Preterm birth and the immature cardiovascular system

Department of Anatomy and Developmental Biology
School of Biomedical Sciences
Monash University, Australia

Jonathan Guy Ablett Bensley
2014

A thesis submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy
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Summary

Preterm birth (defined as birth prior to 37 completed weeks of gestation) affects 8-10% of all live births worldwide. Preterm birth introduces the cardiovascular system of the baby to the *ex utero* environment before cardiovascular development and maturation is complete. Being born necessarily causes a profound haemodynamic change within the cardiovascular system, including a shift from right ventricular dominance to left ventricular dominance, closing of the ductus arteriosus and foramen ovale, and an increase in heart rate and arterial blood pressure. The broad aim of this thesis was to understand how preterm birth affects the development of the immature cardiovascular system. To achieve this aim I have used an ovine model of preterm birth, and autopsy material from human infants. Due to the imposition of *ex utero* life on an immature and therefore ill-prepared cardiovascular system, it was hypothesised that preterm birth would lead to an early termination of cardiomyocyte proliferation, abnormal cardiomyocyte maturation, increased interstitial collagen deposition, and thicker aortic blood vessels with a reduced lumen diameter.

Prior to the commencement of the studies described in this thesis, relatively little was known about how preterm birth affected the development and maturation of the cardiovascular system.

The aim of the first experimental chapter in this thesis (Chapter 2) was to examine the effect of preterm birth on the development and maturation of the cardiomyocytes (heart muscle cells). Preterm lambs were born at a time-point approximating moderately preterm birth (ie. 133 days of the 147 day gestation in sheep (0.9 of term); this is developmentally equivalent to ~32 weeks of human gestation. Lambs born at term were used as controls. The hearts of the lambs were examined at 9 weeks post-term equivalent age. To determine whether or
Summary

not preterm birth had an effect on the development and maturation of cardiomyocytes, I utilised stereology to examine the number of cells within the heart, confocal microscopy to measure the size, nuclearity and ploidy (the number of genome copies per nucleus) of the cardiomyocytes, and picrosirius red staining to examine the amount and distribution of collagen within the heart. This study demonstrated that at 9 weeks post-term equivalent age, lambs born preterm, compared with controls, had hearts with enlarged cardiomyocytes in both the left and right ventricles, abnormal maturation of the cardiomyocytes (remaining mononucleated but became polyploid) and increased interstitial collagen deposition. The publication arising from this study was the first published study to examine the effect of preterm birth on the development and maturation of cardiomyocytes.

The aim of the second experimental chapter (Chapter 3) was to examine how preterm birth affects the morphometry and composition of the great vessels (the aorta and pulmonary artery), using the same ovine model of preterm birth as in the previous chapter. This is important as the aorta and pulmonary artery change their roles dramatically at the time of birth. The aorta in utero is exposed to relatively low systolic/diastolic pressures, but high systolic/diastolic pressure after birth. The pulmonary artery in utero supplies a large proportion of the blood to the whole body (via the ductus arteriosus) and experiences systemic arterial pressure, whereas ex utero it supplies only the pulmonary circulation and its pressure is considerably lower than systemic arterial pressure. The results of this study showed that preterm birth led to thickening of the aortic wall coupled with a narrower lumen, but without any changes in pulmonary artery morphometry. The composition of vessel walls was also altered, in that the aortae from the preterm lambs had increased elastin and reduced smooth muscle; the pulmonary artery only had increased elastin
Summary

deposition. The most concerning finding were areas of wall injury in the aortae from preterm lambs, which was not observed in lambs born at term.

Chorioamnionitis is an inflammatory process, usually resulting from a microbial infection, affecting the chorion, amnion, umbilical cord and amniotic fluid; it is the most common antecedent of human preterm birth. The aim of the third experimental chapter (Chapter 4) was to determine how chorioamnionitis affects the development and maturation of cardiomyocytes, using an ovine model of acute chorioamnionitis. On day 120 of the 147 day gestational period (0.82 of term), LPS (lipopolysaccharide, a protein from the outer coat of gram negative bacteria) was injected under ultrasound guidance into the amniotic sac. Seven days later (127 days, 0.86 of term), the fetus was humanely killed and the heart removed for examination. I used confocal microscopy to examine the number, size, maturation and ploidy of the cardiomyocytes, immunohistochemistry to measure cell proliferation and picrosirius red staining to measure the amount and distribution of interstitial collagen within the heart. Lambs exposed to LPS, compared to controls, had smaller mononucleated (immature) but larger binucleated (mature) cardiomyocytes, a reduction in estimated cardiomyocyte number and increased capillary density. The reduction in estimated cardiomyocyte number was offset by an 8-fold rise in cardiomyocyte proliferation in LPS exposed lambs.

The aim of the fourth experimental chapter (Chapter 5) was to assess the effects of preterm birth on development of the human heart. To achieve this aim I obtained archival heart tissue from the Women’s and Children’s Hospital in Adelaide, South Australia; the fixed infant cardiac tissue was obtained at perinatal autopsies following written consent from the parents. I analysed heart tissue from 13 infants born preterm, but who subsequently died
having lived for at least one day. As controls I analysed heart tissue from 17 stillborn infants who died in utero from acute causes. I used confocal microscopy to examine cardiomyocyte volume, maturation, and ploidy. Cell proliferation was measured using immunohistochemistry and interstitial collagen deposition was measured using picrosirius red staining and image analysis. The results of this study demonstrated that there was no effect of preterm birth on heart weight (absolute or relative to bodyweight) or on cardiomyocyte volume, maturation and ploidy. Cardiomyocyte proliferation was negligible in all but one of the preterm born infants, as early as 24 hours of birth. There was a small, but statistically significant increase in collagen deposition in the hearts of preterm born infants.

In order to perform the cardiac analyses necessary for these studies, I developed an improved method for morphometrically analysing cardiomyocytes in thick paraffin sections (Chapter 6). Ordinarily, it is almost impossible to identify the boundaries of cardiomyocytes in paraffin sections stained with haematoxylin and eosin. Wheat germ agglutinin (a lectin) bound to a fluorescent dye has previously been used to delineate cell boundaries in many tissues. I used wheat germ agglutinin-Alexa Fluor 488 to stain the cardiomyocyte cell membranes and a nuclear dye (e.g. DAPI, YOYO-3) to identify the nuclei. Utilising confocal microscopy and image analysis I was able to quantitatively analyse cardiomyocytes in thick (≥40µm) paraffin sections including measurement of cell volume, nuclearity (number of nuclei per cell) and ploidy (number of genome copies per nucleus). This improved technique enabled the studies described in this thesis to proceed, and will be helpful to others in better characterising heart tissue embedded in paraffin, such as autopsy specimens.
Summary

In conclusion, the results of my studies demonstrate that preterm birth, and one of the major risk factors for preterm birth (intrauterine inflammation), has potentially serious adverse effects on the development and maturation of the heart and major arteries. My findings suggest that people born preterm are likely to be at increased risk of developing cardiovascular disease in later life; hence it is important in future studies to closely examine the postnatal growth and structure and function of hearts and arteries from adults who were born moderately, very and extremely preterm. The monitoring of cardiovascular health throughout life in individuals born preterm would enable early intervention and possibly prevention of overt cardiovascular disease.
General Declaration

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

I hereby declare that approximately one-third of the stereology data (assessment of cardiomyocyte number) reported in Chapter 2 was included in my honours thesis entitled “The effect of premature birth on the structure of the heart” (2007). Additional analyses were conducted in the 1st, 2nd and 3rd years of my PhD to build on these interim findings.

Except as noted above, 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 three original papers published in peer reviewed journals and two unpublished publications. The core theme of the thesis is the effect of preterm birth on the development and maturation of the immature cardiovascular system. The ideas, development and writing up of the published papers comprising Chapters 2 and 3, and the cardiac structural analyses sections of the paper in Chapter 4 were the principal responsibility of myself, the candidate, working within the Department of Anatomy and Developmental Biology, School of Biomedical Sciences, under the supervision of Associate Professor M Jane Black, Professor Richard Harding and Dr Robert De Matteo.
The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. In chapter 4, I had no involvement in the animal or physiological studies. In chapter 5, the autopsies were performed by Professor Lynette Moore and/or her colleagues at the Women’s and Children’s Hospital in Adelaide, South Australia, Australia.

