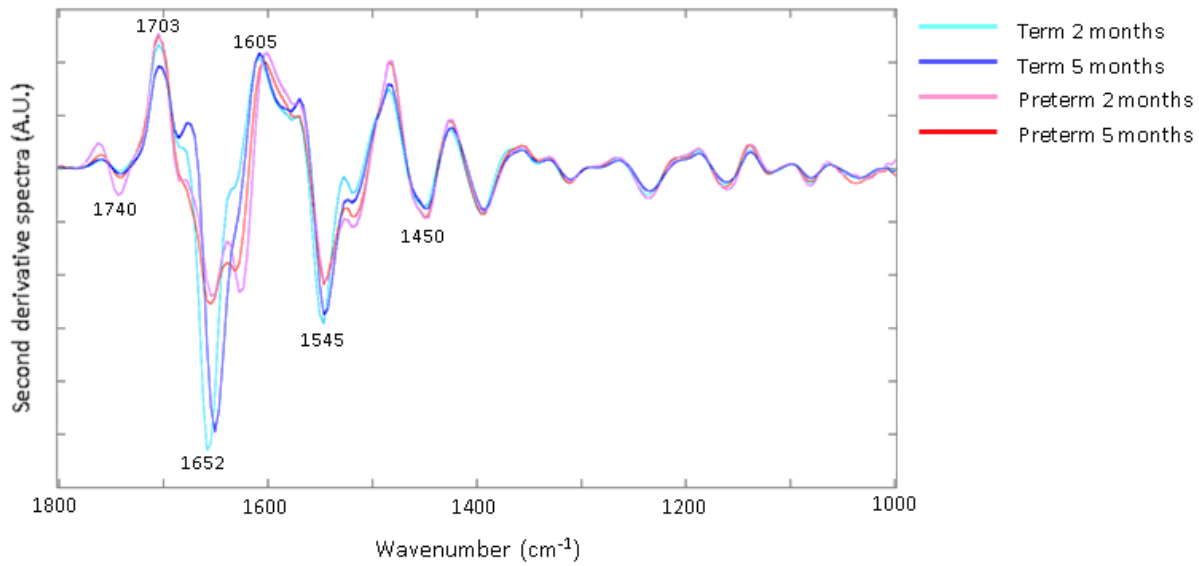

Figure 1. Group-averaged second derivative FTIR spectra of left ventricular myocardium from lambs born at term and lambs born preterm, assessed at timepoints 2 and 5 months term equivalent age.

3.2. Changes in the biochemical profile of the myocardium in lambs born preterm versus term

PCA compared spectral profiles of the LV myocardium between the former-preterm and term lambs at the two time points, 2 months TEA (Fig. 2) and 5 months TEA (Fig. 3).

3.2.1. Spectroscopic analysis at two months TEA

Figure 2A shows the PCA scatter plot of spectra from the term and former-preterm groups at 2 months of age, with clear differentiation observed between groups along PC1. PC1 explained more than 61% of variability and PC2 explained approximately 16% of variability. The 2nd derivatives of the preterm 2 month spectra were negatively loaded along PC1, whereas the 2nd derivatives of term 2 month spectra were positively loaded along PC1 (Fig. 2A).

Positive bands in the loadings plot for PC1 (bands above the dotted line; Fig. 2B) were associated with spectra from former-preterm lambs at 2 months; this means that the absorption levels at these wavenumber values were greater in the preterm 2 month samples than in the term 2 month samples. Strong positive bands were found at 1672 cm^{-1} (amide I anti β-sheet [10, 29]) and 1622 cm^{-1} (amide I β-sheet [30]). These results indicate that the proteins in the preterm 2 month myocardium adopt a predominately β-sheet conformation, compared to the term 2 month myocardium. Another positive band was found at 1516 cm^{-1}, suggesting that tyrosine is upregulated in the preterm 2 month myocardium [30, 31]. Tyrosine, found in cardiac sympathetic nerves, is involved in the biosynthesis of catecholamines that augment myocardial contractility [32].

Collagen content was upregulated in the former-preterm lambs at 2 months. Small positive bands seen at 1450, 1312, 1238, and 1160 cm^{-1} are associated with collagen's signature triple helical structure [10, 26, 27, 29]. In support of these findings, previous histological analyses of the LV myocardium in lambs born moderately preterm showed increased collagen deposition at 9 weeks TEA [9]. Increased collagen

content within the LV may represent a structural adaptation of the immature preterm heart to the haemodynamic transition at birth and subsequent postnatal haemodynamics. Over time, however, excessive collagen deposition may render individuals born preterm susceptible to impaired cardiac function later in life. Indeed, young adults born preterm have been reported to have impaired LV systolic function [7] and an impaired LV response to physiological stress when subjected to physical exercise [33], compared to adults who were born at term. Whether excessive myocardial collagen deposition contributes to this LV dysfunction is yet to be elucidated.

Positive peaks associated with triglycerides based on ester carbonyl (1743 cm^{-1}) and phospholipids (1080 cm^{-1}) were increased in the former-preterm lambs at 2 months TEA (Table 1; Fig. 2B) [20, 28, 29]. This increase in myocardial triglycerides may also be a contributing factor to the impaired cardiac function in preterm individuals later in life. Notably, increased myocardial triglyceride content is commonly recognised as a risk factor for acute heart failure, myocardial infarction, and stroke in humans [34-38]. Further studies are required to establish whether the abnormally high myocardial triglyceride content contributes to cardiac dysfunction in individuals born preterm. If so, this could be used as a potential indicator for cardiovascular disease risk in people who were born preterm, particularly with the help of modern *in vivo* imaging techniques, such as proton magnetic resonance spectroscopy.

In contrast, negative bands (those below the dotted line) in the loadings plot for PC1 (Fig. 2B) were associated with the term 2 month spectra; this means that the absorption levels at these wavenumber values were greater in the term 2 month samples than in the preterm 2 month samples. Strong negative bands were found at 1649 and 1543 cm^{-1}. These peaks are characteristic for the wavenumber values corresponding to the α-helix structure in amide I and amide II, respectively [10-12, 20, 21, 29]. The band position 1304 cm^{-1} is associated with proteins found in myofibres [13]. Interestingly, the PC1 loadings plot showed no band at 1304 cm^{-1}, suggesting no differences in myofiber protein content between

groups at 2 months TEA. In support of this finding, cardiomyocyte hypertrophy and proliferation have previously been shown to be unaffected in preterm lambs at 9 weeks TEA [9].

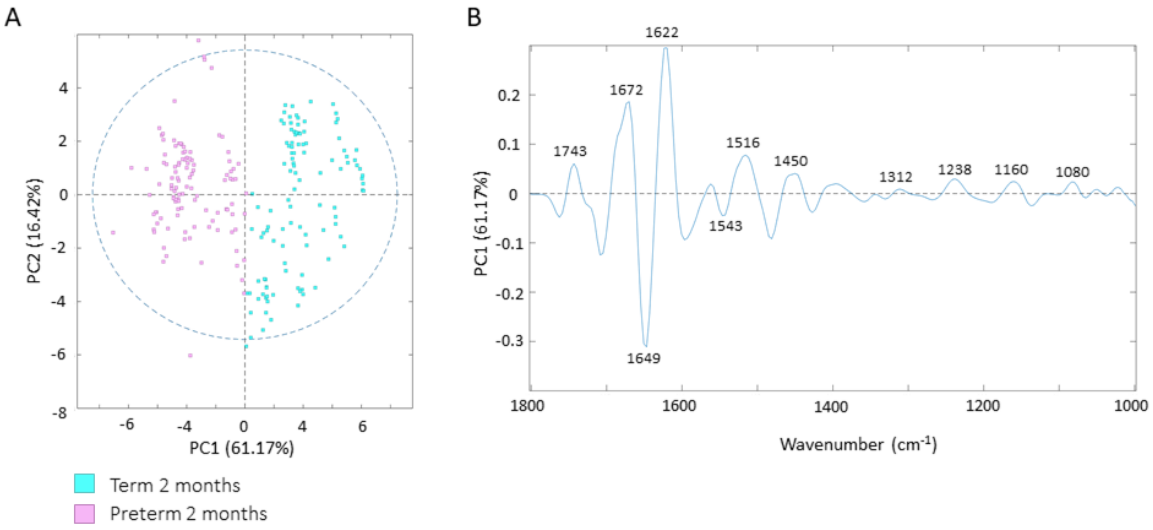

Figure 2. The results of PCA showing a PC1 vs PC2 two-dimensional scores scatter plot (dashed circle represents the 95% confidence interval) (A) and the corresponding PC1 Loadings plot (B) of the left ventricular myocardium from lambs born at term (negatively loaded) and lambs born preterm (positively loaded), assessed at 2 months term equivalent age.

3.2.2. Spectroscopic analysis at five months TEA

The PCA scatter plot between PC1 and PC2 in Figure 3A again shows clear differentiation between the preterm and term lambs at 5 months TEA along PC1. PC1 explained more than 70% of the explained variance and PC2 explained over 12%. The PCA scores plot shows greater discrimination between preterm and term at 5 months TEA compared to that at 2 months TEA (Fig. 2), indicating that cardiac differences between preterm and term become more prominent with age. The 2nd derivatives of spectra from former-preterm lambs at 5 months are negatively loaded along PC1, while 2nd derivatives of spectra from term-born lambs at 5 months are positively loaded (Fig 3A).

Positive bands (those above the dotted line) in the loadings plot for PC1 (Fig. 3B) were associated with preterm 5 month spectra, meaning the absorption levels at these wavenumber values were greater in the preterm 5 month samples compared to term 5 month samples. All the spectral trends observed in the analysis at 2 months TEA between the former-preterm and term-born lambs (Fig. 2B) were repeated at 5 months TEA (Fig. 3B), except that no difference in tyrosine levels were found between the former-preterm and term-born lambs at 5 months TEA. The loadings plot for PC1 (Fig. 3B) showed strong positive bands in the preterm 5 month samples at 1680 cm^{-1} and 1627 cm^{-1}. These bands are characteristic for the wavenumber values corresponding to the amide I structure, specifically anti β-sheet and β-sheet (Table 1) [10, 30]. These results confirm that the proteins in the former-preterm myocardium adopt a predominantly β-sheet conformation at both 2 and 5 months TEA, when compared to the term myocardium at the same time points. Smaller, broader positive bands in the former-preterm lambs at 5 month TEA at 1458, 1384, 1235 and 1161 cm^{-1} are associated with collagen's signature triple helical structure (Table 1) [10, 26, 27, 29], with higher levels in the former-preterm lambs compared to the term-born lambs at 5 months TEA, which is consistent with the analysis at 2 months TEA. A positive peak at 1743 cm^{-1} was associated with higher levels of triglycerides and phospholipids (Table 1) [28, 29] in former-preterm lambs at 5 months TEA compared to the term-born lambs at 5 months of age.

In contrast, negative bands (those below the dotted line) in the loadings plot for PC1 (Fig. 3B) were associated with the term 5 month spectra, meaning the absorption levels at these wavenumber values were greater in the term 5 month samples than in the preterm 5 month samples. Negative bands were found at 1658 and 1550 cm^{-1}, which are characteristic for the wavenumber values corresponding to amide I α-helix and amide II α-helix structure [10-12, 20, 21, 29].

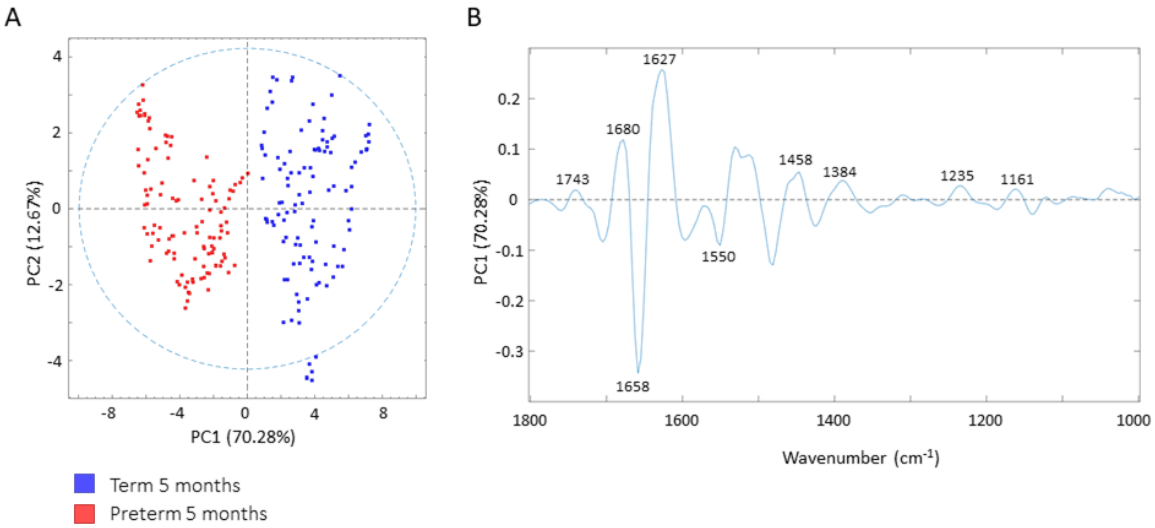

Figure 3. The results of PCA analysis showing a PC1 vs PC2 two-dimensional scores scatter plot (dashed circle represents the 95% confidence interval) (A) and the corresponding PC1 Loadings plot (B) of the left ventricular myocardium from lambs born at term (negatively loaded) and lambs born preterm (positively loaded), assessed at 5 months term equivalent age.

3.3. Biochemical alterations of the myocardium with postnatal age

In addition to comparing the myocardial biochemical profile between the lambs born preterm or at term at the two postnatal timepoints, we extended the PCA analysis to investigate how postnatal age affects the biochemical composition of the LV myocardium between 2 and 5 months TEA in lambs born at term (Fig. 4) and lambs born preterm (Fig. 5).

3.3.1. Comparison of the spectroscopic analysis of the myocardium in term lambs at 2 versus 5 months of age

Figure 4A shows clear differentiation between the 2 and 5 month term lambs along PC1. PC1 explained more than 73% of variance and PC2 explained roughly 17%. In this analysis, the 2nd derivatives of spectra from the term-born lambs at 2 months scored positively along PC1, while 2nd derivatives of spectra from the term-born lambs at 5 months scored negatively (Fig. 4A).