In the case of Chapters 2-6 my contribution to the work involved the following:

<table>
<thead>
<tr>
<th>Thesis chapter</th>
<th>Publication title</th>
<th>Publication status</th>
<th>Nature and extent of candidate’s contribution</th>
</tr>
</thead>
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<tr>
<td>2</td>
<td>Cardiac remodelling as a result of pre-term birth: implications for future cardiovascular disease</td>
<td>Published</td>
<td>Performed experimental analyses (including a small contribution to the animal studies), data analysis and wrote manuscript. Total Contribution: 75%</td>
</tr>
<tr>
<td>3</td>
<td>Preterm birth with antenatal corticosteroid administration has injurious and persistent effects on the structure and composition of the aorta and pulmonary artery</td>
<td>Published</td>
<td>Performed experimental analyses (including a small contribution to the animal studies), data analysis and wrote manuscript. Total Contribution: 75%</td>
</tr>
<tr>
<td>4</td>
<td>Exposure to intrauterine inflammation leads to impaired function and altered structure in the preterm heart of fetal sheep</td>
<td>Published</td>
<td>Performed fibrosis, capillarisation, cardiomyocyte volume, nuclearity, ploidy and maturation component of the study. Performed some data analysis and co-authored manuscript. Total Contribution: 40%</td>
</tr>
<tr>
<td>5</td>
<td>Preterm birth adversely impacts cardiomyocyte proliferation in the developing human heart</td>
<td>Unpublished</td>
<td>Performed experimental analyses (except autopsies), data analysis and wrote manuscript. Total Contribution: 90%</td>
</tr>
<tr>
<td>6</td>
<td>An improved method for 3D measurement of cardiomyocyte volume, nuclearity and ploidy in thick histological sections</td>
<td>Unpublished</td>
<td>Performed experimental analyses and wrote manuscript. Total Contribution: 90%</td>
</tr>
</tbody>
</table>
General Declaration

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: [Redacted]

Date: 27/7/15
Acknowledgements

This thesis would not have been possible without the love, help, support and care of extraordinary people.

Foremost are my supervisors, Associate Professor Jane Black, Professor Richard Harding and Dr Robert De Matteo. Thank you for all your generosity, time, advice, help and support throughout my honours and PhD.

I owe an immense debt of gratitude to my parents, Stuart and Barbara. I will never be able to repay their faith in me, and their love, kindness, generosity and understanding (and cooking, and waiting up for me). Without them, this thesis would have been impossible to complete. I’m immensely grateful to my late grandmother, Roma, for her love and for always believing in me. Thank you to my brothers, Nicholas and Andrew, for their support.

I must thank Ian Boundy and Camilla Cohen, at Monash Histology Platform, for employing me and allowing me to learn the art of histology. Thank you to my friends and colleagues, Sue Connell, Kasia Eassom, Julie Hickey, Associate Professor Jeffrey Kerr, Dr Jelena Kezic, Andrew Lim and Stefania Tombs.

I have also received invaluable technical support and advice from the staff of Monash Micro Imaging and wish to thank Dr Judy Callaghan, Steven Firth, and Professor Ian Harper. I am also thankful to Dr Lucy D’Agastino from Invitrogen/Life Technologies for her expertise.

I must acknowledge the huge help from the technical support teams at the following organisations: ANSYS, Information Sciences Institute (University of Southern California, USA), PerkinElmer Improvision, Bitplane, Invitrogen, Sigma-Aldrich, Nikon, Leica, Olympus, Aperio, Molecular Devices, MathWorks, Wolfram Research, IBM SPSS, CellProfiler (Carpenter
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Lastly, I must thank my wonderful friends. I’m exceedingly lucky and grateful to have such wonderful people in my life. So a massive thank you to Jenny Krohn, Dr Ryan Wood-Bradley, Dr Chantal Cincotta, Dr Megan Sutherland, Anne O’Connor, Sarah Henry, and Benjamin Barzel.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>ANCOVA</td>
<td>Analysis of Co-Variance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AOTF</td>
<td>Acousto-Optical Tuneable Filter</td>
</tr>
<tr>
<td>AR</td>
<td>Adrenoreceptor</td>
</tr>
<tr>
<td>BPM</td>
<td>Beats Per Minute</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>dGA</td>
<td>Days of Gestational Age</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra-Cellular Matrix</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
</tr>
<tr>
<td>IR</td>
<td>Ischaemia Reperfusion</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LV+S</td>
<td>Left Ventricle (with Septum)</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimetres of mercury</td>
</tr>
<tr>
<td>MP</td>
<td>Multiphoton</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kB</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>Partial pressure of arterial Carbon Dioxide</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Partial pressure of arterial Oxygen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier Tube</td>
</tr>
<tr>
<td>PNA</td>
<td>Post-Natal Age</td>
</tr>
<tr>
<td>PTEA</td>
<td>Post-Term Equivalent Age</td>
</tr>
<tr>
<td>RV</td>
<td>Right Ventricle</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine Receptor</td>
</tr>
<tr>
<td>SaO₂</td>
<td>Saturation (arterial) Oxygen</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>TEA</td>
<td>Term Equivalent Age</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor α</td>
</tr>
<tr>
<td>TTZ</td>
<td>Triphenyltetrazolium</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>WGA-AF488</td>
<td>Wheat Germ Agglutinin-Alexa Fluor 488</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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</tbody>
</table>


Conference Abstracts


7. **Bensley, J. G.,** Stacy, V. K., De Matteo, R., Harding, R. & Black, M. J. Maladaptive cardiac remodelling as a result of preterm birth: Implications for future
cardiovascular disease. Developmental Origins of Health and Disease Congress (DoHAD 2009) – Santiago, Chile. (Poster presentation)


16. Bensley, J.G., Moore, L., De Matteo, R., Harding, R., **Black, M.J.** Cardiomyocyte growth and maturation during mid to late gestation and the effect of preterm birth. Fetal and Neonatal Physiological Society 2011 – Palm Cove, Queensland, Australia (Poster presentation)
Chapter 1

Literature Review
1.1 Preterm Birth

At the present time, approximately 10% of babies born worldwide are born preterm\(^1\). Preterm infants are born at a time when their organ systems are immature. To date, relatively little is known about how being born before term affects the development of the immature cardiovascular system; this is of particular importance given that it is now well recognised that the antecedents of cardiovascular disease may originate very early in life.

<table>
<thead>
<tr>
<th>Completed Weeks of Gestation</th>
<th>CDC Classification (USA)</th>
<th>WHO Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>34 to 37</td>
<td>Late/Near Preterm</td>
<td>Moderately Preterm</td>
</tr>
<tr>
<td>32 to 33</td>
<td>Moderately Preterm</td>
<td>Very Preterm</td>
</tr>
<tr>
<td>28 to 31</td>
<td>Very Preterm</td>
<td>Extremely Preterm</td>
</tr>
<tr>
<td>22 to 27</td>
<td>Extremely Preterm</td>
<td>Incompatible with Life</td>
</tr>
<tr>
<td>0 to 22</td>
<td>Incompatible with Life</td>
<td>Incompatible with Life</td>
</tr>
</tbody>
</table>

Table 1: Classifications of preterm birth by completed weeks of gestation. CDC = Centers for Disease Control and Prevention (United States of America)\(^2\). WHO = World Health Organisation (United Nations, Switzerland)\(^3\).

1.1.1 Preterm Birth – Defining preterm birth

Preterm birth (also termed premature birth) is defined as birth prior to 37 completed weeks of gestation (timed since a woman’s last menstrual period) and can be further sub-classified into moderately preterm (32-37 weeks), very preterm (28-31 weeks) and extremely preterm (22-27 weeks), as described in Table 1. The majority of preterm infants (84% of all preterm births) are born moderately preterm (32-37 weeks)\(^4\).

The classification of late/near preterm was added by the CDC (Centers for Disease Control and Prevention, United States of America) because of evidence demonstrating that, although infants born at that age (34 to 37 weeks) are usually treated in the same way as term infants, they do appear to have long-term adverse effects as a result of being born.
slightly early\textsuperscript{5,6}. Throughout this introduction the WHO (World Health Organisation, United Nations, Switzerland) classification of preterm birth will be used, except as noted.

1.1.2 Preterm Birth – Limit of viability

Birth prior to 23 completed weeks of gestation is associated with extremely poor outcomes in regards to survival (<1% surviving to discharge)\textsuperscript{7}, especially survival without neurological defects (e.g. cerebral palsy) or behavioural disturbances (0% having no measurable defect or disturbance)\textsuperscript{8,9} and is hence termed the limit of viability\textsuperscript{10}. Because of these poor outcomes, the American Academy of Pediatrics and the American Heart Association recommend that active resuscitation not be attempted on neonates prior to 23 completed weeks of gestation and the neonate be given palliative care\textsuperscript{11}.

Although in the past few decades the limit of viability has been lowered to gestational ages previously thought impossible, at the present time a gestational age of 23 weeks appears to be the lowest that can be achieved in the absence of an artificial placenta\textsuperscript{12}. The inability to survive prior to 23 weeks relates to many organ systems, especially the lung, being too immature to sustain independent life.

1.1.3 Preterm Birth – Epidemiology

Over the past few decades, the incidence of preterm birth has been generally increasing in developing countries, as shown in Table 2. In 2011, the preterm birth rate was 11.7% in the United States of America (USA) and 8.3% in Australia. It is important to note that the incidence of preterm birth differs according to ethnicity; for example, rates are higher in African Americans, Aboriginal Canadians and Aboriginal Australians compared to those with other ethnic backgrounds in the USA, Canada and Australia, respectively. Statistics on
Chapter 1 – Literature Review

Preterm birth among Aboriginal Canadians are unreliable, primarily due to poor reporting\textsuperscript{13}, and are not included in Table 2.