Positive bands in the loadings plot for PC1 (those above the dotted line; Fig. 4B) were associated with term 5 month spectra, meaning the absorption levels at these wavenumber values were greater in the term 5 month samples than in the term 2 month samples. Amide I α-helix structure (1643 cm^{-1} [11-13, 21]) and, to a weaker extent, amide II β-sheet structure (1535 cm^{-1} [29]) were upregulated in the term 2 month myocardium. A weak negative band at 1708 cm^{-1} (corresponding to the C=O stretching vibrations of the nucleoside side of nucleic acids [13, 29]) suggests that the relative content of nucleic acid is greater in the term-born lambs at 2 months compared to term-born lambs at 5 months. This may be because nuclear density decreases with age as cardiomyocytes undergo hypertrophy in the developing hearts of sheep [39] and humans [40].

In contrast, negative bands in the loadings plot for PC1 (bands below the dotted line; Fig. 4B) were associated with term 2 month spectra, meaning the absorption levels at these wavenumber values were greater in the term 2 month samples than in the term 5 month samples. The term 5 month myocardium showed a clear increase in collagen, due to a strong positive band at 1662 cm^{-1}, characteristic for the wavenumber corresponding to the triple helix structure found in collagen [15, 29]. This finding was expected because myocardial collagen content naturally increases with age in healthy mammals [41]. Furthermore, a positive band at 1558 cm^{-1} associated with amide II α-helix structure increased in the term 5 month spectra [29].

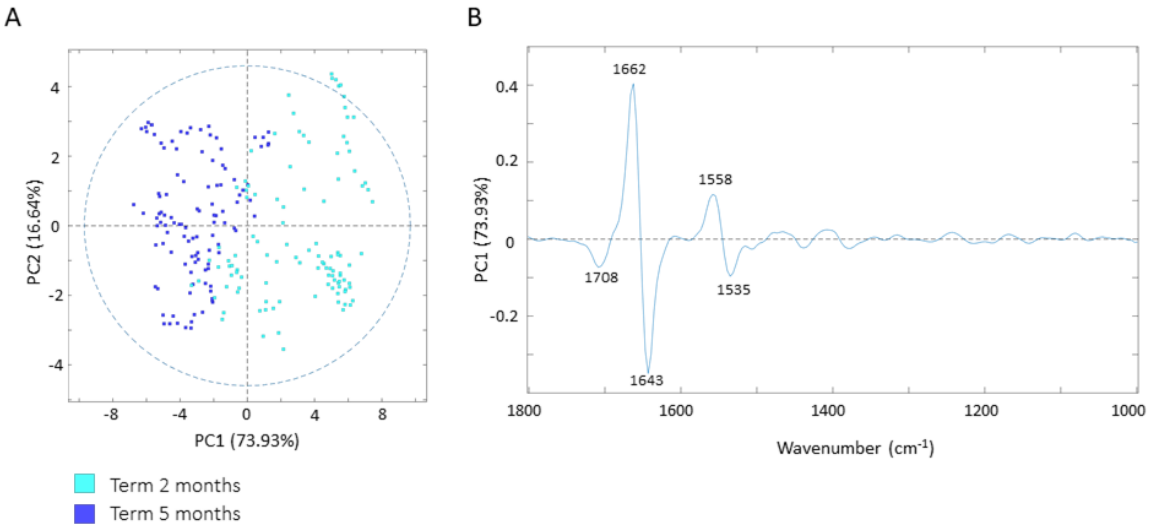

Figure 4. The results of PCA analysis showing a PC1 vs PC2 two-dimensional scores scatter plot (dashed circle represents the 95% confidence interval) (A) and the corresponding PC1 Loadings plot (B) of the left ventricular myocardium from term lambs at 2 months of age (negatively loaded) and 5 months of age (positively loaded) in the 1800 - 1000 cm^{-1} region.

3.3.2. Comparison of the spectroscopic analysis of the myocardium in former-preterm lambs at 2 versus 5 months TEA

A slight differentiation between the 2 and 5 month former-preterm lambs was found along PC2, not PC1, and is much less prominent than was observed in the term lambs (Fig. 5A), indicating that the differences between 2nd derivatives of spectra of former-preterm lamb at 2 and 5 months are smaller than that of the term lambs between time points. The preterm 2 month data scored positively along PC2, whilst the preterm 5 month data scored negatively along PC2 (Fig. 5A).

Negative bands in the loadings plot for PC2 (bands below the dotted line; Fig. 5B) were associated with the preterm 2 month spectra, meaning the absorption levels at these wavenumber values were greater in the preterm 2 month samples than in the preterm 5 month samples. Interestingly, the changes in the secondary structure of proteins within the LV myocardium in lambs born preterm between 2 and 5

months TEA are similar to that found in lambs born at term, albeit to reduced intensity (Fig. 5B). Like the term-born lambs at 2 months compared to their 5 month counterpart (Fig. 4B), the preterm 2 month myocardium also had higher levels of proteins with amide I α-helix structure (1651 cm^{-1} [11-13, 21]) and amide II β-sheet structure (1539 cm^{-1} [29]), when compared to its preterm 5 month counterpart. The preterm 2 month myocardium also had a higher content of nucleic acids (1720 cm^{-1} [15]), which, again, suggests that the nuclear density of cardiomyocytes decreases with age. The former-preterm lambs at 2 months TEA had higher levels of protein with amide I β-sheet structure (1620 cm^{-1} [30]) than the former-preterm lambs at 5 months TEA; this finding was not seen in the term groups.

In contrast, positive bands in the loadings plot for PC2 (bands above the dotted line; Fig. 5B) were associated with the preterm 5 month spectra, meaning the absorption levels at these wavenumber values were greater in the preterm 5 month samples than in the preterm 2 month samples. The preterm 5 month myocardium had higher levels of protein with amide II α-helical structure (1562 cm^{-1} [10, 20, 29]) and Type I collagen (1169 cm^{-1} [10, 29]), when compared to the preterm 2 month group. Positive bands corresponding to lipid structure were seen at 1747 cm^{-1} (ν(C=O) of ester carbonyl groups of phospholipids and triglycerides [28, 29]), 1465 cm^{-1} (CH_2 scissoring: lipids [20]), and 1242 cm^{-1} (antisymmetric stretch of phosphate found in phospholipids [13]). These higher lipid levels were also seen in the term 5 month myocardium when compared to the term 2 month myocardium. One finding that was unique to the preterm cohorts was that the myocardium at 5 months TEA had a strong positive band at 1674 cm^{-1}, associated with amide I anti β-sheet structure [30].

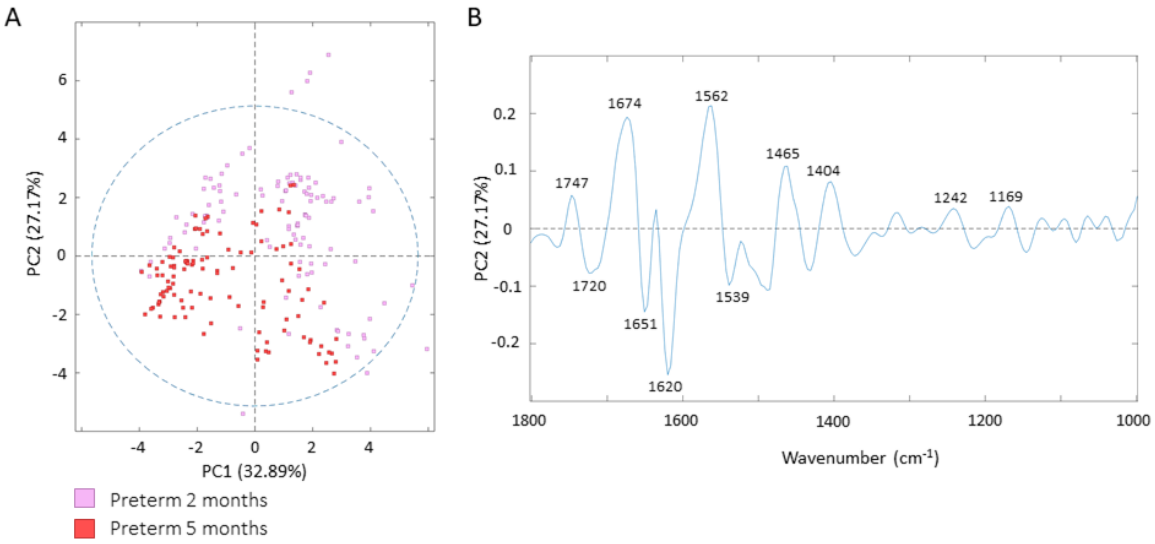

Figure 5. The results of PCA analysis showing a PC1 vs PC2 two-dimensional scores scatter plot (dashed circle represents the 95% confidence interval) (A) and the corresponding PC2 Loadings plot (B) of the left ventricular myocardium from preterm lambs at 2 months term-equivalent age (negatively loaded) and 5 months term-equivalent age (positively loaded) in the 1800 - 1000 cm^{-1} region.

4. In Conclusion

The findings of this clinically relevant sheep study, using FTIR micro-spectroscopy, clearly show that the biochemical composition of the LV myocardium is markedly altered in juvenile life (equivalent to childhood in humans) following preterm birth, with alterations in protein secondary structures and increases in collagen deposition within the myocardium. These changes in the biochemical composition of the myocardium following preterm birth likely contribute to the increased vulnerability to cardiac dysfunction later in life.

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Conflicts of Interest

None to disclose.

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Chapter 5

Microarchitecture of the hearts in term and former-preterm lambs using diffusion tensor imaging

Microarchitecture of the hearts in term and former-preterm lambs using diffusion tensor imaging

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Abstract

Diffusion tensor imaging (DTI) is an MRI technique that can be used to map cardiomyocyte tracts and estimate local cardiomyocyte and sheetlet orientation within the heart. DTI measures diffusion distances of water molecules within the myocardium, where water diffusion generally occurs more freely along the long axis of cardiomyocytes and within the extracellular matrix, but is restricted by cell membranes such that transverse diffusion is limited. DTI can be undertaken in fixed hearts and it allows the 3-dimensional mapping of the cardiac microarchitecture, including cardiomyocyte organisation, within the whole heart. The objective of this study was to use DTI to compare the cardiac microarchitecture and cardiomyocyte organisation in archived fixed left ventricles of lambs that were born either preterm (n = 5) or at term (n = 7), at a postnatal timepoint equivalent to about 6 years of age in children. Although the findings support the feasibility of retrospective DTI scanning of fixed hearts, several hearts were excluded from DTI analysis because of poor scan quality, such as ghosting artefacts. The preliminary findings from viable DTI scans (n=3/group) suggest that the extracellular compartment is altered and that there is an immature microstructural phenotype early in postnatal life in the LV of lambs born preterm. Our findings support a potential time-efficient imaging role for DTI in detecting abnormal changes in the microstructure of fixed hearts of former-preterm neonates, although further investigation into factors that affect scan quality is required.

Key words: Preterm birth, cardiac remodelling, diffusion tensor imaging, cardiovascular magnetic resonance, left ventricle.

1. Introduction

The 3D microstructure of the myocardium is complex and highly organised, consisting of a syncytium of cardiomyocytes arranged in long, branching fibres, embedded in a predominantly collagen matrix (Pope et al., 2008). The unique organisation of the cardiomyocytes has a profound impact on both gross cardiac morphology and function (Rademakers et al., 1994; McGill et al., 2016). To date, histology remains the primary method for characterising myocardial structural development; however, this technique can be time-consuming and requires extensive tissue sampling. Therefore, non-invasive imaging methods are highly desirable. In this regard, diffusion tensor imaging (DTI) is a magnetic resonance imaging (MRI) technique that can map cardiac fibre tracts and estimate local cardiomyocyte and sheetlet orientation.

The primary organisation of the left ventricle (LV) myocardium consists of the cardiomyocyte syncytium, wrapping around the ventricle, such that the cardiomyocytes are arranged in a left-handed helix towards the epicardium, a circumferential orientation in the mesocardium, and a right handed-helix towards the endocardium (McGill et al., 2016). This highly complex cardiomyocyte orientation is optimal to allow the ventricles to twist and untwist during each contraction. The secondary organisation of the myocardium consists of laminar sheetlets, 5 to 10 cardiomyocytes thick, that are sandwiched between collagen-lined shear layers (Ferreira et al., 2014). These sheetlets are the dominant mediator of the radial thickening and longitudinal shortening of the LV during contraction (LeGrice et al., 1995; Takayama et al., 2002; Axel et al., 2014). Overall, this cardiac microstructure is essential for efficient cardiac output and stable cardiomyocyte ATP consumption (Vendelin et al., 2002). Importantly, cardiomyocyte microstructure changes throughout development (Notomi et al., 2006; Abdullah et al., 2016), throughout the cardiac cycle (Ferreira et al., 2014), and is altered in diseased hearts (Abdullah et al., 2014; Ferreira et al., 2014; Winklhofer et al., 2014; Gotschy et al., 2019).

DTI measures diffusion distances of water molecules within the myocardium, where water diffusion generally occurs more freely along the long axis of cardiomyocytes and within the extracellular matrix

but is restricted by cell membranes (Garrido et al., 1994), thus providing a measure of cardiomyocyte shape and orientation. It is an effective way of imaging (both *in vivo* and *ex vivo*) the 3-dimensional structure of the heart and determining how the cardiomyocyte laminar sheetlets form the ventricular walls. DTI is a useful imaging modality that can be applied to gain an understanding of how the microstructure of the heart may be influenced by early life factors, such as preterm birth, and this is the aim of the present study (with the LV the primary focus). Importantly, in this regard, DTI can be performed retrospectively in stored fixed organs, and the utility of this approach in comparing the 3-dimensional structure of the left ventricle in fixed hearts from lambs born preterm or at term is explored in this study.

Adults born preterm are at increased risk of developing cardiac dysfunction later in life (Crump et al., 2011; de Jong et al., 2012; Kowalski et al., 2016; Carr et al., 2017; Crump et al., 2019; Skudder-Hill et al., 2019), which may be linked to abnormal postnatal remodelling of the preterm heart (Le et al., 2018). Functional MRI studies show that young adults born preterm have impaired LV systolic and diastolic function, which is associated with abnormal LV morphology (Lewandowski et al., 2013a; Kowalski et al., 2016). Furthermore, histological studies have shown that lambs born preterm have increased levels of fibrosis within the LV myocardium (Bensley et al., 2010).