The vast majority of all preterm births fall into the moderate preterm birth (32-37 weeks) (Table 1) category. The USA is the only large country which publishes detailed records of how many births fall into each gestational age category, and these statistics appear in Table 3 (presented as percentages of total preterm births). Canada provides data on the total preterm birth rate and moderate preterm birth rate but, very and extremely preterm birth are pooled; this data appears in Table 4 (presented as percentages of total live births). The percentage of preterm births falling into each category has not changed appreciably over the 2000 to 2010 period\textsuperscript{13,14}. Data from other jurisdictions does exist, but is complicated by the inclusion of some pregnancy terminations (e.g. State of Victoria, Australia), exclusion of small numbers to protect privacy, and unreliable data collection. Data for Canada does not include any births that took place in the province of Ontario, but does include births from women resident in Ontario that gave birth in any other Canadian province or territory. There is also a considerable delay between data collection and publication of preterm birth data/statistics for whole countries; delays are up to 10 years in some cases or in other cases, the data is published every 2-5 years. The most recent data, encompassing the 2011 calendar year, for the United States and Australia was published in 2013.
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<tbody>
<tr>
<td>World All</td>
<td>Births All</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9.6%</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Africa All</td>
<td>Births All</td>
<td>-</td>
<td>-</td>
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<td>11.2%</td>
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</tr>
<tr>
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<tr>
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<tr>
<td>Britain All</td>
<td>Live Births All</td>
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</tbody>
</table>

Table 2: Percentages of all births that occurred preterm in that calendar year. “All births” in the Australian data means that the data includes live births, stillbirths, and abortions (in certain jurisdictions of Australia for terminations after 20 weeks gestation due to lethal fetal malformations or threat to maternal life). Data for Canada does not include Ontario. Stratified by race.
Table 3: Percentages of all preterm births which occurred in the categories of near/late + moderate (pooled), very and extremely preterm. Stratified by race and year. Data may not add up to 100% due to rounding. CDC Classification system used. Near/Late + Moderate (pooled) equates to Moderate in the WHO Classification System. Data calculated from raw data extracted from National Vital Statistics System (Centers for Disease Control and Prevention, United States of America).

<table>
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</thead>
<tbody>
<tr>
<td></td>
<td>Near/Late + Moderate (32-37 weeks)</td>
<td>All</td>
<td>83.6%</td>
<td>83.9%</td>
<td>84.1%</td>
<td>84.3%</td>
<td>84.2%</td>
<td>84.3%</td>
<td>84.2%</td>
<td>84.1%</td>
<td>83.9%</td>
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<tr>
<td></td>
<td></td>
<td>White</td>
<td>85.6%</td>
<td>85.7%</td>
<td>85.8%</td>
<td>85.9%</td>
<td>85.9%</td>
<td>86.0%</td>
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<td>85.7%</td>
<td>85.4%</td>
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<tr>
<td></td>
<td></td>
<td>Black</td>
<td>77.0%</td>
<td>77.5%</td>
<td>77.6%</td>
<td>78.2%</td>
<td>78.1%</td>
<td>78.2%</td>
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<tr>
<td></td>
<td>Very (28-31 weeks)</td>
<td>All</td>
<td>10.4%</td>
<td>10.2%</td>
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<td>10.0%</td>
<td>10.1%</td>
<td>9.9%</td>
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<tr>
<td></td>
<td>Extremely (22-27 weeks)</td>
<td>All</td>
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</table>

Table 4: Percentages of all live births in Canada (except Ontario), which were preterm (22 to 37 weeks gestation), moderately preterm (32-37 weeks) and very + extremely preterm (pooled) (22-32 weeks). Data may not add up due to rounding. This data does not contain births from the province of Ontario. Data prepared from Perinatal Health Indicators for Canada 2013, published by the Public Health Agency of Canada, Canadian Perinatal Surveillance System.  

<table>
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<th>Group</th>
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<th>2004</th>
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<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
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<td></td>
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<tr>
<td>All Preterm (22-37 weeks)</td>
<td>7.5%</td>
<td>7.6%</td>
<td>7.9%</td>
<td>8.20%</td>
<td>7.9%</td>
<td>8.0%</td>
<td>7.7%</td>
<td>7.9%</td>
<td>7.6%</td>
<td>7.7%</td>
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<tr>
<td>Moderate (32-37 weeks)</td>
<td>6.4%</td>
<td>6.5%</td>
<td>6.7%</td>
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<td>6.8%</td>
<td>5.9%</td>
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<td>6.8%</td>
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<td>Very and Extremely (22-32 weeks)</td>
<td>1.1%</td>
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<td>1.1%</td>
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<td>1.2%</td>
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</tbody>
</table>

Table 3: Percentages of all preterm births which occurred in the categories of near/late + moderate (pooled), very and extremely preterm. Stratified by race and year. Data may not add up to 100% due to rounding. CDC Classification system used. Near/Late + Moderate (pooled) equates to Moderate in the WHO Classification System. Data calculated from raw data extracted from National Vital Statistics System (Centers for Disease Control and Prevention, United States of America).
1.1.4 Preterm Birth – *Induced and spontaneous preterm delivery*

There are two primary causes of preterm birth. The first is medically indicated (induced) preterm birth, which is the induction of birth or caesarean delivery due to a fetal or maternal disease process, or where continuation of the pregnancy is impossible or poses high risk to the health of the mother and/or offspring. The most common reasons for medically indicated preterm birth based on maternal health are eclampsia, pre-eclampsia, gestational diabetes, diabetes (Types 1 and 2), HELLP (Haemolysis, Elevated Liver enzymes, Low Platelet count) syndrome, malignancy and physical trauma/violence. Pregnant women who have a congenital cardiac defect or acquired cardiac disease are also sometimes induced to deliver preterm to relieve haemodynamic stress which could otherwise endanger the mother’s life.

Fetal indications leading to induced preterm birth include fetal distress, congenital abnormalities not immediately incompatible with life, intra-uterine growth restriction (IUGR), chorioamnionitis, intrauterine infection, placental infarction, placental abruption, placental insufficiency, placenta previa and conditions incompatible with life (e.g. bilateral renal agenesis, anencephaly). Induced preterm birth removes the fetus from a hostile or unfavourable intra-uterine environment that is no longer able to support the pregnancy. In the case of conditions incompatible with life, it is often considered appropriate to terminate the pregnancy or deliver the fetus preterm as survival of the fetus *ex utero* is impossible.

Within the last 10 years, the rate of induced or medically indicated preterm birth has risen much faster than the rate of spontaneous preterm birth. The reasons for this are multifactorial, but may partially relate to the more litigious and therefore more risk-averse practice of obstetrics in most developed countries.
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The second primary cause is spontaneous preterm birth, where preterm birth occurs without medical intervention. Depending on the background population, spontaneous preterm birth accounts for 60-85% of all preterm births\textsuperscript{63}.

1.1.5 Preterm Birth – \textit{Risk factors for spontaneous preterm birth}

In the majority of spontaneous preterm deliveries, no definitive cause of the preterm birth is recorded. This is likely due to preterm birth being multi-factorial in origin and the absence of an investigation into the cause of individual preterm births. Although the aetiology of preterm birth is not fully understood, there are a number of risk factors for preterm birth that have been identified and some of the frequently reported factors associated with preterm delivery are shown in Table 5. The presence of chorioamnionitis, a bacterial infection of the chorion, amnion and placenta, is often associated with the induction of spontaneous preterm delivery, especially in those born very and extremely preterm.
<table>
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<th>Risk Factor</th>
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<tbody>
<tr>
<td>Alcohol Consumption (Moderate to Heavy)</td>
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<tr>
<td>Bacterial Vaginosis</td>
<td>69-71</td>
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<tr>
<td>Body Mass Index (Underweight or Overweight)</td>
<td>72-76</td>
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<td>Chorioamnionitis</td>
<td>56, 77, 78</td>
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<td>Cocaine Use</td>
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<tr>
<td>Depression, Anxiety, Domestic Violence and Psychological Distress</td>
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<tr>
<td>Family History of Preterm Birth (Genetic)</td>
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<td>Fetal Malformation/Congenital Defect</td>
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<td>Gestational Diabetes/Pre-existing Diabetes</td>
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<td>HIV Infection</td>
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<td>IUGR (Intra-Uterine Growth Restriction)</td>
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<td>IVF (In Vitro Fertilisation)/Assisted Reproduction</td>
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<td>Limited/No Antenatal Care</td>
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<td>Low Socio-Economic Group</td>
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<td>Maternal Heart Disease (Acquired/Congenital)</td>
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<td>Maternal/Paternal Race (African American/Aboriginal)</td>
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<td>Maternal/Paternal Smoking</td>
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<td>Multiple Gestation (Twins or greater)</td>
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<td>Older Maternal Age (&gt;35 Years)</td>
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<td>Physically Demanding Occupation</td>
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<td>Poor Oral Hygiene</td>
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<td>Pre-Eclampsia/Eclampsia</td>
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<td>Prior Preterm Delivery</td>
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<td>Sexually Transmitted Infections</td>
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<td>Short Cervix</td>
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<tr>
<td>Young Maternal Age (&lt;12-16 Years)</td>
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</tr>
</tbody>
</table>

Table 5: Risk factors associated with the induction of spontaneous preterm birth (arranged in alphabetical order).
1.1.6 Preterm Birth – Chorioamnionitis

In cases where an investigation into the cause of a preterm birth event takes place, the most frequently identified risk factor is chorioamnionitis\textsuperscript{172}. Chorioamnionitis is the only risk factor and putative cause of preterm birth for which the underlying mechanism is understood. The most frequently identified organisms are \textit{Gardnerella vaginalis}, \textit{Escherichia coli}, \textit{Ureaplasma urealyticum}, \textit{Mycoplasma hominis}, \textit{Neisseria gonorrhoeae}, \textit{Chlamydia trachomatis} and \textit{Streptococcus agalactiae} (Group B Streptococcus)\textsuperscript{55, 56}.

The diagnosis of chorioamnionitis is often complicated by a number of factors; it can also often remain undetected throughout gestation and may only be evident at birth (either at term or preterm), or go entirely unnoticed. The gold standard for diagnosing chorioamnionitis is amniotic fluid culture, Polymerase Chain Reaction (PCR) and placental histology; however, obtaining a sample suitable for culture is challenging. After rupture of the fetal membranes, a sample is likely to become contaminated with organisms present in the vagina. A particular pathogen may be present in the vagina, producing bacterial vaginosis, but may never have been present in amniotic fluid. It is particularly important to emphasise this because 29.2\% of reproductive age women in the United States have bacterial vaginosis; however, only 15.7\% of those women have any overt symptoms\textsuperscript{173}. Ideally, the sample should be obtained during caesarean section, but this is only possible at some caesarean deliveries. PCR analysis is an important and sensitive diagnostic tool\textsuperscript{174, 175} as it can demonstrate clearing infections (very low organism numbers), poorly growing organisms and viral infections. Diagnosis can also be achieved on other samples: fetal bronchiolar alveolar lavage fluid (BALF), the placenta, fetal membranes and cord blood\textsuperscript{176}.

There is also evidence from high-throughput sequencing that there are many more species
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of microbes, some previously unknown, which may be involved in chorioamnionitis\textsuperscript{177}. Histology of the placenta and fetal membranes generally provides a good indication as to the severity and natural history of the infection\textsuperscript{178-180}.

The administration of antibiotic therapy after confirmed chorioamnionitis, or after PPROM (Preterm Premature Rupture Of Membranes) is accepted therapy and increases the time to delivery\textsuperscript{181-183}.

The effect of antibiotic administration during pregnancy to reduce the risk of preterm birth is a subject of considerable debate. Trials to evaluate the effectiveness of antibiotic therapy to treat abnormal vaginal flora and bacterial vaginosis during pregnancy have been problematic, and trials have demonstrated both positive\textsuperscript{184, 185} and negative\textsuperscript{186-189} results. The most recent meta-analysis by the Cochrane Collaboration suggests no benefit of antibiotic therapy during mid to late gestation\textsuperscript{190}. This may relate to the high recurrence rates for abnormal vaginal flora (69% at one year) and bacterial vaginosis (58% at one year)\textsuperscript{159}.

1.1.7 Preterm Birth – Genetics of preterm birth

Single Nucleotide Polymorphisms (SNP) are single nucleotide changes in genes that can affect gene regulation, function and downstream signalling. Using SNP microarrays and high throughput sequencing, it has been determined that several SNPs are associated with an increased probability of preterm delivery. Interestingly, almost all of the SNPs identified are related to cytokine gene expression, innate immune function and cell surface markers\textsuperscript{87, 88, 90, 91}. These SNPs may affect a woman’s susceptibility to chorioamnionitis and the immune response to the infection. SNPs may also partly explain the heritability of preterm birth.
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Women who are born preterm are more likely to deliver a preterm baby themselves; being of African American or Aboriginal (Australian, Canadian) maternal/paternal race also greatly increases the risk of preterm birth\textsuperscript{93}.

1.1.8 Preterm Birth – Survival and costs

Due to a number of innovations, particularly the administration of antenatal corticosteroids, the current survival rate for a neonate born at 32 weeks is almost identical to that of a term infant\textsuperscript{191}. Survival to hospital discharge improves substantially between 23 and 32 weeks of gestation (Figure 1). This improvement in survival for preterm neonates has not come without significant costs for health systems but also in quality of life for those born preterm, and for parents and caregivers\textsuperscript{192}.

The resources required to care for neonates born as early as 23 weeks gestation are enormous\textsuperscript{193}. The cost to the US health system in 2005 in caring for preterm babies was US$26 billion, with most of this cost relating to care in the NICU\textsuperscript{194}. This cost does not include ongoing care for disabilities or other problems that occur after hospital discharge.

Individuals born preterm, especially those born extremely and very preterm, are frequently more vulnerable to lifelong disease processes, directly related to preterm birth (e.g. brain injury) and the interventions necessary to keep them alive (e.g. ventilation); this subsequently leads to increased lifelong health costs. Other deleterious effects of preterm birth, or medical interventions around preterm birth, may not become apparent until adolescence or adulthood, and of particular relevance to this thesis are the potential long-term deleterious consequences of preterm birth on the cardiovascular system, leading to an elevated risk of cardiovascular disease.
Figure 1: Percentages of preterm infants born between 22 and 32 weeks gestation that survived to discharge, or died in the delivery suite or were admitted to the NICU. Data was collected across nine administrative regions of France in 1997. Graph prepared based on data from Larroque et al, Survival of very preterm infants: Epipage, a population based cohort study. Arch Dis Child Fetal Neonatal Ed 2004 89: F139-F144.¹⁹⁵
Cardiovascular disease is defined as any disease process or malformation affecting the heart and/or vasculature. This encompasses a large number of conditions, ranging from abdominal aortic aneurisms (AAA) to varicose veins. Cardiovascular disease can be broadly divided into congenital and acquired cardiovascular disease. Congenital cardiovascular disease is any cardiovascular disease process or malformation apparent in utero, at, or soon after birth. Acquired cardiovascular disease is any cardiovascular disease process that occurs after birth. As will be discussed later, this definition may be misleading as acquired cardiovascular disease can have its beginnings during very early life.\textsuperscript{196}

1.2.1  \textbf{Cardiovascular Disease – The leading cause of morbidity and mortality}

Acquired cardiovascular disease is of singular importance as the leading cause of death world-wide and it is the leading cause of death in almost every country in which records are kept. Globally, in 2008, 7.25 million people died of ischaemic heart disease (12.8\% of all deaths) and 6.15 million people died of cerebrovascular disease (10.8\% of all deaths).\textsuperscript{197} In Australia, in 2011, diseases of the circulatory system accounted for 31.0\% of all deaths; ischaemic heart disease was the leading cause of death, accounting for 14.6\% of all deaths.\textsuperscript{198} Cerebrovascular disease accounted for 7.7\%, heart failure for 2.4\%, hypertension for 1.2\% and cardiac arrhythmias for 1.1\% of all deaths in Australia in 2011. For comparison, the combined deaths from prostate and breast cancer accounted for 4.2\% of all deaths in Australia in 2011.\textsuperscript{199}
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Congenital abnormalities of the heart are the most common congenital defects, affecting up to 1% of all live births. In the first year of life, congenital defects of the heart are the leading cause of congenital defect related death\textsuperscript{200}.

1.2.2 Cardiovascular Disease – Pathogenesis

It is a commonly held view that cardiovascular disease, apart from congenital cardiovascular disease, is the result of an unhealthy lifestyle and is primarily a disease of smokers, the obese, unfit, and the elderly. However, it is important to recognise that cardiovascular disease often takes decades to develop, and it is now recognised that antecedents of cardiovascular disease can occur early in life. Hence, unhealthy lifestyle choices are likely to accelerate and/or exacerbate pathological processes that have been initiated very early in life\textsuperscript{201-203}.

The work of McNamara et al\textsuperscript{204} provides evidence that cardiovascular disease is a slow developing disease that may remain silent for several decades before manifesting into overt symptoms. McNamara et al examined hearts from American soldiers who died due to combat injuries in the Vietnam War\textsuperscript{204}, enabling the examination of hearts from apparently healthy young men aged from 18 to 37 years old (mean of 22.1 years old); 45% of subjects examined had atherosclerosis in at least one coronary artery and 5% of subjects had severe atherosclerosis in at least one coronary artery. This is unlikely to be an effect of the changes in the American diet around the early 1960s-early 1970s, as there are comparable findings from American soldiers who died due to combat wounds in the Korean War (combat operations from 1950-1953)\textsuperscript{205}. In the Korean War study, 77.3% of the soldiers had evidence of coronary atherosclerosis and their age distribution was very similar to that of the Vietnam War cohort (mean age of 22.1 years at death). A more recent study performed in
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2,876 autopsy cases\textsuperscript{206} (due to what the authors describe as “external causes”, mostly traumatic death) from 1987 to 1994 in the USA demonstrated that every person examined, aged 15-19 years, had at least one intimal lesion and at least one fatty streak in the thoracic and abdominal aorta, and 50-60\% also had at least one intimal lesion and at least one fatty streak in their right coronary artery. In a study of autopsied human fetuses there was clear evidence of small atherosclerotic lesions in most of the fetuses examined\textsuperscript{207}.

1.3 Developmental Origins of Health and Disease

The pathological processes, such as atherosclerosis, associated with cardiovascular disease usually develop over decades before symptoms become overt. The disease process may begin before birth as there is now mounting epidemiological evidence linking the \textit{in utero} environment with long-term health and disease susceptibility. This idea was first articulated by David Barker and his colleagues in 1989\textsuperscript{208}, and has since evolved as the ‘developmental origins of health and disease’ hypothesis (DOHAD)\textsuperscript{203, 208, 209}.