To date, however, little is known of the long-term effects of preterm birth on cardiomyocyte organisation in the LV, the major pumping chamber of the heart postnatally. One previous *ex vivo* DTI study has found that cardiomyocyte organisation changes throughout development in sheep (Abdullah et al., 2016). Given that gross cardiac structure is altered as a result of preterm birth (Lee et al., 1992; Mann et al., 2010; Lewandowski et al., 2013a; Lewandowski et al., 2013b; Kowalski et al., 2016; Aye et al., 2017; Cox et al., 2019), it is likely that the 3-dimensional microstructural architecture of the heart is also altered; this is addressed in the present study, where we have undertaken *ex vivo* DTI in fixed hearts from lambs at 5 months term-equivalent age (approximately equivalent to 6 years of age in children) that were born either preterm or at term. Importantly, the studies utilise a clinically relevant sheep

model of preterm birth, where the lambs were delivered prematurely and placed into a neonatal intensive care facility where they were provided respiratory support for about a week, and clinical care that replicates the contemporary care of preterm newborn babies.

2. Methods

2.1 Ethical approval:

The lamb studies adhered to the American Physiology Society and National Institutes of Health guidelines for humane use of animals for research and were prospectively approved by the Institutional Animal Care and Use Committee at the University of Utah Health Sciences Center.

2.2 Animal model:

Date-mated Rambouillet x Columbia ewes were randomly assigned for caesarean-section delivery of preterm lambs at ~128 days' gestation (female = 2, male = 3) or were spontaneously born at term (~150 days' gestation) (female = 5, male = 2). Details of the perinatal procedures have been reported by our group (Dahl et al., 2018). Briefly, the ewes chosen for preterm delivery received an intramuscular injection of dexamethasone phosphate (6 mg; Vedco, Inc., St. Joseph, MO, USA) 48 and 24 hours before operative delivery. At ~128 days' gestation, the preterm fetuses were exposed via midline hysterotomy and administered calfactant (6 ml, Infasurf; generously provided by ONY Biotech, Inc, Amherst, NY, USA). Once the umbilical cord was milked, clamped, and cut, the preterm lambs were resuscitated with a t-piece resuscitator (NeoPuff; Fisher & Paykel, Auckland, New Zealand). Lambs were weighed, then mechanically ventilated (Dräger ventilator; model VN500, Lubeck, Germany). All preterm lambs were treated with a loading dose of caffeine citrate (15 mg/Kg, given over 90 min, Sagent Pharmaceuticals, Schaumburg, IL; iv) within 30 min of delivery, followed by maintenance treatment (5 mg/Kg) every 24h for 6d. Preterm lambs were transitioned to non-invasive respiratory support at 72h after birth, which was maintained for 3 days. Term lambs did not receive any form of respiratory support.

At 5 months after term-equivalent age (TEA), the lambs were weighed, then euthanised via administration of Beuthanasia (0.25mL/Kg, Intervet Inc., Madison, NJ) followed by potassium chloride (10 mEq; Hospira, Illinois, USA) intravenously. The full terminal tissue collection procedure is reported by our group (Dahl et al., 2018). The hearts were excised, arrested in diastole via coronary perfusion

with potassium chloride (10 mEq; Hospira, Illinois, USA), then immersion fixed in 10% buffered formalin at 4°C.

2.3 MRI acquisition and analysis

Each heart was placed in a container filled with a susceptibility matching fluid (Fomblin; Solvay Solexis, NJ, USA). DTI used a standard 3D spin-echo echo-planar imaging (900 µm isotropic resolution, 4-shot acquisition, 111 x 111 x 111 matrix size, TE/TR = 41.5/1000 ms) on a 7T Bruker Biospec 70/30 instrument (Bruker Biospin, Ettlingen, Germany) equipped with 600 mT/m peak amplitude gradient sets. The diffusion time was 20 ms. The diffusion encoding duration was 7 ms. Each DTI dataset consisted of 1 non-diffusion weighted (b0) image and 12 diffusion-weighted images with b values of 1000 s/mm^2 in 12 non-coplanar directions per slice.

2.4 Cardiac morphology measurements

Only measurements of the LV (the major pumping chamber of the heart postnatally) were included in this study. Seg3D (Scientific Computing and Imaging Institute (SCI); Utah, USA) (Institute) was used to manually segment the LV and determine LV wall volume, LV cavity volume, LV length, and LV lateral wall width. The interventricular septum was included in the LV measurements. LV mass was calculated by multiplying LV wall volume by the specific gravity of heart muscle, otherwise known as myocardial density (1.053 g/ml) (Vinnakota and Bassingthwaighte, 2004). LV length was measured in a straight line from the centre of the mitral annulus to the apical endocardium. LV lateral wall width was measured at the level of the mitral valve leaflet tips.

2.5 MATLAB image processing

The raw DTI data were converted into standard DICOM images, using ParaVision 6.0.1 software (Bruker; Ettlingen, Germany). Due to the poor quality of some scans, six hearts were excluded from the final DTI analysis, resulting in three hearts per group. For each heart, the isotropic 3D DICOM data were initially rotated and truncated in order to have a stack of short-axis slices covering the LV. The endocardial and epicardial walls were manually segmented for each slice in order to build a 3D model of the LV. The

diffusion tensors (involving measures of 3 eigenvectors and 3 eigenvalues) were calculated for each voxel with a linear least square fit of the measured signal intensities. Measures of helix angle, transverse angle, absolute sheetlet angle, and rotational invariant measures of the eigenvalues (fractional anisotropy and mean diffusivity) were extracted from the tensors. Each measure was then averaged for the whole LV, and per region of the LV (interior, lateral, inferior and septal).

The primary eigenvector (E1) represents the direction of greatest diffusivity, thus aligning with the long axis of cardiomyocytes. The secondary eigenvector (E2) represents the direction of greatest diffusivity perpendicular to E1. Both E1 and E2 align with the sheetlet plane orientation. The tertiary eigenvector (E3) is perpendicular to E1 and E2, thus aligning perpendicular to the myocyte long-axis and perpendicular to the sheetlet plane. Eigenvalues D_1, D_2 and D_3 reflect the diffusion coefficients for the primary, secondary and tertiary eigenvectors, respectively. The eigenvalues were used to obtain two scalar rotational invariant diffusion parameters: fractional anisotropy and mean diffusivity. Fractional anisotropy represents the degree of anisotropy, where 0 is completely isotropic (all eigenvalues are equal, meaning water can diffuse equally in all directions, i.e. the cardiomyocyte has the same length and width) and 1 is completely anisotropic (two out of three eigenvalues are zero, meaning water can only diffuse in one axis). Mean diffusivity was calculated by averaging all eigenvalues, thereby representing the mean magnitude of diffusion within a tensor.

Helix angle was defined as the angle between the projection of E1 onto the tangent plane of the epicardium and the circumferential direction, with a range of -90° (rotating clockwise toward the base) to 0° (wrapping circumferentially around the LV) to 90° (rotating anti-clockwise toward the base) (Ferreira et al., 2014). Transverse angle was defined as the angle between E1 and the projection of E1. Absolute sheetlet angle was defined as the absolute angle between the projection of E2 in the radial cross-myocyte plane and the cross-myocyte direction, tangential to the epicardial wall (Ferreira et al., 2014; Ferreira et al., 2018).

2.6 Statistical analysis

Body weights and measurements of cardiac morphology in former-preterm and term lambs (Table 1) were analysed with unpaired t-tests, using Prism v7.01 (GraphPad, La Jolla California, USA). Statistical significance was assessed at $p<0.05$.

Data for the DTI rotational invariant scalar parameters (fractional anisotropy, mean diffusivity, D_1-D_3) are presented as mean ± 95% confidence interval. The measured helix angle and absolute sheetlet angle values were binned (bin width = 5°) and plotted as histograms for the entire LV and for each LV region. These DTI measures were not statistically compared between groups due to low sample size.

3. Results

3.1 Body weights and cardiac morphology

Body weights, heart weights, and heart to body weight ratios did not differ between term and former-preterm lambs at 5 months TEA (Table 1). Parameters of LV morphology also did not differ significantly between groups. Lateral wall width was thinner in the LV of former-preterm lambs than the term lambs, but this difference just failed to reach statistical significance (p = 0.053).

Table 1. Body weights, heart weights, and measures of LV morphology in term and former-preterm lambs at 5 months TEA.

	Term (n = 7)	Former-preterm (n = 5)	p-value
Body weight (kg)	54.9 ± 4.0	50.6 ± 2.0	0.46
Heart weight (g)	226.0 ± 17.9	231.9 ± 9.3	0.80
Heart weight:body weight (g:kg)	4.13 ± 0.15	4.63 ± 0.29	0.11
Left ventricular mass (g)	123.50 ± 10.35	95.48 ± 6.65	0.10
Left ventricular cavity volume (ml)	24.96 ± 2.15	25.44 ± 2.51	0.89
Mass-volume index (g/ml)	5.16 ± 0.57	3.94 ± 0.24	0.12
Lateral wall width (mm)	14.65 ± 0.40	13.37 ± 0.41	0.053
Left ventricular length	67.25 ± 1.67	63.07 ± 1.99	0.14

3.2 DTI analysis

Ex vivo DTI imaging was successfully conducted in some of the fixed hearts. Several hearts, however, were excluded from the DTI analyses because of poor scan quality, such as ghosting artefacts. The artefacts could not be minimized by post-hoc imaging corrections. Consequently, the present study reports results for 3 hearts per group; therefore, only a visual description of the data is presented without statistical analysis.

DTI scalar parameters

Figure 1 shows representative cross-sectional DTI maps of a term lamb heart and a former-preterm lamb heart. The figure shows LV segmentation and measurements of each of the DTI scalar parameters. These DTI maps represent group-averaged measurements taken from the anterior, lateral, inferior, and septal regions of the LV (first panel of Figure 1).

Fractional anisotropy refers to the shape of the space in which water can freely diffuse. High fractional anisotropy in each voxel indicates that water is freely diffusing along one axis and is restricted in the other two axes, which indicates that the general shape of the cardiomyocytes is long and thin; lower fractional anisotropy indicates a relative increase in cardiomyocyte width. DTI parametric fractional anisotropy maps appeared similar between the term and former-preterm hearts (Figure 1). Regionally, fractional anisotropy appeared higher in the centre of the myocardial wall, slightly lower towards the epicardium, and least towards the endocardium, a finding also previously reported in healthy human and sheep hearts (Jiang et al., 2007; McGill et al., 2015). Overall, mean fractional anisotropy was similar between the former-preterm and term groups (former-preterm: 0.335, term: 0.341) (Figure 2A). Eigenvalues show that the average shape of the tensors (known as glyphs; Figure 3) appeared to be cylindrical (E1: former-preterm = 0.98, term = 0.92), with an elliptical cross-section (E2: former-preterm = 0.63, term = 0.58; E3: former-preterm = 0.52, term = 0.47) in both groups.

The map for mean diffusivity and the corresponding DTI principal diffusivities (eigenvalues D_1 to D_3) appeared to have higher water diffusivity in the preterm LV, particularly towards the epicardium (Fig. 1). Increases in overall diffusion (mean diffusivity) is typically consistent with increased extracellular space, and, therefore, higher diffusion rates. Mean diffusivity appeared to be greater in the LV of preterm lambs compared to term lambs (preterm: 0.711 $x10^{-3}$ mm^2/s, term: 0.657 $x10^{-3}$ mm^2/s) (Figs. 2B). All three eigenvalues appeared greater in the preterm LV (Figs. 2C-D). No clear regional differences were observed in any scalar parameters (Fig. 2).

Cardiomyocyte organisation

Helix angle, absolute sheetlet angle, and transverse angle measurements (Figure 4) inform of the way that cardiomyocytes are organised within the ventricular wall. Helix angle maps of the LV appeared similar between the two groups of lambs, where helix angle was positive towards the endocardium, zero at the centre of the myocardial wall (due to circumferential cardiomyocytes), and negative towards the epicardium in the LV. These qualitative maps are supported by group-averaged quantitative measurements shown in Figures 5, 6, and 7, respectively.

The group-averaged histogram of helix angle suggested a right-skewed distribution in the former-preterm LV, meaning that more cardiomyocytes appeared orientated in the right-handed helix ($30° < \text{helix angle} \leq 90°$) relative to the term LV (Figures 5A-C). Conversely, the LV of the former-preterm lambs appeared to have fewer circumferential cardiomyocytes ($-30° \leq \text{helix angle} \leq 30°$) relative to the term LV (Figures 5B and C). This skewed distribution relationship was more obvious in the relative frequency graphs for anterior, lateral, inferior, and septal regions of the heart of both groups of lambs (Figures 5D-G).

The appearance of the absolute sheetlet angle maps in the LV of term lambs (Figure 4) was consistent with previous DTI studies in healthy hearts (Chen et al., 2005; Cheng et al., 2012; Ferreira et al., 2014; Abdullah et al., 2016). The absolute sheetlet angle map for the of the LV in former-preterm lambs appeared to be less, relative to the term-born lambs (Figure 4). The group-averaged histogram shows that the relative frequency of sheetlet angle was similar for the LV in former-preterm lambs and term-born lambs (Figures 6A-C), consistent with the tensor orientation maps in Figure 3. This distribution relationship was more obvious in the relative frequency graphs for anterior, lateral, inferior, and septal regions of the heart of both groups of lambs (Figures 6D-G).

Transverse angle provides information about positively- and negatively-angled cardiomyocytes (Figure 4). The absolute transverse angle map of the LV in former-preterm lambs appeared to have a higher

frequency of positively and negatively angled cardiomyocytes relative to the LV in term-born lambs (Figure 4). The group-averaged histogram shows that the relative frequency of transverse angle was similar for the LV of former-preterm lambs and term-born lambs, being centred around zero (Figures 7A-C). In the LV of term-born lambs, the relative frequency for the transverse angle appeared negatively skewed in the anterior wall of the LV and positively skewed in the lateral wall (Figs. 7D and 6E), a finding supported by a study in healthy rat hearts (Teh et al., 2016b).

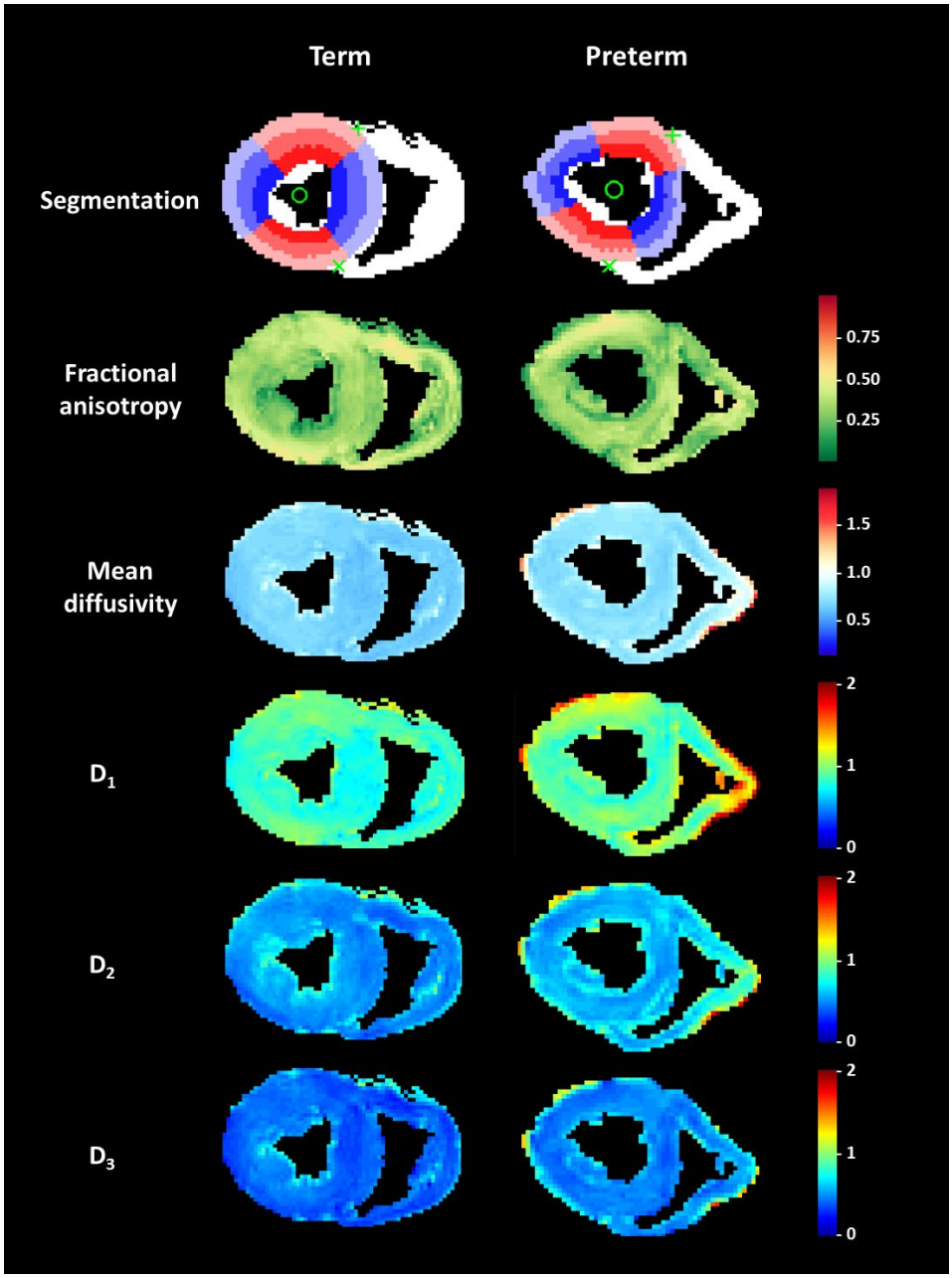

Figure 1. Representative DTI maps of a heart from a term-born lamb at 5 months of age (left) and the heart of a lamb born preterm at 5 months TEA (right). From top to bottom: segmentation of the left ventricle (anterior, lateral, inferior, septal), fractional anisotropy, mean diffusivity, primary eigenvalue (D_1), secondary eigenvalue (D_2), and tertiary eigenvalue (D_3). Units of diffusivities are $x10^{-3}$ mm^2/s.

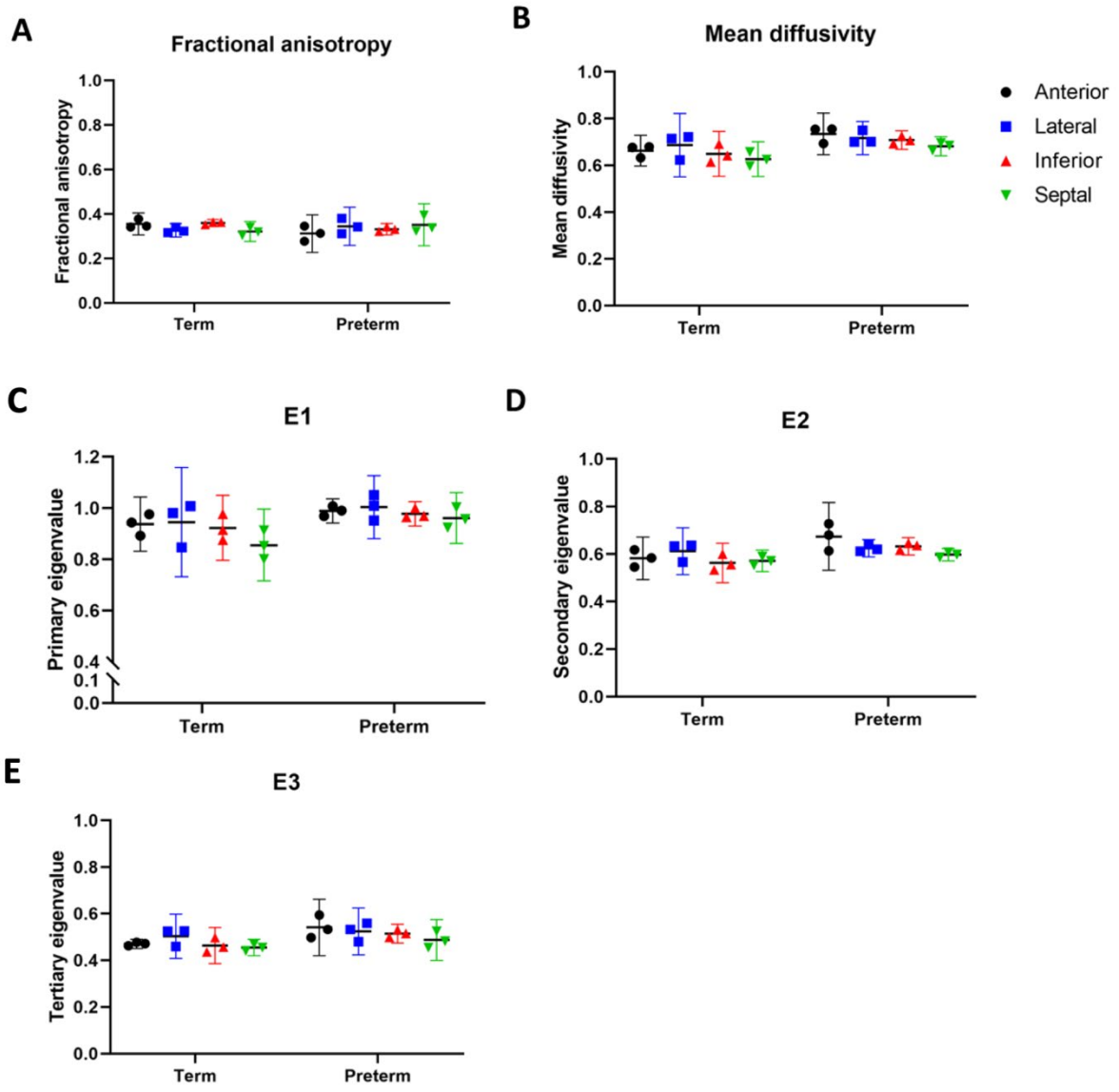

Figure 2. DTI measurements (mean ± 95% confidence interval) taken from the anterior, lateral, inferior and septal regions of the left ventricle in each lamb born at term or preterm. Units of diffusivities are $x10^{-3}$ mm^2/s. n=3/group.

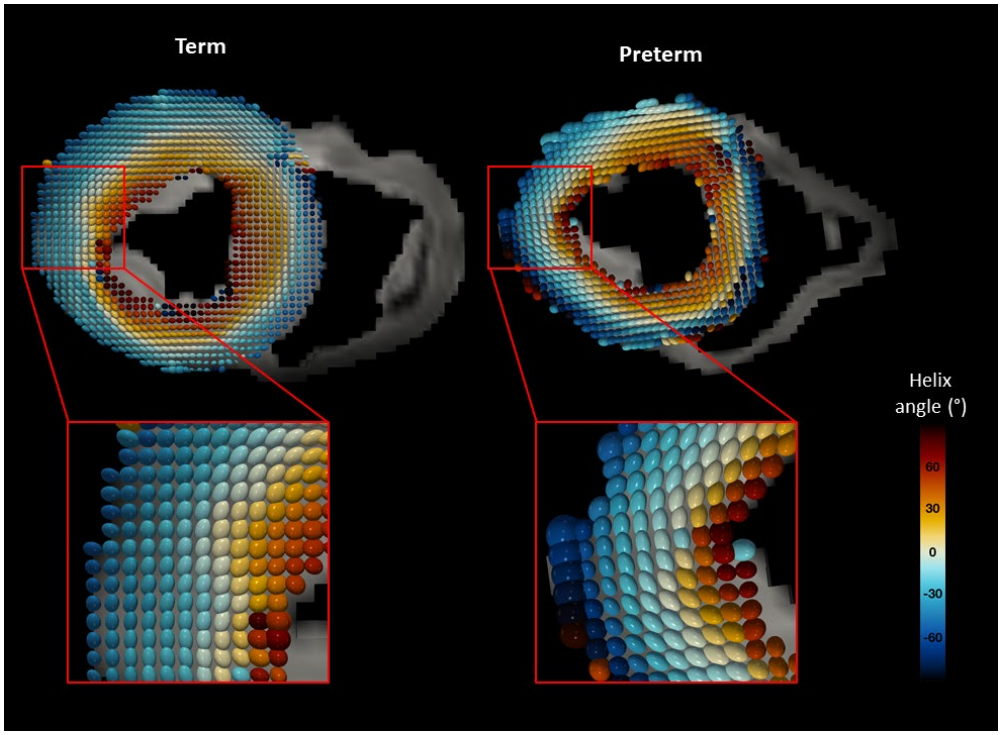

Figure 3. Representative diffusion tensors in the left ventricle from a term-born lamb at 5 months of age (left) and the heart of a lamb born preterm at 5 months TEA (right). The diffusion tensor in each voxel is represented by an ellipsoidal glyph, colour coded to helix angle (°).

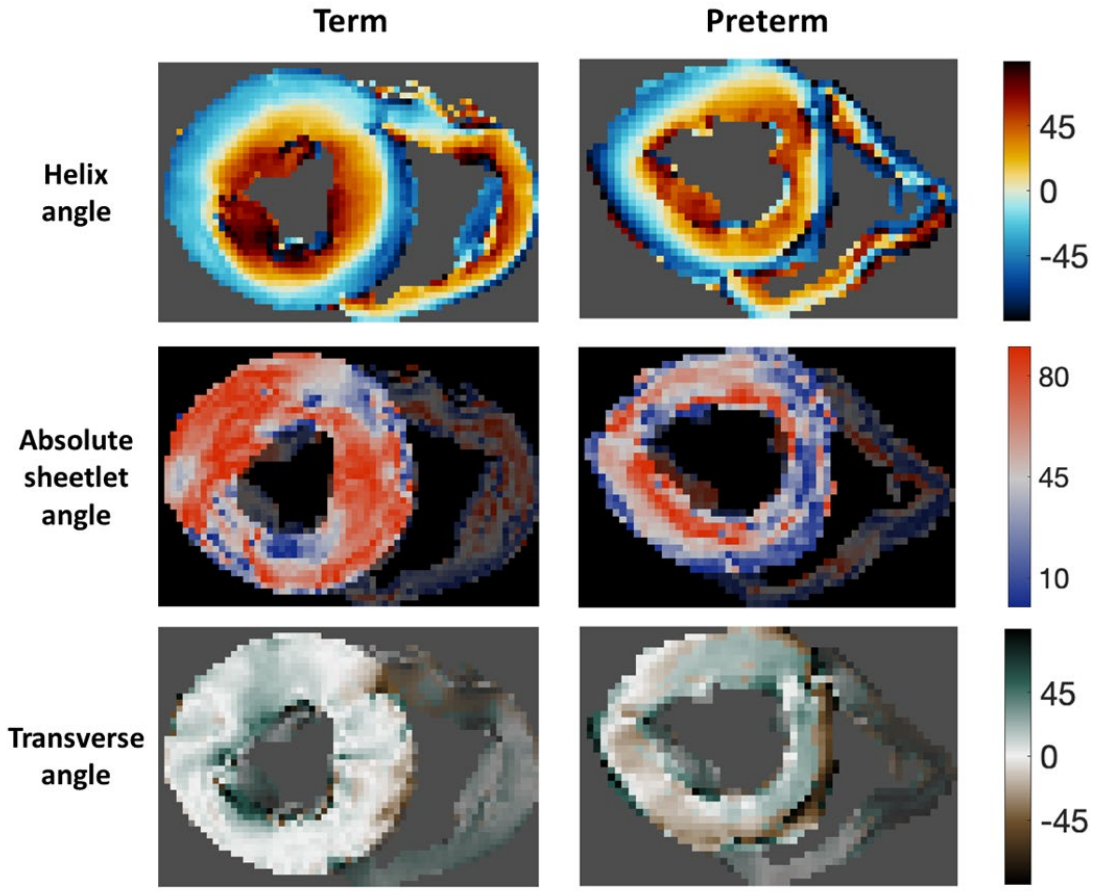

Figure 4. Representative tensor orientation maps of a term-born lamb at 5 months of age (left) and the heart of a lamb born preterm at 5 months TEA (right). From top to bottom: helix angle, absolute sheetlet angle (E2A), and transverse angle. Units are degrees.