The DOHAD hypothesis proposes that a sub-optimal or abnormal environment during early life alters the biochemistry, epigenetics and/or physiology of an individual in such a way as to increase disease risk later in life\textsuperscript{208, 209}. This is often referred to as developmental programming, although it is not predicting a definite outcome but rather refers to an increased risk of an adverse event occurring.

The underlying basis of developmental programming is that an impaired intrauterine environment (e.g. fetal hypoglycaemia, hypoxemia), whether acute or chronic and regardless of causation, can induce adaptive responses in the fetus that allow it to survive the challenge. If the challenge is sufficiently severe, the fetus may not be able to survive.
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Fetal responses to an impaired intrauterine environment include physiological responses such as redistribution of blood flow, anatomical alterations (e.g. altered organ development) or changes to the epigenome. Together, these adaptive changes allow the fetus to survive an intrauterine challenge, but may induce developmental alterations which are long lasting, such as altered organ development or epigenetic alterations. If this process works well, the fetus will survive to be born, but will carry deficits that can affect its later development or vulnerability to disease processes later in life.

1.3.1 Developmental Origins of Health and Disease – In Utero Growth Restriction (IUGR)

Birth weight, length and head circumference are considered to be good indictors of how an infant grew in utero\textsuperscript{203, 210}. As fetal growth is largely determined by the intrauterine environment (i.e. placenta size, nutrient and oxygen availability), which in turn may be influenced by the maternal environment (i.e. maternal nutrition, living at high altitude, disease), fetal growth can be used as a proxy for the health of the intrauterine environment. When charting fetal growth from large cohorts, tables have been drawn up for male and female fetuses/infants to project the normal growth trajectory that can be expected in body weight, length (height) and head circumference\textsuperscript{211}. When the weight of a fetus or infant falls below the 10\textsuperscript{th} percentile for their sex and age group, the fetus or infant is determined to be growthrestricted\textsuperscript{212}. When this occurs in utero it is called intra uterine growth restriction (IUGR) (contrasted with ex utero growth restriction if growth is stunted after birth).

The original study by Barker et al examined the relationship between birth weight, birth at one year of age, and the risk of cardiovascular disease risk later in life\textsuperscript{208}. This study was performed in 5,654 men born during 1911 to 1930. There was a strong relationship between body weight at birth, body weight at one year of age, and the risk of death from
cardiovascular disease. The lightest babies had the highest risk of death. Weeks of gestation at birth and weight at death were not recorded for this study.

There have been many other epidemiological studies following up on the initial findings of Barker and his colleagues, in particular examining the presence of ‘catch up’ growth. Overwhelmingly, these studies have demonstrated an association between low birth weight and an increased risk of cardiovascular disease. These correlations remain even when confounding factors such as economic status, maternal smoking status and other factors are taken into account. There are many studies that have demonstrated that it is accelerated or ‘catch up’ growth in IUGR infants that leads to greatest long-term cardiovascular disease risk. Catch-up growth may be harmful following IUGR because nutrient availability may exceed the ability of the infant to metabolise them; excess nutrients may be deposited as adipose tissue.

Other effects of catch-up growth are related to IUGR retarding organ growth. For instance, the development of nephrons (the functional unit of the kidney) only occurs during mid to late gestation, no new nephrons are formed after term birth. Nephron endowment is strongly related to birth weight, with IUGR babies having a reduced endowment at birth. If an IUGR born baby remains small (short in stature, low in body weight) throughout life, the nephron endowment may be sufficient for their needs; however, if the IUGR baby catches up in growth with normally grown babies, there are fewer nephrons per kilogram of body weight, and body surface area, than a baby born of normal weight with a normal nephron endowment. As no nephrons are formed in post-natal life, with a natural depletion of nephrons throughout life, IUGR born babies that caught up in growth with normally grown babies may not have sufficient nephrons to last their entire lifetime with normal
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renal function\textsuperscript{215,217}. IUGR born babies with catch-up growth may have an increased rate of nephron loss, owing to increased filtration demand compared with a kidney having a normal nephron endowment at birth, leading to an increased risk of hypertension in particular\textsuperscript{215-217}.

1.3.2 Developmental Origins of Health and Disease – Preterm Birth

Low birth weight can result from preterm delivery as well as IUGR. Many of the early epidemiological studies may include some moderately preterm infants, as accurate assessments of gestational age at birth were not available. However, very preterm and extremely preterm infants are unlikely to be included in these studies as these infants would not have survived.

Importantly, being born preterm and/or the causes of preterm delivery (e.g. IUGR, chorioamnionitis) may have the potential to predispose to disease processes in adulthood. The effects of preterm birth on an immature cardiovascular system and the long-term consequences for cardiovascular structure and function are relatively poorly understood. It is therefore important to gain an understanding of how the immature cardiovascular system of the preterm infant adapts to being born early and whether these adaptive changes may lead to an increase in long-term cardiovascular disease risk.

1.4 Consequences of Preterm Birth

Over recent decades, with advances in neonatal care, there has been a marked improvement in the survival of preterm infants. Preterm delivery also has the potential to lead to developmental programming, with gestational age at birth (and thus degree of organ immaturity) likely to adversely influence long-term health outcomes.
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Preterm birth introduces an underdeveloped fetus into the *ex utero* environment, with all its challenges, before most of the organ systems are fully mature. Unlike some laboratory animals, such as the rat, mouse, and rabbit, many of the pivotal events in organ maturation in the human take place *in utero*. Preterm birth requires many organs, such as the brain\textsuperscript{218, 219}, lungs\textsuperscript{220, 221}, kidney\textsuperscript{222, 223} and heart\textsuperscript{224, 225}, to function differently compared to their function in the intrauterine environment before organ maturation is completed. As a result, these organs may undergo adaptive mechanisms to cope with the altered functional requirements and this may render them prone to altered development or injury. Collectively, these functional changes at the time of preterm birth can have deleterious consequences to long-term organ function and increased disease risk.

1.4.1 **Consequences of Preterm Birth – The Brain**

Preterm birth can adversely affect the development of the central nervous system, as the preterm brain is very immature and sensitive, particularly in the setting of extremely and very preterm birth\textsuperscript{226}. Even moderate preterm birth has been shown to lead to inferior neurodevelopmental outcomes compared to people born at term\textsuperscript{218, 227}. Preterm birth also carries substantial risks of immediate injury, such as peri-ventricular and intra-ventricular haemorrhage\textsuperscript{228, 229}, white matter injury\textsuperscript{230-232} and hypoxic ischaemic injury\textsuperscript{233, 234}. There are also risks of later aberrant effects on brain function: reduced cognitive ability\textsuperscript{235-238}, cerebral palsy\textsuperscript{239, 240}, neurodevelopmental delay\textsuperscript{236, 241, 242}, epilepsy\textsuperscript{243}, attention deficit hyperactivity disorder (ADHD)\textsuperscript{244} and psychological problems\textsuperscript{245}.
1.4.2 **Consequences of Preterm Birth – The Respiratory System**

The lungs have also been the focus of a large research effort into how preterm birth affects their development and functional maturation. The immediacy of the requirement for a functioning respiratory system, at birth when the lungs are in an immature state of development, underlies this effort. *In utero*, the lungs are filled with liquid, and this provides the physical stretch required to promote normal development of the respiratory tree. The lungs also receive relatively little blood flow from the heart *in utero*, as the lungs do not need to perform a gas exchange role. *In utero*, the pulmonary vasculature is a high resistance, low flow circuit\(^{246}\). As the placenta is the site of gas exchange in the fetus, the foramen ovale (which connects the right and left atria) and the ductus arteriosus (which connects the pulmonary artery and the aorta), take ventricular output away from the pulmonary circulation and into the systemic circulation. From ~22 weeks to ~26 weeks of gestation the human lung is still in the canalicular stage of lung maturation\(^2^{220,247}\). Saccular development occurs from ~26 weeks gestation onwards towards birth. Some alveolar development occurs before birth, but most alveolar development in the human occurs after birth\(^247\).