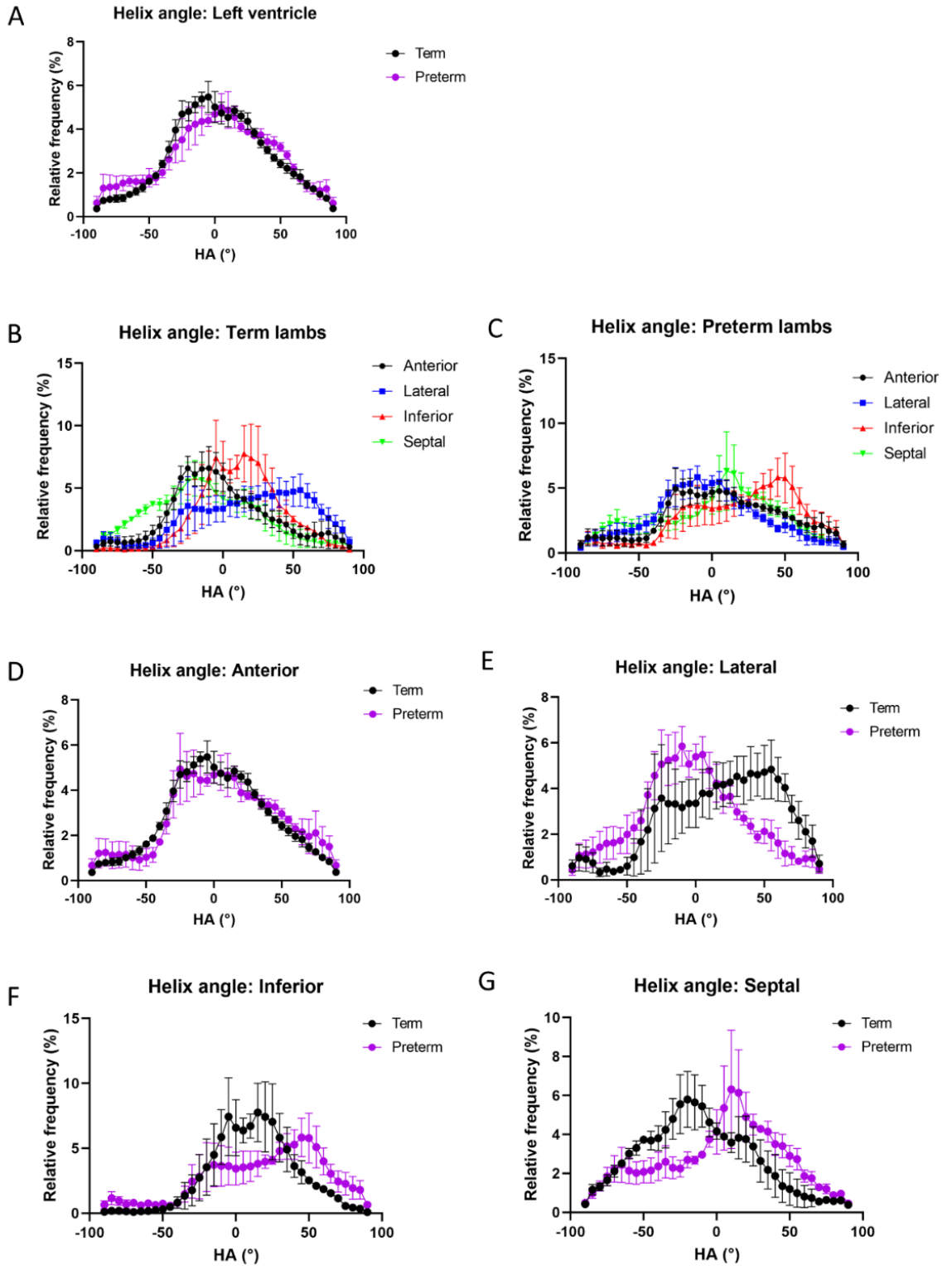

Figure 5. Group-averaged histograms of helix angle in term-born and former-preterm lambs. Data are presented as mean ± standard error of the mean. n=3/group.

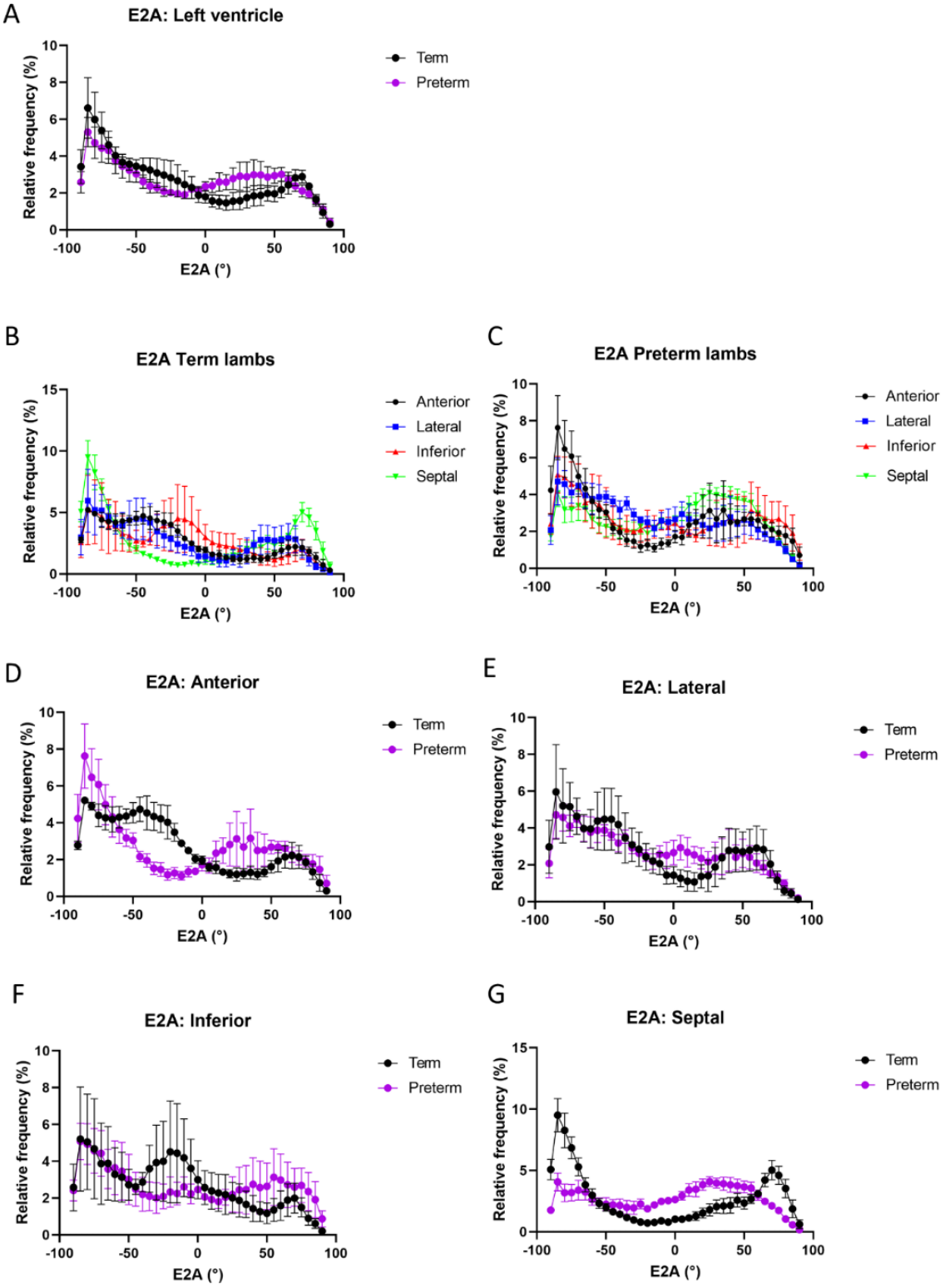

Figure 6. Group-averaged histograms of absolute sheetlet angle (E2A) in term-born and former-preterm lambs. Data are presented as mean ± standard error of the mean. n=3/group.

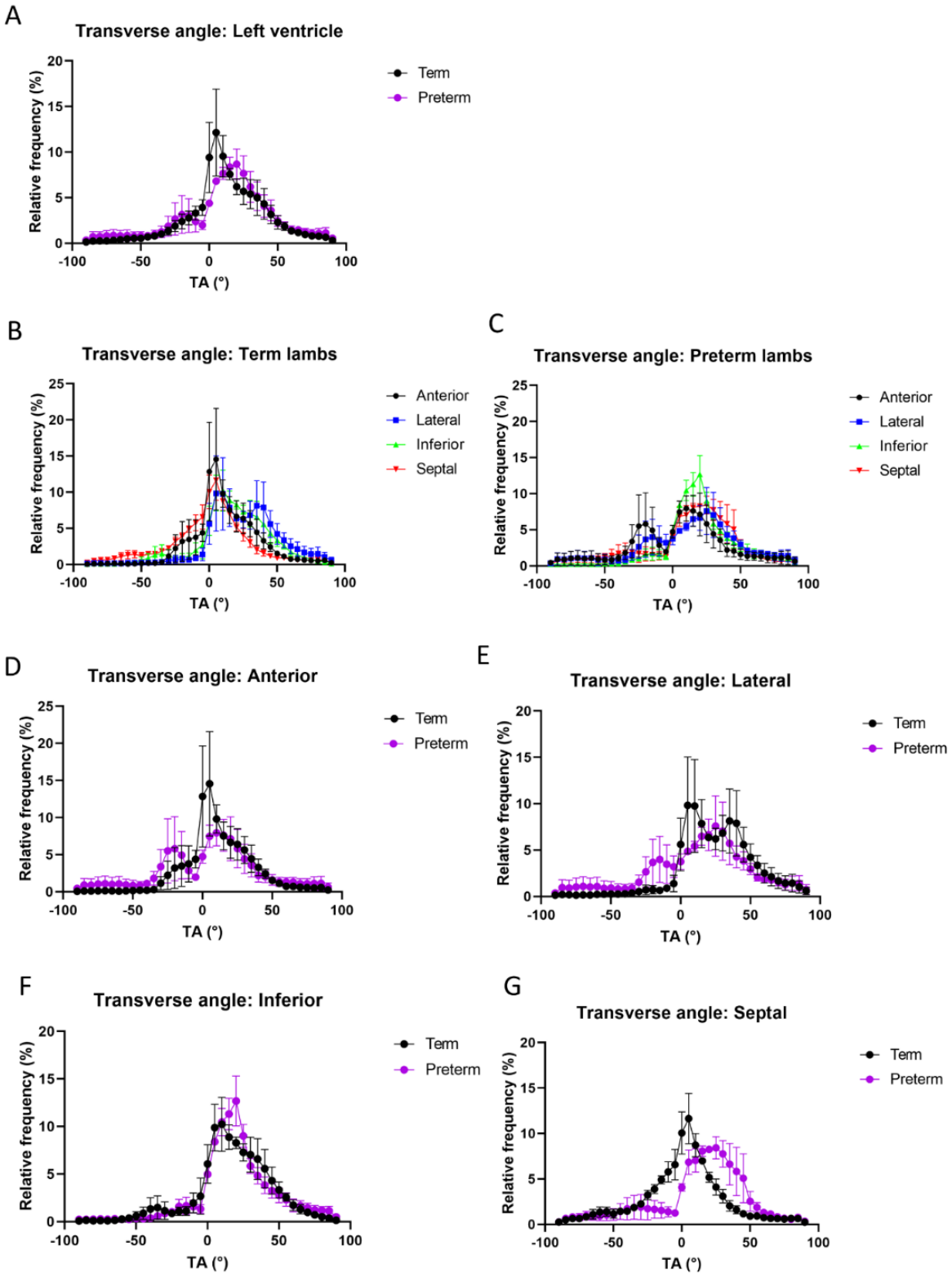

Figure 7. Group-averaged histograms of transverse angle in term-born and former-preterm lambs. Data are presented as mean ± standard error of the mean. n=3/group.

4. Discussion

The findings of the present study support the feasibility of retrospectively conducting DTI to image cardiac microarchitecture in archived fixed hearts, a technique that is likely to be of benefit to many researchers. Notably, however, technical difficulties were encountered in the imaging of some of the archived hearts. Several hearts were excluded from quantitative analyses because of poor scan quality (such as ghosting artefacts), the causes of which were not apparent. Given the limited number of viable scans, the experimental findings are at this stage preliminary, but do support the view that the microarchitecture of the LV in early life is altered following preterm birth.

Specifically, preterm birth appeared to be associated with greater water diffusivity in the myocardium. We speculate that this is linked to an increase in the extracellular compartment within the myocardial tissue, and supports previous histological findings of excessive collagen deposition within the LV myocardium in early life following preterm birth (Bensley et al., 2010). Furthermore, DTI techniques revealed a shift towards an immature orientation of cardiomyocytes in the LV of lambs born preterm, thus suggesting that an immature cardiac microarchitecture persists into juvenile life following preterm birth.

Fractional anisotropy is associated with cardiomyocyte shape

Normal postnatal development of cardiomyocytes is characterised by cellular hypertrophy, affecting both the size and shape of cardiomyocytes. Preterm birth did not appear to affect fractional anisotropy, a proxy for cardiomyocyte shape, in the LV. Normal transmural heterogeneity of fractional anisotropy was seen in the LV, as reported in previous studies (Jiang et al., 2007; McGill et al., 2015); however, no regional differences were found. The fractional anisotropy values in this study are consistent with previous findings in term lambs at 5 months of age (Abdullah et al., 2016). Previous DTI studies of developing prenatal and postnatal hearts have shown that fractional anisotropy changes throughout development, potentially due to changes in cell size and shape (Chen et al., 2005; Abdullah et al., 2016), although the direction in which this change occurs is unclear. It is likely that fractional anisotropy

increases during gestation (Mekkaoui et al., 2013; Pervolaraki et al., 2013), then decreases throughout postnatal life (Abdullah et al., 2016), due to other confounding factors that influence fractional anisotropy such as cardiomyocyte shape (Abdullah et al., 2016). Early in fetal life, hearts are highly isotropic (Mekkaoui et al., 2013; Pervolaraki et al., 2013) due to their lack of cardiomyocyte organisation, as early fetal cardiomyocytes have not yet aligned into distinct tracts (Mekkaoui et al., 2013). Fractional anisotropy gradually increases with gestational age as the myofiber architecture in the LV develops (Wu and Wu, 2009; Zhang et al., 2013) and their cardiomyocyte length-to-width ratio increases (Burrell et al., 2003; Jonker et al., 2015). In early postnatal life, physiological cardiomyocyte hypertrophy becomes the main determinant of cardiac growth, whereby the length-to-width ratio of cardiomyocytes decreases (Burrell et al., 2003; Jonker et al., 2015); this results in a decrease in fractional anisotropy throughout postnatal life (Chen et al., 2005; Abdullah et al., 2016).

The findings of fractional anisotropy in this study suggest that preterm birth does not influence cardiomyocyte shape in the LV of lambs by 5 months TEA. Since physiological cardiomyocyte hypertrophy is associated with a decrease in cardiomyocyte length-to-width ratio (and therefore a change in cell shape and isotropy) (Burrell et al., 2003; Jonker et al., 2015), it is reasonable to suggest that preterm birth is not associated with cardiomyocyte hypertrophy in the LV at this time-point.