One of the pivotal factors in lung maturation, of relevance to preterm birth, is the development and maturation of alveolar epithelial type II pneumocytes, which are the source of surfactant in the lung\(^2^{248,249}\). The surfactant lowers surface tension within the lung, making breathing easier and minimising damage to the lung. The production of substantial quantities of surfactant is a late event in human gestation, occurring past ~32-34 weeks gestation.
Some of the seminal achievements in neonatal care facilitating the survival of preterm babies are: the administration of corticosteroids, administration of surfactant and the development of gentler ventilation strategies. The administration of corticosteroids aids in rapidly maturing fetal lungs and increasing the production of surfactant within the lung. Betamethasone and dexamethasone are the two most commonly used corticosteroids in obstetrics. The administration of corticosteroids, both to the mother in the setting of threatened preterm birth, and to the infant after birth, lowers the incidence of respiratory distress syndrome (RDS), death from RDS and death from all causes. As an additional benefit, corticosteroid administration also lowers the risk of intra-ventricular haemorrhage in the brain. Administering either bovine origin or recombinant surfactant directly into the lungs via the intubation tube further enhances the beneficial effect of corticosteroids and has the advantage of working almost immediately. Older ventilation strategies had primarily been harsh mechanical ventilation strategies and also lacked the precision to deliver the small volumes required for preterm babies. More modern techniques, such as CPAP (Continuous Positive Airway Pressure) and NIPPV (Nasal Intermittent Positive Pressure Ventilation), help to minimise damage to the preterm lung, by preventing the lung from collapsing at the end of each respiratory cycle, and by responding to and supporting the baby’s own breathing movements. Injury to the lung is still common in very and extremely preterm infants, both immediately in the form of bronchopulmonary dysplasia (requiring supplemental oxygen at 34 weeks of gestational age) and later on in life as reduced lung function, increased asthma incidence and inferior exercise performance.
1.4.3 **Consequences of Preterm Birth – The Kidneys**

The kidney is also sensitive to preterm birth as it is undergoing development at this time. Nephrons, the individual functional units of the kidney, are formed primarily in late gestation and there are no new nephrons formed after birth\(^{222}\). Nephrons form from week 9 to approximately 36 weeks gestation and this is referred to as the nephrogenic period\(^{222}\). The nephron endowment reached by the end of the nephrogenic period is the apex of endowment. It has been demonstrated in human neonatal autopsy specimens that nephrogenesis does continue after preterm birth but the width of the nephrogenic zone (where new nephrons are formed) is markedly reduced in the kidney of preterm infants\(^{269}\). There is also evidence that many preterm kidneys have markedly abnormal nephrons\(^{222,269}\) (although variable between patients) which will probably never become functional. If nephrogenesis is terminated early in preterm babies, it is likely that they will start out life with a reduced endowment of nephrons, which will only decline with age\(^{222,269}\).

Of special significance for the kidney are the side-effects of certain antibiotic agents, such as the aminoglycosides antibiotics (e.g. gentamicin), which are nephrotoxic\(^{270}\), but are frequently a necessity for prophylaxis against and/or treatment of certain gram-negative bacterial infections\(^{271}\) and this will further harm the preterm kidney.

1.4.4 **Consequences of Preterm Birth – The immature cardiovascular system**

Prior to the commencement of my studies for this thesis, relatively little attention had been focused on how preterm birth affects the developing cardiovascular system and the potential to influence lifelong cardiovascular health and disease risk. Although there is clinical experience and research in managing low ventricular output, instability, low arterial
pressure and arrhythmias in preterm babies\textsuperscript{272-276}, very little attention has been focused on how the immature cardiovascular system of the preterm newborn adapts to the major hemodynamic transition at birth and whether there are long-term effects on cardiovascular structure and function which may result in an increased risk of cardiovascular disease in adulthood.

To date, evidence as to whether or not preterm birth is linked to long-term cardiovascular disease has been derived primarily from data on arterial pressure, blood vessel morphometry and peripheral vascularisation. This is because these parameters are easy to measure; measurements can be performed in living subjects and are considered to be predictors of future cardiovascular disease risk, even when overt cardiovascular symptoms are not present. It is important to re-emphasise that cardiovascular disease is a slow-developing disease and overt cardiovascular pathology usually takes decades to manifest. Survival after very preterm and extremely preterm birth is a relatively recent phenomenon; at the present time the oldest survivors of preterm birth are only now reaching their mid-30s\textsuperscript{277}. Hence, even though these preterm subjects may be prone to cardiovascular disease, overt symptoms may not yet be apparent. The accumulating epidemiological data to date suggest that these individuals may be more prone to cardiovascular disease. A recent study conducted using the Swedish Medical Birth Register, for all infants born in Sweden between January 1\textsuperscript{st} 1983 and December 31\textsuperscript{st} 1995 (n = 1,306,943), demonstrated that young adults (aged 18 to 30) born prior to 32 weeks had a rate of cerebrovascular disease 2-fold higher than young adults born at term\textsuperscript{278}. There was no effect on cardiovascular disease, as there were no ischaemic cardiovascular events in young adults born prior to 32 weeks gestation; there were 180 ischaemic heart events in the 1,306,943 people for whom records were
available. A total rate of ischaemic heart disease of 0.014% indicates that the people included in this study are much too young to begin to show a change in ischaemic heart disease, if one exists, in people born preterm.

Over recent years there has been a multitude of studies reporting an elevation in arterial blood pressure in subjects who were born preterm\textsuperscript{277, 279-288}. This is of major concern, given that elevated arterial pressure is a major risk factor for the development of cardiovascular disease and adverse cardiovascular events\textsuperscript{289, 290}. Importantly, very small changes in arterial pressure can have a very large effect on cardiovascular disease risk; for example, it has been estimated that a 4.7mmHg drop in blood pressure lowers stroke risk by 32% and ischaemic heart disease risk by 20%\textsuperscript{291}.

Numerous studies have now documented that preterm birth is a strong risk factor for increased arterial pressure in children\textsuperscript{279-281}, adolescents\textsuperscript{282-284}, young adults\textsuperscript{285-287} and older adults\textsuperscript{277, 288}. These studies have described an inverse relationship between gestational age at birth and systolic blood pressure. An Auckland based study of 458 preterm born subjects, examined at 30 years of age\textsuperscript{277}, found an increase in systolic blood pressure of 0.55mmHg for each week less than term birth (<37 weeks gestation). Another study performed in Sweden (which has compulsory military service for males) measured blood pressure in 329,495 male conscripts between 1993 and 2001\textsuperscript{286}, on average, an increase in systolic blood pressure of 0.31mmHg for each week less than term birth (<37 weeks gestation) was observed in these men.

The developing vascular tree is also adversely affected by preterm birth. Using ultrasound and laser Doppler, Edstedt Bonamy and her colleagues demonstrated that adolescent girls born preterm had narrower but more compliant abdominal aortae and lower peripheral skin
blood flow\textsuperscript{282}. Following up from these findings, they examined the aortae of adolescents born preterm using MRI; they demonstrated a 16\% narrowing in the thoracic aorta and a 19\% narrowing of the abdominal aorta in individuals born preterm, after correcting for body size and gender\textsuperscript{283}. Using intra-vital microscopy, the initial findings of lower peripheral skin blood flow were further examined. This study demonstrated that preterm born adolescents had a 7\% mean reduction in capillaries per square millimetre of skin\textsuperscript{279}.

1.5 \textbf{Heart Development}

In order to gain an understanding of how preterm birth affects the immature heart, it is important to understand the processes of cardiac development during normal gestation and the haemodynamic transition that takes place at birth.

Cardiac development begins very early in gestation, with the heart being the first organ to form and function in practically all animals with hearts. In order to meet the functional demands of the growing human embryo, the linear heart tube begins to pump blood at three weeks after fertilisation\textsuperscript{292} and by 10 weeks of gestation the heart is in a relatively mature form.

Development of what will become the human heart begins at 18-19 days after fertilisation. The initiating events in heart development are the formation of the two primary endocardial tubes in the cardiogenic area of the primitive embryo. Following lateral and cephalic folding of the primitive embryo, the tubes are brought into the midline and fuse to form a simple linear heart tube by 21 days gestation. Subsequently the linear heart tube undergoes a process of looping and folding which brings the primitive atria, ventricles and outflow tract into the spatial alignment of the mature heart\textsuperscript{293}. The heart then undergoes septation to
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divide the atria from the ventricles and to form the right and left atria and ventricles. Shunting of blood directly from the right atria to the left atria is permitted by the formation of an opening (the foramen ovale) between the two atria; this foramen usually closes soon after birth\textsuperscript{294}. Simultaneously, there is a division of the outflow tract of the heart with spiralling of the dividing septa aligning the aorta with the left ventricle and the pulmonary artery with the right ventricle. Whilst these developmental processes are occurring the atrioventricular valves are also formed between the atria and ventricles and the aortic valve between the aorta and left ventricle and the pulmonary valve between the pulmonary artery and right ventricle\textsuperscript{295}.

Given that the fetal heart forms very early in development (often before a woman knows she is pregnant) it is particularly vulnerable to developmental insults very early \textit{in utero} and subsequent congenital defects. Importantly, in the case of the preterm infant, given that the limit of viability is around 22 weeks of gestation, the heart is fully formed at the time of preterm delivery, even in the extremely preterm infants. However, the heart muscle is still growing and therefore it is highly likely that the growth of the cardiac muscle in the myocardium is altered as a result of preterm birth, especially owing to the marked changes in systemic and pulmonary haemodynamics at the time of birth. In this thesis I examine how the growth of the cardiac muscle changes as a result of preterm birth.

1.6 \textbf{The Cardiomyocyte}

Cardiomyocytes are the cardiac muscle cells which comprise the functional units of the heart, providing all of the contractile power of the heart. Over the lifetime of any animal, the heart muscle must adapt to normal growth, pathology and physical demands (e.g. exercise and pregnancy).
Mammalian cardiomyocytes all share some common characteristics associated with being a contractile cell. All cardiomyocytes require the presence of myosin and actin filaments; these are what enable the cell to contract. As a direct result of this, the nucleus or nuclei are located in the middle of the cell, with the contractile filaments enveloping them as they cannot pass through the nuclei. The contractile filaments within the cardiomyocytes are arranged in a precise manner in the form of sarcomeres and it is the presence of sarcomeres that give the cells a striated appearance under the light microscope. There is also a complex electrical conduction system within each cardiomyocyte, between cardiomyocytes and within the heart as a whole.

In general, growth of the heart during early gestation is due to proliferation of cardiomyocytes. Towards late gestation the cardiomyocytes undergo a maturation process whereby they become terminally differentiated in preparation for the haemodynamic transition at birth. In the case of rodents this maturation process occurs in the first two weeks after birth. In almost all species (except primates, pigs, zebrafish and a few others) the cardiomyocyte is a mononucleated diploid cell in its immature form and in the mature form it is a binucleated cell with diploid nuclei.

Immature cardiomyocytes in the rat, mouse and sheep heart can re-enter the cell cycle during the *in utero* period. In contrast, mature cardiomyocytes rarely re-enter the cell cycle. The development of binucleation is timed such that maturation is completed at or soon after birth. In the mouse, cardiac regenerative ability is lost by 2 days after birth.

In this thesis, extensive use has been made of a sheep model of moderate preterm birth. The development and maturation of sheep cardiomyocytes has been well studied compared to other species. The sheep has a relatively long gestational period of 147 days. Until day
~110 of gestation, almost all the cardiomyocytes (in both the left and right ventricle) are mononucleated (immature), with progressive maturation occurring such that at ~145 days gestation, >80% of cardiomyocytes will be binucleated (mature)\textsuperscript{207}.

Certain *in utero* factors can be induced experimentally in the sheep, which can alter the rate and nature of cardiomyocyte maturation and proliferation. For example, elevated thyroid hormone levels increased the percentage of binucleated (mature) cardiomyocytes by 31% in the left ventricle but reduced the proliferation rate by 39%\textsuperscript{302}. IUGR induced by placental embolization, between days 110 and 130 of gestation, led to a marked decrease in the percentage of binucleated cardiomyocytes in both ventricles\textsuperscript{303}. Reducing systolic pressure, with an Angiotensin Converting Enzyme (ACE) inhibitor, during late gestation (127-135 days of gestation) decreased cardiomyocyte proliferation in both ventricles by 80-90%\textsuperscript{304}. After inducing severe anaemia in the fetal lamb, between day 129 and 138 of gestation, increased the percentage of binucleated cardiomyocytes by 8% in the right ventricle, but did not alter cardiomyocyte proliferation characteristics\textsuperscript{305}.

Cardiomyocyte maturation in human and non-human primates it is quite distinct from other animals, and it happens over a longer timeframe. The human embryonic heart is composed overwhelmingly of mononucleated cardiomyocytes (also with a diploid nucleus), as in most other animals. After birth, for at least the first 12-14 years, this does not change significantly. It is not until approximately 12-14 years of age that human cardiomyocytes undergo their last stage of maturation, whereby many of the mononucleated cardiomyocytes become tetraploid (4n DNA within a single nucleus)\textsuperscript{306}. In the adult human heart, approximately 75% of cardiomyocytes are mononucleated and tetraploid, with the remainder being binucleated (or greater)\textsuperscript{307, 308}. 
1.7 Cardiomyocyte Endowment

The number of cardiomyocytes formed in the heart at birth is influenced by the growth of the heart in utero. As with any organ, there is evidence of a quorum sensing mechanism between the cells that compose that organ, to prevent overgrowth or to have too few cells. In the mouse heart, this endowment is set in late gestation and early post-natal life primarily by the action of Survivin. Survivin expression influences cardiomyocyte endowment by regulating cardiomyocyte mitosis and DNA replication.

The majority of studies examining the post-natal growth of the heart, specifically cardiomyocyte proliferation, have been conducted in the rodent. Interestingly, these studies have very rarely used stereological means to assess cardiomyocyte number, which is the gold standard for assessing cardiomyocyte endowment and change in number. Instead, these studies have relied on immunohistochemistry on fixed tissue, or in vivo lineage tracing, radioactive labels or nucleotide incorporation as a means to determine when new cardiomyocytes were created. A study in the mouse, using Bromodeoxyuridine (BrdU) to identify replicating cells showed that from 2 weeks after birth, mouse cardiomyocytes have almost completely exited from the cell cycle.

Humans appear to follow a different cardiomyocyte endowment pathway to that in the mouse. In humans, cardiomyocyte endowment is not set in place immediately after birth in humans, and the number of cardiomyocytes in the average heart was three times higher at 20 years than at birth.
From 1945 to 1964, above nuclear ground testing released large amounts of Carbon-14, which were then incorporated into cell components. Using mass spectroscopy to measure the incorporation rate, it was estimated that humans have a cardiomyocyte replacement rate per year of approximately 5% at age 15, falling to 0.5% at 60 years of age.306

Interestingly, in the mouse, Mahmoud and colleagues demonstrated that by knocking out Meis1, cardiomyocytes were able to re-enter the cell cycle and repair infarcts. As previously mentioned, cardiac regenerative ability is normally lost by around post-natal day 2 or so in the mouse; in the Meis1 knock-out mice this was extended to the end of the study at post-natal day 21.

There is also evidence to demonstrate that micro RNAs are also involved in the post-natal cell-cycle arrest and the progression towards cardiomyocyte binucleation found in most animals. When a member of the miR-15 family (miR-195) was over-expressed in the mouse, this led to a reduction in ventricular weight, fewer cardiomyocytes undergoing mitosis and more multinucleated cardiomyocytes (terminally differentiated) within the heart. Equally, when miR-15 was inhibited after birth, there was an increase in the number of mitotic cardiomyocytes.

However, it is likely that a markedly lower endowment at the beginning of life would lead to a smaller pool of cardiomyocytes to make up the full endowment as a person heads into young adulthood. If babies born preterm have cardiomyocytes that exit the cell cycle earlier than they should, they would begin life with a smaller base endowment of cardiomyocytes, that would lead to cardiomyocyte hypertrophy earlier in life to try to reach a normal sized heart with a normal cardiomyocyte endowment.
The topic of when and how cardiomyocyte endowment is established, and how cardiomyocyte endowment is maintained throughout life, has been a very controversial topic in the literature for many decades. Partly this relates to the species differences between the mouse and humans in cardiomyocyte growth and development. As mentioned above, humans do not reach their complete cardiomyocyte endowment until approximately 20 years of age, whereas the mouse achieves this in a few weeks after birth. How and why humans are different remains unexplained, as does the cellular mechanism for humans expanding their cardiomyocyte endowment throughout early life and replacing cardiomyocytes throughout their entire lifespan.\(^{306, 311}\)

1.8 The Haemodynamic Transition at Birth

As described in the previous section, cardiomyocytes in the human heart undergo a process of maturation late in gestation, with the majority of cardiomyocytes terminally differentiated by the time of birth. By undergoing maturation in utero, cardiomyocytes are able to cope with the major haemodynamic changes that occurs at the time of birth, whereby the cardiovascular system changes from an \textit{in utero} configuration to an \textit{ex utero} configuration.

Hence, a number of changes to the cardiovascular system are required at the time of birth in order to facilitate life independent of the placenta. The first of these is the physiological closure of the foramen ovale.\(^{275}\) Throughout gestation, the patent foramen ovale allows the shunting of blood from the right atria directly to the left atria, thus allowing blood to bypass the high resistance pulmonary circulation. At the time of birth, the resistance in the pulmonary circulation falls, the lungs are emptied of fluid, and the pressure on the right side of the heart falls dramatically. The corresponding increase in pressure within the left side of
the heart physically closes the valve of the foramen ovale which subsequently becomes structurally and thus permanently closed within a few weeks after birth.

The other major change is the closure of the ductus arteriosus. In utero, the ductus arteriosus connects the pulmonary artery and the aorta, allowing most blood flow entering the pulmonary artery from the ventricles to bypass the lungs. As mentioned previously, the pulmonary circulation is a high resistance and relatively low flow circuit in utero and therefore blood readily flows through the ductus arteriosus and into the aorta. Physiologic closure of the ductus arteriosus is usually achieved within 24 hours after term birth, with complete closure achieved within a month. The primary mechanism for the closure of the ductus arteriosus is the withdrawal of the prostaglandin supply, which ordinarily works to maintain ductal patency. The second mechanism is the rise in oxygen concentrations, which leads to contraction of the ductus, but some details of this mechanism remain unclear.

The ductus arteriosus is of special significance in the setting of preterm birth, as preterm born babies can have delayed closure of a patent ductus arteriosus. This is usually managed using a Non-Steroidal Anti-Inflammatory Drug (NSAID) like Ibuprofen, which inhibits Cyclooxygenase Type 1 and Type 2 (COX-1/COX-2) and this inhibition then down-regulates prostaglandin production and leads to closure. If the ductus arteriosus remains patent, then open surgery or endovascular intervention can be performed to achieve closure.