Mean diffusivity of the myocardium is correlated with the myocardial extracellular compartment

Biological factors such as myocardial fibrosis and extracellular volume can influence mean diffusivity (Chen et al., 2003; Wu et al., 2007; Abdullah et al., 2014; Winklhofer et al., 2014). In this study, mean diffusivity values for the LV in term-born lambs are consistent with previous findings in term-born lambs at the same age (Abdullah et al., 2016). The LV of former-preterm lambs, however, appeared to be less restrictive to water diffusion, which is indicative of altered LV microstructure. In this regard, our previous histological analyses of the hearts of lambs born moderately preterm showed increased collagen deposition within the LV, which may account for the increased diffusivity of the LV of former-preterm lambs in this study (Bensley et al., 2010). Increased collagen deposition within the ventricular

myocardium in early life may represent a compensatory mechanism to the premature haemodynamic transition at birth. Over time, however, further development of cardiac fibrosis may render individuals born preterm susceptible to impaired cardiac function later in life. We speculate that the impaired LV systolic function evident in young adults born preterm (Lewandowski et al., 2013a; Huckstep et al., 2018) is partly due to an increase in collagen deposition.

Cardiomyocyte and laminar sheetlet orientation changes throughout development, contributing to efficient ventricular contraction

DTI analysis provides novel insight into the orientation of cardiomyocytes and laminar sheetlets throughout the myocardium, an anatomical feature unique to the heart that is important for efficient ventricular contraction. Abdullah *et al*., (2016) were the first to use DTI to characterise the microstructure of the developing heart in lambs as it transitions from prenatal to postnatal life; the hearts were examined from the third trimester through to 5 months after birth (Abdullah et al., 2016). They found that during this period, the helix angle distribution in the LV shifts towards a circumferential orientation at the expense of a reduction in left handed cardiomyocytes. They also found an increase in sheetlet angle with increasing age (Abdullah et al., 2016), which was hypothesised to be attributed to ventricular growth (Ferreira et al., 2014).

Based on these previous findings, and according to the frequency distribution of helix angle in our study, the LV of the former-preterm lambs appears to have a more immature myofiber organisation compared to the LV of term-born lambs, such that there appeared to be fewer circumferential cardiomyocytes and more positively angled cardiomyocytes. While the functional implications of this unique myofiber phenotype are still unknown, these results suggest that the LV of former-preterm lambs maintains an underdeveloped myofiber organisation by 5 months TEA in lambs. Histological evidence of delayed or abnormal cardiomyocyte maturation in the preterm heart in early postnatal life supports this hypothesis (Bensley et al., 2010).

The orientation of laminar sheetlets of cardiomyocytes directly contributes to the radial thickening and longitudinal shortening of the LV observed during contraction. Sheetlet angle during the cardiac cycle has been shown to increase from diastole to systole, thereby increasing the thickness of the ventricular wall during contraction; the magnitude of that increase in sheetlet angle dictates how efficiently the ventricle contracts (Hales et al., 2012; Ferreira et al., 2014; Teh et al., 2016a). Our findings support the concept of a persistent underdeveloped phenotype in the heart following preterm birth, whereby sheetlet angle appeared to be reduced in the former-preterm LV (Abdullah et al., 2016). Any changes in absolute sheetlet angle were not associated with differences in ventricular growth, as we found no significant changes in LV mass between former-preterm and term hearts. Deviation from normal sheetlet anatomy has been shown to affect cardiac function in pigs (Ferreira et al., 2018). A study using *in vivo* DTI found that hearts with hypertrophic cardiomyopathy have higher than normal global sheetlet angle throughout the cardiac cycle, which was associated with hypercontraction in systole and failure of relaxation in diastole (Ferreira et al., 2014). The observed decrease in sheetlet angle in the LV of former-preterm lambs is indicative of altered sheetlet structure, which also has the potential to impair ventricular function. Indeed, such changes in sheetlet structure may account for the echocardiography findings of diminished LV function in adults born preterm (Appleton et al., 1987; Harada et al., 1999; Kozak-Barany et al., 2001; Lewandowski et al., 2013a; Schubert et al., 2016).

The transverse angle values of the LV in term lambs in this study reproduce those seen in normal hearts (Chen et al., 2005; Lombaert et al., 2012; Teh et al., 2016b; von Deuster et al., 2016). The frequency distribution for global transverse angle in the LV of term-born lambs in our study reflects a circumferential arrangement of cardiomyocytes when projected onto the local short-axis plane, however transverse angle is slightly higher towards the endocardium. These findings are consistently supported throughout the literature (Chen et al., 2005; Lombaert et al., 2012; Teh et al., 2016b; von Deuster et al., 2016). Little is known about the functional significance of transverse angle. It has been previously shown that transverse angle does not change significantly during contraction in the rat heart

(Chen et al., 2005). DTI studies show that transverse angle is unaffected in dilated cardiomyopathy (von Deuster et al., 2016) and cardiac amyloidosis (Gotschy et al., 2019). Our study showed that the LV of lambs born preterm appears to exhibit a lower proportion of circumferentially arranged cardiomyocytes than in term-born lambs; it is unclear whether this deviation from normal anatomy would affect the efficiency of contraction.

Due to the limited sample size in this study, we were only able to report trends in the DTI parameters. Hence, caution should be taken when interpreting the findings of this study, as results could not be statistically validated. Nevertheless, our findings support a role for *ex vivo* DTI in visualising and quantifying abnormal changes in the complex, three-dimensional cardiac microstructure of archived fixed hearts, which could otherwise only be elucidated through time-consuming histology that requires extensive tissue sampling. Further investigation is required, however, into why the DTI imaging was successful in some archived fixed hearts and not in others. There is the potential that the ghosting artefacts may have been caused by table vibrations during scanning. Indeed, *in vivo* cardiac MRI is greatly affected by motion artefacts caused by movement during the cardiac cycle and respiration (McGill et al., 2016), and these are challenges that need to be overcome when considering the translation of these techniques into the clinic.

Prior to scanning archived fixed hearts, it is also important that the hearts are fixed in the same way, with the same fixative and stored for a similar duration of time as these factors can affect the diffusion properties of biological tissue, including the heart. Other fixation factors, such as the method of fixation (perfusion fixation, immersion fixation or both), the thickness of tissue, preparation of the fixing agent, time interval between tissue extraction and fixation, the ratio of the volume of the specimen to the amount of fixation solution, and the temperature of fixation, also play a role in the fixation process and can ultimately affect the diffusion properties of the heart (Mazumder et al., 2016). For our study, the hearts were fixed by the same method.

Overall, the qualitative findings in this DTI study are consistent with those found in our histological study in the preterm lamb model (where the lambs were born moderately preterm) and build from these findings to suggest that preterm birth leads to altered microstructure of the LV myocardium in early life. Although *in vivo* cardiac DTI is still in the early stages of development for clinical use, this study provides the foundations for the future utility of cardiac DTI in monitoring heart health in individuals born preterm.

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Conflicts of Interest

None to disclose.

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Chapter 6

General Discussion

6.1 Introduction

Over recent decades modern clinical interventions have dramatically improved the survival rates of preterm babies that require ventilatory support after birth. Of concern, however, as a legacy of the improved survival at birth, the long-term adverse health outcomes are now becoming apparent, as the first survivors of very and extremely preterm birth progress into adulthood. Importantly, in this regard there is now accumulating clinical evidence demonstrating elevated risk of cardiovascular disease and abnormal cardiac structure and function in people born preterm [1-7]. It is therefore imperative to gain an understanding of how the heart remodels in response to preterm birth; this has been the focus of this thesis. In developing an understanding of the mechanisms of the altered cardiac growth following preterm birth it is envisaged that interventional strategies can be developed to prevent the adverse impact of preterm birth on the heart. In order to do this, a sheep model of preterm birth has been utilised, whereby the lambs are born at a time when their lungs are very immature (during the saccular stage of lung development).

The findings from this thesis build on the seminal studies in our laboratory where abnormalities in cardiac structure were described in lambs at 9 weeks TEA, that were born moderately preterm at 0.9 of gestation (the earliest timepoint where lambs could be delivered and survive after birth without requiring postnatal respiratory support), compared to age-matched controls [8]. This study found that the cardiomyocytes of both ventricles and the interventricular septum were hypertrophied in the preterm lambs. They also showed a six- to seven-fold increase in collagen deposition in the preterm myocardium, which was accompanied by lymphocytic infiltration. However, in those studies, contrary to what was predicted, cardiomyocyte proliferation and total cardiomyocyte number did not differ between preterm and term lambs.

Following on from that preterm lamb study, our laboratory then conducted a study in adult sheep born preterm without requiring postnatal ventilation, where it was shown that the RV had a reduced total number of cardiomyocytes, with no change in cardiomyocyte size, compared to adult sheep born at

term [9]; the effects on the left ventricle were not reported in that study. Furthermore, a comprehensive autopsy study found a marked reduction in the proliferation of cardiomyocytes in preterm neonates (born between 23 and 36 weeks of gestation) relative to age-matched stillborn fetal controls [10]. These experimental studies in preterm sheep and preterm neonates have set the scene for a multitude of clinical and epidemiological studies published in the past decade showing that preterm birth is associated with structural remodelling of the cardiac ventricles, impaired postnatal cardiac function, and an increased risk of developing cardiovascular disease [5-7, 11-20].

Based on these earlier findings, it was proposed at the commencement of this thesis that the maladaptive cardiac remodelling associated with preterm birth would be inversely proportional to gestational age at birth. Thus, it was expected that the cardiac remodelling would be more severe in the lamb studies in this thesis (where the lambs were born at 0.85 of full term gestation and required neonatal intensive care, including postnatal ventilatory support for postnatal survival) when compared to our earlier sheep studies (where the lambs were moderately preterm at 0.9 of gestation and did not require postnatal respiratory support). Interestingly, the findings of this thesis do not necessarily support this view in relation to cardiomyocyte growth.

Overall, it was found that preterm birth at 0.85 of gestation combined with modern clinical interventions had no observable adverse impacts on cardiomyocyte growth at either 2 or 5 months TEA. There were, however, subclinical alterations in the myocardium in early life which may potentially alter cardiac growth and function into adulthood. Although cardiomyocyte growth was not affected, we found that preterm birth results in an altered protein profile at 5 months TEA. Furthermore, our findings from a preliminary study using DTI suggest that the LV microstructure appears to have an immature phenotype (Chapter 5), as indicated by DTI measurements of helix angle and sheetlet angle. The apparent immaturity of the LV structure may affect the long-term growth of the heart and the adaptive capabilities of the LV when functionally challenged, for example, with hypertension, which is commonly observed in people born preterm. In addition, the spectroscopy analyses showed differences in the biochemical composition of the LV myocardium which implies underlying covert differences in cardiac

structure. Of particular concern, accelerated collagen deposition was found in the LV myocardium following very preterm birth, a finding replicated using three different experimental approaches. These findings suggest that preterm birth combined with perinatal treatments could lead to a reduction in the functional capacity of the myocardium and elevated risk of cardiovascular disease, especially if the increases in interstitial fibrosis become further exaggerated throughout life

6.2 Effects of preterm birth and perinatal treatments on cardiac anatomy and myocardial composition

6.2.1 Changes in gross anatomy and microstructure of the preterm LV

Cardiomyocyte microstructure in the LV was altered in lambs born preterm at 5 months TEA, a timepoint equivalent to a 6-year old child in terms of lung and heart development. Cardiomyocyte and sheetlet orientation in preterm lambs resembled an immature phenotype, similar to that found in fetal lamb hearts [21], when compared to lambs born at term. However, measurements of gross cardiac anatomy (LV wall volume, LV cavity volume, LV length, and LV lateral wall width) were not significantly different in preterm lambs at this timepoint (Chapter 5). These findings suggest that very preterm birth combined with modern clinical treatments results in a sub-clinically underdeveloped LV in early life.

There is currently only one study that has characterised both the gross anatomy and ventricular function of the heart in children born preterm. Six-year-old children born extremely preterm exhibit a unique cardiac phenotype characterised by smaller left ventricles than age-matched children born at term. This morphology was associated with altered systolic and diastolic function [22]. Specifically, LV contraction was more concentric (as opposed to a longitudinal, 'wringing' pattern) in the preterm children. Indeed, efficient wringing contraction of the LV is directly correlated to the highly organised orientation of the ventricular cardiomyocytes and sheetlets. It is currently unclear which biological or clinical factors influence the organisation of cardiomyocytes, in either the context of normal variation in healthy humans or in pathological phenotypes. It is likely that the immature phenotype of the

cardiomyocyte syncytium, observed in preterm hearts in the preliminary study in Chapter 5, contributes to the abnormal systolic and diastolic function observed in preterm children.

We did not find any significant changes in the LV gross geometry of very preterm lambs at 5 months TEA. Interestingly, two echocardiographic studies have shown that the absolute measurements of LV size were smaller in 5-7 year old children born extremely preterm compared to age-matched controls [22, 23]; however, there were no statistically significant differences when adjusted for body weight, thus indicating that cardiac growth was proportional to body size

Subclinical changes in cardiac microstructure are usually subtle and often difficult to interpret. As follow up to the findings of the present study, functional cardiac DTI studies are necessary to determine whether this unique change in LV microstructure is associated with impaired cardiac function. In addition, follow-up studies are required to determine whether these DTI parameters are sensitive and reliable enough to potentially predict and/or diagnose cardiovascular diseases. As not all preterm individuals will develop cardiovascular disease later in life, it is important to understand which phenotypes are more likely to be at risk, and therefore determine which preterm patients require regular heart health check-ups.

6.2.2 Preterm birth does not affect total cardiomyocyte number

Proliferation of cardiomyocytes is the major determinant of cardiac size in early gestation [24]; this tapers off in late gestation and early neonatal life [25-28]. Later in gestation, most cardiomyocytes slowly begin to become terminally differentiated in preparation for the increased work load postnatally [29]. By birth, most cardiomyocytes exit the cell cycle, limiting the heart's potential to regenerate and restore function after significant injury. Cellular hypertrophy and deposition of the extracellular matrix then dominate as the major contributors to postnatal cardiac growth [30-32]; however, cardiomyocytes (or perhaps a subset of cardiomyocytes) retain the ability to proliferate postnatally up until adulthood [33-36]. In this regard, there are a number of studies that suggest that childhood is a critical window of development, when lifestyle factors can directly influence cardiomyocyte proliferation. For example,

exercise during juvenile life in rat studies has been shown to stimulate cardiomyocyte proliferation, thus increasing the complement of cardiomyocytes within the heart.

Cardiomyocyte endowment at birth has long been considered one of the important indicators of life-long cardiac functional capacity, given the limited capacity for cardiomyocytes to divide after birth. This is of concern, in the case of preterm infants given that they are born early and may have not yet reached their full cardiomyocyte number potential. It was hypothesised that preterm lambs requiring postnatal ventilation would have a reduced total number of cardiomyocytes within the LV postnatally and subsequent compensatory cardiomyocyte hypertrophy, rendering the preterm heart susceptible to impaired cardiac function.

Contrary to our hypothesis, there was no difference in total cardiomyocyte number in the LV of preterm and term lambs at 2 or 5 months TEA (Chapter 3). These findings support the results of one study in moderately preterm sheep without requiring postnatal ventilation, where neither the LV nor RV showed changes in total cardiomyocyte number at 9 months TEA [8]. There remains the possibility that hyperplasia of cardiomyocytes continues in the preterm hearts after birth. Further studies are required to determine whether this is the case.

Interestingly, although no differences in cardiomyocyte number between preterm and term lambs were detected in this study or in the previous by Bensley *et al.* (2010) at a postnatal timepoint, equivalent to childhood in humans [8], in another study from our laboratory, the total number of cardiomyocytes in the RV was found to be reduced in adult sheep born preterm; the LV was not examined in that study [9]. It was unclear whether the reduction in the number of cardiomyocytes in the preterm RV in adulthood was due to a reduction in postnatal cardiomyocyte proliferation or an increase in postnatal cardiomyocyte apoptosis or necrosis. To address this research question, an autopsy study in human neonates born preterm (between 23 and 36 weeks of gestation) found a marked reduction in the proliferation of cardiomyocytes relative to age-matched stillborn neonate controls [10]. This suggests that preterm birth is, indeed, a perinatal insult that can affect total cardiomyocyte number in the heart

later in life. Therefore, it may be the case that total cardiomyocyte number will differ between our preterm and term-born lambs once they reach adulthood. This highlights a clear need to investigate postnatal cardiomyocyte development at later timepoints in our model of preterm birth.

6.2.3 Changes in the myocardial interstitium

Given that cardiac fibrosis has such an adverse effect on the structure and function of the heart, it is imperative to determine the underlying causes of the fibrosis, which can then be targeted therapeutically. Importantly, in this regard, given that maladaptive increases in fibrosis have been reported in the myocardium following preterm birth [8], a major focus of this thesis was to conduct an in-depth structural investigation of the myocardial extracellular compartment of the former-preterm LV (the major pumping chamber of the heart postnatally). Using three complementary techniques (histology, diffusion tensor imaging, and FTIR micro-spectroscopy), we found that collagen is upregulated in the preterm LV myocardium (Chapters 3 and 4) and that the extracellular compartment appeared to be increased in the preterm LV (Chapter 5). While the levels of collagen (detected histologically) in the LV were low at this relatively early stage in life, the effect of preterm birth on collagen deposition was highly significant ($p = 0.0015$) (Chapter 3). If this accelerated deposition of collagen persists and becomes further exaggerated in postnatal life, this may have major adverse impacts on cardiac function.

Cardiac fibrosis is a major contributor to cardiac dysfunction and disease. Collagen is a major component of the extracellular matrix that ensures the structural integrity of adjoining cardiomyocytes. Historically, the extracellular matrix was thought to be a static component of biological tissue, but it is now well recognised that the collagen network is a dynamic structure which plays a fundamental role in myocardial adaptation. The myocardial interstitium is highly controlled and organised whereby small disruptions in composition or content can lead to altered myocardial systolic and/or diastolic performance by impairing elastic recoil of the myocardium as the myocytes relax [37]. Excessive deposition of collagen, known as fibrosis, is a response to pathological stress regulated by cardiac

fibroblasts [38]. Fibrosis is a well-established feature of many cardiac diseases such as heart failure and myocardial infarction [39], but is poorly understood in the context of preterm birth. One echocardiography study found that six-year-old children (a timepoint equivalent to our preterm lambs at 5 months TEA) born extremely preterm had an impaired diastolic filling pattern, indicative of a stiffer LV wall, compared to their age-matched term-born counterparts [22]. This pathophysiology is potentially a result of excess collagen deposition in the LV. The findings of this thesis and other studies [8] support this concept.

The underlying mechanisms of cardiac fibrosis are as diverse as the etiologies that give rise to cardiovascular disease, since there are a number of ways in which mediators can stimulate cardiac fibroblasts to produce collagen (e.g. angiotensin II, aldosterone, and catecholamines) [38]. Fibrosis can be grossly divided into two types: reparative (where collagen deposition replaces damaged myocardium as scar tissue) and reactive (where diffuse collagen deposition occurs in the absence of cell death) [38]. The distinction between the two is important, as these processes have different triggers, mechanisms, and consequences and this is an important area for future research. In the present study, it was not possible to differentiate between these two mechanisms, as to which was responsible for the increased interstitial collagen in the LV myocardium. Both mechanisms are plausible, and it is conceivable that both mechanisms come into play in the neonatal heart. Given that the preterm heart is prematurely exposed to the hemodynamic transition at birth it is likely that there may have been loss of cardiomyocytes in the immature preterm myocardium leading to reparative fibrosis. Although we found no evidence of cardiomyocyte fibrosis at 2 or 5 months TEA in the preterm hearts, these findings do not inform of the potential adverse impacts on cardiomyocytes in the early neonatal period.

Alternatively, the premature exposure to postnatal hemodynamics may have led to reactive collagen deposition to adapt to the increased cardiac demands. Reactive fibrosis occurs homogenously throughout the myocardium (interstitial fibrosis), however it may also develop in the tissue surrounding intracardiac blood vessels (i.e., perivascular fibrosis), depending on the stimulus. While we did not

measure perivascular fibrosis in our histological study, Bensley *et al.* (2010) found no differences in perivascular fibrosis (adventitia area/luminal area) between lambs born moderately preterm and at term at 9 weeks TEA [8]. Pathologies that increase pressure load and wall stress on the heart, such as aortic stenosis [40] or systemic hypertension [41], have been shown to trigger diffuse reactive fibrosis in the heart. Indeed, the preterm LV is structurally underdeveloped at birth, and must overcome a sudden increase in pressure caused by the haemodynamic transition combined with mechanical ventilation, vasopressive drugs, and/or volume replacement treatment soon after birth. These stressors may be the cause of diffuse, reactive fibrosis in the preterm LV in early life. It is important to determine the underlying causes and pathogenic mechanisms of the early abnormal deposition of collagen in the preterm heart in future studies.

Furthermore, diseases or conditions that trigger an inflammatory response, either systemically or locally, can cause reactive fibrosis to develop [38]. Antenatal glucocorticoids, commonly used to accelerate lung development in preterm babies, have anti-inflammatory properties [42], which could potentially have beneficial anti-fibrotic effects on the heart. Our preterm lamb model includes a clinically relevant dose of dexamethasone, so it is possible that very preterm birth alone would have elicited a more severe fibrotic response in the heart. Further studies are required to elucidate this hypothesis.

6.3 Preterm birth: an emerging risk factor for cardiovascular disease

Maladaptive structural remodelling of the heart has clinical implications beyond impaired cardiac function. Long-term sequelae such as heart failure and chronic kidney disease may develop if impaired cardiac function persists without preventative care or treatment. Our findings suggest that preterm birth combined with modern perinatal treatments results in early structural remodelling of the left ventricle, thus rendering preterm individuals susceptible to developing a multitude of cardiovascular diseases in adulthood (Fig. 1). This highlights the need to further characterise the unique structure of

the preterm heart to understand the underlying mechanisms which make preterm adults at a higher risk of developing cardiovascular diseases.

To gain a comprehensive picture of preterm birth as an emerging risk factor for cardiovascular disease, future research should look beyond the heart. Structural remodelling following preterm birth also occurs in other organs, including the lungs [43], kidneys [44], and brain [45]. It is currently unclear how multiple organ systems interplay in preterm individuals. The addition of non-cardiac diseases associated with preterm birth (such as pulmonary hypertension, bronchopulmonary dysplasia, glomerulosclerosis, chronic kidney disease, and cerebral palsy) could have additional negative effects on cardiac growth and function, and thus could also contribute to the development of cardiovascular disease.

Moreover, the addition of commonly known lifestyle risk factors of cardiovascular disease, including physical inactivity, smoking, alcohol consumption, and obesity, could further render preterm individuals more susceptible to poor cardiovascular outcomes. Regular evaluation and long-term monitoring of general and cardiac health may be necessary in this patient population. This highlights the need for medical records to include - and medical experts to consider - gestational age at birth as a key factor that contributes to overall health outcomes.

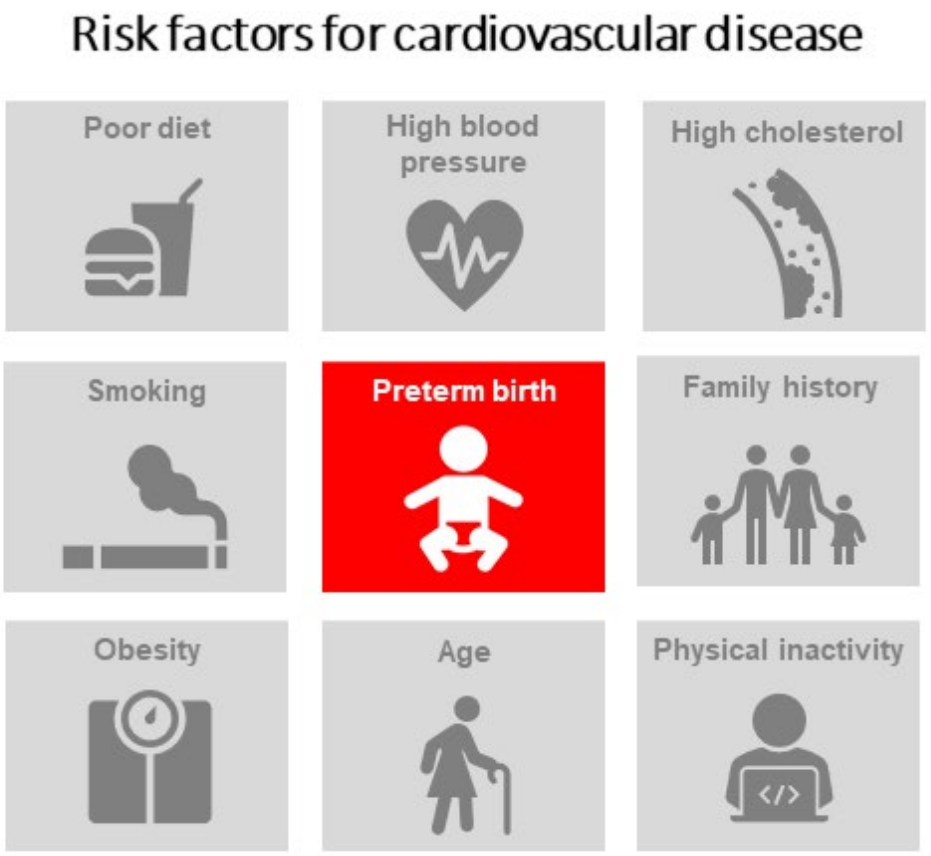

Figure 1. Preterm birth is a novel risk factor for cardiovascular disease, alongside other commonly known risk factors.

6.4 Limitations

Previously, studies exploring the effects of preterm birth on the heart have used animal models of moderate/late preterm birth, where the animal has reached a stage of lung development that does not require postnatal respiratory support. These studies are the first to use a clinically relevant model of moderate preterm birth in lambs to characterise cardiac development and structure at 2 and 5 months TEA. The advantages of this model include the novel consideration of perinatal treatments that are currently being used in neonatal intensive care units for preterm babies; this includes both mechanical and non-invasive respiratory support, administration of antenatal glucocorticoids, exogenous surfactant treatment, and administration of caffeine citrate.

As with all animal models, however, there are limitations that need to be considered when interpreting the findings of these studies. The control groups consisted of lambs born at term without requiring any antenatal treatments. Therefore, we cannot conclude that preterm birth alone was the cause of the cardiac remodelling seen in these studies. Current clinical interventions used to facilitate lung function in preterm neonates may further exacerbate cardiac remodelling following preterm birth. One MRI study has shown that postnatal ventilation in young adults born preterm accounted for some RV structural remodelling, but ultimately did not affect RV function [6]. It is currently unclear whether gentler forms of postnatal ventilation, such as non-invasive respiratory support, has any effect on the RV. As yet, no studies have examined whether postnatal ventilation affects the structure of the developing LV in preterm individuals. Similarly, the long-term impact of antenatal glucocorticoid treatment on cardiac structure and function has not yet been elucidated.

Other limitations of these studies include the technical restrictions involved with using formalin-fixed tissue. As we only had access to archived tissue, we were unable to perform experiments which require fresh, frozen tissue, such as western blot, flow cytometry, RNA sequencing, proteomics, and genomics, which would allow us to further validate our histological findings. However, we were able to support our findings of increased collagen in the LV using alternative techniques such as Fourier-transform infrared micro-spectroscopy and, to a lesser extent, diffusion tensor imaging. Another study that was excluded from this thesis involved a novel imaging system called HistoIndex (HistoIndex® Pte. Ltd, Singapore), which utilises second harmonic generation microscopy and an automated image analysis software to measure collagen content and structural composition. Unfortunately, the validation of this technique in cardiac tissue was unsuccessful (further discussed in subsection 6.6).

Another major limitation of this thesis is the lack of functional outcomes. My thesis included the retrospective analysis of fixed hearts collected since 2008 and I was not involved with the functional measurements of these animals. While alterations in cardiac structure and biochemical composition are often accompanied by changes in physiology, we do not know whether these preterm lambs

experience any impairment in cardiac function by 5 months TEA. Commonly used techniques to measure cardiovascular function in sheep include echocardiography, pulse oximetry, sphygmomanometry, pulmonary artery catheterisation, ultrasound, pulmonary capillary wedge pressure test, and functional MRI. The addition of these techniques in our preterm lamb model would allow us to determine typical parameters of cardiac function, such as heart rate, systolic and diastolic function, cardiac output, and cardiac contractility; importantly, this would allow us to compare our experimental results to clinical data in former-preterm children [22, 46, 47].

Finally, a limitation of Chapter 5 is the low number of animals included in the DTI analyses. There were two key factors which led to the exclusion of 6 hearts from this study. Firstly, we were unable to extract accurate diffusion tensors from the voxels in several hearts due to ghosting artefacts in the dataset, potentially caused by an offset in the resonance frequency. Secondly, portions of the RV and the apex of the LV were cut out of the scan due to an inexperienced user operating the DTI scanner. As a result, we had to exclude the right ventricle from the DTI study completely. Due to the low number of animals with complete LV scans (n = 3/group), we consulted with a biostatistician from Monash University and agreed to not statistically compare the DTI measurements between groups. Therefore, Chapter 5 is considered a preliminary study that supports a potential time-efficient diagnostic role for DTI at autopsy in detecting abnormal changes in the microstructure of the preterm heart, which could otherwise only be elucidated through destructive and time-consuming histology.

6.5 Future directions

The novel findings and limitations of this thesis have highlighted the need for future directions of research. In order to build from our findings, five major areas need to be explored in order to elucidate the biological mechanisms underlying the development of cardiovascular disease in individuals born preterm.

6.5.1 Characterising cardiac development in preterm lambs at later postnatal timepoints

Considering we found evidence of early structural remodelling in the former-preterm heart, the next obvious step is to determine whether this remodelling worsens with age. Recent studies have shown that impaired cardiac function becomes clinically apparent in preterm-born adults [4-7, 13]. However, the pathophysiological mechanisms of adverse long-term cardiac outcomes in preterm individuals remain poorly understood. Future studies using later timepoints in our ovine model of preterm birth would help elucidate the timing and mechanisms underlying the pathogenesis of cardiac diseases associated with preterm birth.

6.5.2 Proteomic analyses of the ventricular myocardium following preterm birth

The micro-spectroscopic findings from Chapter 4 show drastic changes in the biochemical profile of the LV myocardium in preterm lambs at 2 and 5 months TEA. In particular, the secondary structure of proteins in the preterm LV was significantly shifted towards a β-sheet confirmation. While FTIR micro-spectroscopy is a technique that cannot determine which proteins with β-sheet confirmation are being upregulated, this important finding suggests that myocardial remodelling occurs in the preterm heart at a stage of life as early as 2 years postnatal age in humans. It is necessary, therefore, to employ proteomic techniques, such as mass spectrometry-based methods or immunoassays, to determine overall protein signatures of the myocardium and identify key proteins that could contribute to impaired ventricular function following preterm birth. In particular, cardiac contractile proteins and proteins involved in the biochemical pathways leading to collagen deposition are of interest.

6.5.3 Elucidating the impacts of perinatal treatments on cardiac development following preterm birth

As mentioned in Section 6.4, a major limitation of the studies in this thesis is the lack of control groups that could account for the perinatal treatments used in the preterm lamb group. Consequently, the findings from this thesis cannot conclude that preterm birth alone results in the cardiac remodelling reported in our studies. The addition of two experimental groups - preterm lambs without perinatal

treatments and term lambs with perinatal treatments – in future experiments could, in theory, address this important limitation. However, preterm lambs delivered at this gestational age develop respiratory failure and cannot survive without perinatal respiratory support, making this experimental design practically unfeasible.

A possible alternative is to include a group of preterm lambs that are noninvasively supported from birth to determine whether intubation and mechanical ventilation are contributing factors to the cardiac remodelling seen in these studies. Furthermore, varying the dosage and number of repeated courses of dexamethasone could elucidate the role of antenatal glucocorticoids on cardiac development and structure. However, it is already known that the effects of antenatal corticosteroids on lung maturation are dose dependent [48]. Furthermore, the short and long-term effects on other organ systems, particularly the central nervous system, after different doses and repeat courses of glucocorticoids require further research. Therefore, it would be difficult to know whether glucocorticoids per se are directly contributing to cardiac remodelling or whether they indirectly affect cardiac development via other organs. These research questions are currently being addressed by another member of our laboratory.

6.5.4 Measuring functional outcomes in the preterm heart

Based on our findings from this thesis, we hypothesise that an increase in collagen deposition within the LV myocardium would initially result in a stiffer ventricle and diastolic dysfunction in preterm lambs. If excessive collagen deposition in the LV continues throughout the life of preterm lambs, this would lead to cardiac fibrosis. In turn, the activation of matrix-degrading pathways would be triggered, eventually resulting in systolic failure [49]. To test this hypothesis, functional measurements are required at multiple postnatal timepoints in the preterm lambs. Specifically, monitoring systolic and diastolic function from 2 months TEA to adulthood via echocardiography and/or MRI would be feasible in our preterm lamb model. This would also allow us to determine whether the preterm lambs develop cardiovascular complications that are commonly seen in humans born preterm, such as heart failure or

ischaemic heart disease [4]. Furthermore, blood pressure measurements throughout the postnatal life of the preterm lambs are necessary to see if hypertension, another cardiovascular outcome common in preterm adults, develops.

Interestingly, one echocardiographic study has shown that young adults born preterm had an impaired LV response to physiological stress when subjected to physical exercise [13]. When exercising on a seated stationary cycle ergometer, ejection fraction and cardiac output reserve was lower in preterm subjects compared to term-born controls. Hemodynamic exercise testing can be performed in sheep using a motorised treadmill to determine pulmonary and cardiac function [50]. This methodology could, therefore, be performed in our preterm lamb model to determine whether myocardial functional capacity is reduced.

Preliminary findings from Chapter 5 suggest that the microstructure of the LV myocardium in preterm lambs appears to have an immature phenotype, in terms of cardiomyocyte helix and sheetlet angle, compared to lambs born at term. The functional implications of this unique myofiber phenotype are still unknown. *In vivo* DTI has previously been used in humans and sheep to determine how DTI parameters relate to cardiac function [51-54]. In this regard, future studies using *in vivo* DTI are required to explore how the immature microstructure of the preterm heart affects cardiac function, and whether this phenotype persists or worsens with age.

6.5.5 Investigating the impact of preterm birth at earlier gestational ages on cardiac remodelling

The preterm lambs included in this thesis were delivered at the earliest gestational age at which the lambs were viable (0.85 of gestation, equivalent to approximately 32 weeks' gestation in humans). However, extremely preterm babies born as early as 22 weeks' gestation (0.59 of gestation) are now surviving, thanks to modern clinical treatments [55, 56]. As we currently cannot extend the limit of viability beyond 128 days' gestation in our preterm lamb model, alternative large-animal models need to be considered.

The ideal animal model of human preterm physiology should produce viable neonates with maturation characteristics similar to a very preterm human infant, and have a body size that allows comparative monitoring, blood sampling, and clinical interventions, such as mechanical and non-invasive assisted ventilation. Sheep are the most common large-animal model of preterm birth used in postnatal ventilation studies. The youngest a postnatally ventilated lamb has been delivered is 125 days , however survival was less than 3 hours [57]. This is because lung development in sheep and other non-primate mammals (such as pigs, rodents, and dogs) at full term is immature in comparison to humans [58-60].

Non-human primates are advantageous in biomedical research thanks to their phylogenetic closeness to humans. Chronically ventilated baboons have previously been used to study lung, kidney, and brain development following preterm birth [61-63]. Preterm baboons can be delivered as early as 0.65 of gestation with lung development at full term similar to that in humans [58]. Similarly, macaques have been used in preterm birth studies to explore lung development and systemic inflammation [64, 65], although these animals were not ventilated. Unfortunately, analyses of cardiac development or structure were not performed in any of these non-human primate studies. The lack of clinically-relevant non-human primate models of preterm birth is due to the high expenses involved in maintaining a 24 hour intensive care research facility and the ethical concerns surrounding non-human primate research. However, more clinically-treated infants are now surviving after extremely preterm birth and their long-term health outcomes are currently unknown. Thus, there remains a strong rationale and value for using non-human primate models in preterm birth research.

6.5.6 Reducing long-term cardiovascular risk in adults born preterm

When preterm birth cannot be prevented, it is important to consider potential preventative approaches throughout the postnatal period that could mitigate some of the long-term cardiac remodelling that occurs as a result of preterm birth. One study has shown that breast milk consumption for preterm infants can reduce long-term cardiovascular risk. Young adults that were born preterm and were exclusively fed human milk during infancy had increased LV and RV end-diastolic volume (+9.73%, p =

0.04 and +18.2%, $p < .001$) and stroke volume index (+9.79%, $p = 0.05$ and +22.1%, $p = 0.01$) compared with preterm-born young adults who were exclusively formula fed as infants [66]. Essential growth factors, such as vascular endothelial growth factor, in breast milk may have protective effects on cardiac and vascular development, particularly during this early postnatal period when angiogenesis and vasculogenesis is occurring. Heart mass in adulthood could also be influenced by exercise training in childhood. Adult rats (24 weeks old) that underwent four weeks of exercise training during juvenile life (5-9 weeks of age; equivalent to childhood or adolescence in humans) had sustained increases in LV mass and wall thickness, compared to adult rats that led a sedentary life [67]. It is possible that early exercise training in individuals born preterm could attenuate long-term cardiac remodelling, thus improving myocardial functional reserve in adulthood, however this hypothesis is yet to be tested.

6.6 Experimental challenges in my PhD

As is the case in many scientific investigations, there were many challenges encountered during my PhD. Two major studies were excluded from this thesis due to technical issues in the laboratory. The first study involved using stereology to assess capillary density, total capillary length, and the media:lumen ratio of intramyocardial arteries in the cardiac ventricles of each lamb group. The second study was a collaborative project with Associate Professor Chrishan Samuel and Sadman Bhuiyan from the Pharmacology Department at Monash University. This study involved a novel imaging technique called Second Harmonic Generation microscopy to determine collagen distribution and structure within the myocardium of lambs born preterm and at term.

6.6.1 Issues with tissue fixation

Although every effort was made to ensure that every heart was arrested in diastole and perfusion fixed immediately after the heart was excised, some areas of the ventricles in some heart samples were poorly fixed. Sections stained with toluidine blue viewed via light microscopy revealed that some blood

vessels within the myocardium were collapsed, and others were filled with erythrocytes. As a result, we were unable to stereologically assess capillary density or total capillary length in our samples.

Furthermore, we were unable to determine cardiomyocyte volume and cardiomyocyte nuclearity using a novel technique developed by a previous member of our laboratory [68], since staining with Wheat Germ Agglutinin-Alexa Fluor 488 to visualise cell membranes in thick histological sections (40 µm thick) resulted in patchy staining in the myocardial areas that were poorly fixed. Despite this setback, we were able to determine cross-sectional area of cardiomyocytes using thin tissue sections (5 µm thick) stained with Wheat Germ Agglutinin-Alexa Fluor 488 (results reported in Chapter 3).

6.6.2 Using Second Harmonic Generation microscopy to quantify changes in collagen content and structure

Following on from the findings in Chapter 3 regarding excessive collagen deposition in the LV myocardium following preterm birth, we began a collaborative study with Associate Professor Chrishan Samuel and Sadman Bhuiyan from the Fibrosis Laboratory in the Department of Pharmacology at Monash University. The aims of the study were to use second harmonic generation microscopy to: 1) validate our histological findings, and 2) compare the distribution and structural assembly of collagen in the LV myocardium between preterm and term-born lambs.

Fibrillar collagen is a biomolecule with a unique structure, in that it is made of proteins organised in a triple helix that is not centrosymmetric [69]. Non-centrosymmetric materials, such as fibrillar collagens, have a unique optical property, in that they can uniquely emit strong second harmonic generation light when excited with an intense laser light. Second harmonic generation is a nonlinear optical phenomenon, whereby two photons with the same frequency (produced from an intense laser light source) combine when passing through non-centrosymmetric material (fibrillar collagen), generating a new photon with double the frequency, or half the wavelength, of the initial photons (collected by a light detector) [70]. Second harmonic generation microscopy has, therefore, become a promising imaging technique that can detect collagen in unstained biological tissue. By eliminating the use of

staining, this method removes the risk of false positive results caused by artefacts or background staining occasionally observed in collagen stains (such as picrosirius red, Masson's Trichrome, Van Gieson's stain) and collagen immunohistochemistry.

In relation to this, a novel imaging system has been developed called HistoIndex (HistoIndex® Pte. Ltd, Singapore), which combines second harmonic generation microscopy with a novel automated image analysis software called FibroIndex. This method has been successfully used to quantify collagen content and characterise collagen structure in fixed liver and skeletal muscle tissue [71-73]. The HistoIndex can determine several characteristics of collagen distribution, including percentage area, average fiber density, branching and orientation. Given the impacts on collagen deposition I had observed using my other methodologies, in collaboration with Prof Samuel and Masters student Sadman Bhuiyan, I embarked on a comprehensive series of studies that utilised the HistoIndex system to determine whether these collagen characteristics differ in the LV myocardium of preterm versus term-born lambs. Unfortunately, however, although I spent considerable effort and time in conducting and trouble-shooting these techniques, I was unable to obtain meaningful results and, hence, the findings were not suitable for publication.

Firstly, we aimed to validate the HistoIndex findings against traditional light microscopy. Briefly, unstained, paraffin-embedded LV tissue sections were dewaxed, dehydrated, then mounted onto glass slides. The sections were scanned (Genesis 200, Histoindex, Singapore), then analysed using the image analysis software FibroIndex to determine the percentage area of collagen (among other collagen parameters). The same sections were then stained with picrosirius red, and analysed using ImagePro Plus software to determine the percentage area of collagen (full details of this protocol are described in Chapter 3). We used a linear regression analysis to compare the results obtained via HistoIndex with the results obtained using light microscopy. The correlation between the two results was extremely low (R^2 = 0.06) due to autofluorescence issues in the cardiac tissues when conducting the Histoindex

analyses. As a result, we did not perform any further statistical analyses to compare the preterm and term groups.

To date, the computational algorithm in the Fibroindex software has not been validated in cardiac tissue. While it is unclear why the heart tissue reacted differently to other biological tissues that were validated in the HistoIndex system, we believe the presence of cardiac myosin, which is known to cause low levels of autofluorescence, could have contributed to the inaccurate HistoIndex results. Cardiac myosin filaments also have a non-centrosymmetric structure, meaning they can also generate a second harmonic generation signal, albeit to a much lesser extent compared to collagen [74]. As we were unable to retrieve accurate results from the HistoIndex and complete the second aim of this study, we ultimately decided to exclude this project from this thesis.

6.7 Conclusions

In conclusion, this thesis has shown that preterm birth combined with modern perinatal treatments result in structural and biochemical alterations in the LV myocardium in lambs. While cardiomyocyte growth itself was not affected in either ventricles, alterations in cardiomyocyte orientation and the biochemical composition of the LV myocardium suggest that the preterm heart is compromised in early life, thus rendering preterm individuals susceptible to developing cardiovascular diseases. The findings from this thesis add to a growing recognition that preterm birth disrupts the growth and development of many organs, resulting in adverse long-term health consequences. These studies not only provide evidence of cardiac remodelling following preterm birth and its associated clinical treatments, but also insights into the potential mechanisms by which this occurs.

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