Importantly, as a consequence of the differences in systemic and pulmonary haemodynamics before and after birth, the growth (and dominance) of the ventricles within the heart is markedly differently in utero versus ex utero. In utero, the dominant ventricle is the right ventricle, which supplies the majority of the systemic circulation. In utero, the
lungs only require minimal perfusion as it is not involved in gas exchange; most of the right ventricular output either goes through the pulmonary artery, through the ductus arteriosus and into the aorta or passes through the foramen ovale into the left atria, left ventricle and finally to the aorta. In addition, there is a marked rise in systemic arterial blood pressure and heart rate at the time of birth which also influences ventricular dominance and postnatal growth of the ventricles. Mean systolic pressure is low throughout normal gestation, from around 25 mmHg at 20 weeks to 45 mmHg at 38 weeks gestation. In the human fetus, heart rate generally declines as gestation advances, from a mean of 175 beats per minute (bpm) (5%: 154 bpm, 95%: 195 bpm) at 9 weeks to an accepted normal range of 110-160 bpm during the 2nd and 3rd trimester. At birth, the placenta is removed and the low resistance circuit it provides is removed; the lung becomes emptied of liquid and becomes a low resistance, high flow circuit (the ex utero cardiovascular configuration). Systemic arterial blood pressure needs to rise to at least 60/40 mmHg (systolic/diastolic) soon after birth to provide sufficient perfusion of major organs. This gradually rises to approximately 85/50 mmHg (systolic/diastolic) by the 3rd week of life. At birth, the heart rate also dramatically rises. For example, during vaginal birth, the heart rate rises to 120-200 beats per minute (bpm), and settles at around 120 bpm within the first hour after birth.

Being born induces major changes in the cardiovascular system, switching the dominant ventricle from right to left, closing the ductus arteriosus/venosus, closing the foramen ovale, increasing systemic blood pressure and other challenges. These changes arguably present a significant burden to the developing heart and arteries. Being born preterm places this burden on an ill prepared and immature cardiovascular system and may be the basis for the
adverse outcomes seen in preterm babies, such as increased blood pressure and changes in the vascular tree.
1.9 **Hypothesis**

As the heart and major blood vessels are still developing during late gestation it was hypothesised that preterm birth would affect their development and maturation; I tested this hypothesis in an ovine model of preterm birth and in human neonatal autopsy tissue. It also hypothesised that chorioamnionitis, the most common antecedent of preterm birth, would affect cardiomyocyte endowment, maturation, proliferation and collagen deposition within the heart. To facilitate these studies, I developed an improved method for measuring cardiomyocyte morphometry in thick paraffin sections.
Chapter 1 – Literature Review

1.10 Aims

To address the hypotheses, the specific aims of this thesis were:

1 – Chapter 2 – To determine the effect of moderate preterm birth, in an ovine model, on the developing heart at 9 weeks post-term equivalent age; in particular we wished to examine cardiomyocyte endowment, volume, maturation, and ploidy, and extra-cellular matrix deposition within the myocardium.

2 – Chapter 3 – To examine the effect of moderate preterm birth, with antenatal corticosteroid administration, on the morphometry and composition of the aorta and pulmonary artery, in the moderately preterm born lamb, at 9 weeks post-term equivalent age.

3 – Chapter 4 – To examine the effect of chorioamnionitis, the leading cause of preterm birth in humans, on the developing fetal sheep heart; in particular we wished to examine cardiomyocyte density, volume, maturation, ploidy, proliferation, and extra-cellular matrix deposition.

4 – Chapter 5 – To examine normal cardiomyocyte growth and development in the human heart during mid-to-late gestation, particularly in relation to cardiomyocyte size and growth, and proliferation. Using these data, to compare with tissue collected from neonates who died after being born preterm, to assess how preterm birth affects the structure of the human heart.

5 – Chapter 6 – To develop an improved method to simultaneously measure cardiomyocyte size, ploidy and nuclearity in thick paraffin sections. This method was used in Chapters 2, 4 and 5.
1.11 References


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Chapter 1 – Literature Review


Chapter 1 – Literature Review


Chapter 2 – Cardiac remodelling as a result of pre-term birth: implications for future cardiovascular disease

Chapter 2

Cardiac remodelling as a result of pre-term birth: implications for future cardiovascular disease
Chapter 2 – Cardiac remodelling as a result of pre-term birth: implications for future cardiovascular disease

Declaration by candidate

Chapter 2 was accepted on the 2nd of February 2010 by the European Heart Journal. Reprinted in this thesis is a copy of the final printed manuscript. Bensley, J. G., et al. (2010). "Cardiac remodelling as a result of pre-term birth: implications for future cardiovascular disease." Eur Heart J 31(16): 2058-2066. The final version may be obtained at: http://dx.doi.org/10.1093/eurheartj/ehq104

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performed experimental analyses (including a small contribution to the animal studies), data analysis and wrote manuscript.</td>
<td>75%</td>
</tr>
</tbody>
</table>

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%) for student co-authors only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Victoria K Stacey, Robert de Matteo, Richard Harding, M Jane Black</td>
<td>Conducted animal studies, designed the study, obtained funding, and assisted in writing the manuscript</td>
<td>25%</td>
</tr>
</tbody>
</table>

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work.

<table>
<thead>
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<th>Main Supervisor’s Signature</th>
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Cardiac remodelling as a result of pre-term birth: implications for future cardiovascular disease

Jonathan G. Bensley, Victoria K. Stacy, Robert De Matteo, Richard Harding†, and M. Jane Black*†

Department of Anatomy and Developmental Biology, School of Biomedical Sciences, Monash University, Clayton, VIC 3800, Australia

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Aims

Pre-term birth affects 10–12% of live births and occurs when the myocardium is still developing; therefore, the final structure of the myocardium could be altered. We hypothesized that, in response to pre-term birth, structural remodelling occurs within the myocardium which enables the immature heart muscle to adapt to the haemodynamic transition at birth but results in persistent alterations in its structure. Our objective was to determine how pre-term birth alters the final structure of the myocardium.

Methods and results

Using sheep, pre-term birth was induced at 0.9 of term; hearts were examined at 9 weeks after term-equivalent age, when cardiomyocyte proliferation and maturation have ceased. In pre-term lambs, we found that cardiomyocytes of both ventricles and the interventricular septum were hypertrophied. Cardiomyocyte maturation in pre-term lambs was altered in that there was a greater proportion of mononucleated, polyploid (4n) cardiomyocytes in both ventricles compared with controls; importantly, induction of polyploidy is associated with irreversible stress-related changes in DNA. We also found a six- to seven-fold increase in collagen deposition, usually accompanied by lymphocytic infiltration.

Conclusion

We conclude that pre-term birth leads to remodelling of the myocardium that alters its final structure. This may programme for long-term cardiovascular vulnerability.

Keywords

Cardiomyocyte • Pre-term birth • Developmental programming • Paediatrics • Risk factors

Introduction

Pre-term birth, defined as birth before 37 weeks of gestation, occurs in 10–12% of all live births.1,2 Today, pre-term infants born as early as 23 weeks of gestation are able to survive, but the majority are born between 32 and 36 completed weeks of gestation.3 It is now recognized that pre-term birth can increase the risk of respiratory disease and hypertension later in life.4–6 Although pre-term birth occurs when the heart is still developing, little is known about its effects on the myocardium.

Cardiomyocytes (the functional units of the heart muscle) undergo proliferation until late gestation when they progressively become terminally differentiated.7 As pre-term birth causes an abrupt and premature shift in the circulation from the foetal to post-natal configuration, we hypothesized that it would accelerate cardiomyocyte maturation. In the foetus, the right ventricle (RV) is responsible for 66% of cardiac output and has a thicker wall at birth,8 this is largely due to the RV receiving the bulk of venous return as the pulmonary circulation is a low-flow, high-resistance circuit resulting in a reduced venous flow to the left heart. After birth, the functioning of both ventricles is changed such that they each have the same output but against different pressures. The RV supplies only the pulmonary circulation which, soon after birth, becomes a high-flow, low-resistance circuit.9 With the loss of the low-resistance placental vascular bed, systemic arterial pressure is elevated by ~75%,10 increasing the load on the left ventricle (LV). It is possible that other changes associated with the transition from foetal to post-natal life, such as altered oxygenation, temperature, and nutrition, could also influence the development of the heart muscle.

The functional transition in cardiac function at birth usually takes place with a relatively mature heart capable of handling the necessary cardiac performance for ex utero life; however, little is known as

*†Mj. Black and R. Harding are joint senior authors.
†Corresponding author. Tel: +61 3 9902 9112; Fax: +61 3 9902 9223, Email: jane.black@med.monash.edu.au

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to the effects of placing the demands of ex utero life on a relatively immature, pre-term heart. We reasoned that because pre-term birth necessarily results in a premature haemodynamic transition at birth, it may lead to accelerated cardiomyocyte maturation and subsequently a reduced cardiomyocyte endowment in the pre-term neonate, as well as increased extracellular matrix (ECM) deposition. Such a reduced complement of cardiomyocytes and augmented ECM could adversely impact on the adaptive capabilities of the heart later in life, as cardiomyocytes have very limited proliferative potential after birth. We have also examined cardiomyocyte volume to determine whether cardiomyocyte hypertrophy has occurred. In addition, we hypothesized that cardiomyocyte ploidy may increase in response to the stress associated with pre-term birth. As such a change in ploidy is considered irreversible and has been linked to DNA damage, it may have adverse long-term implications. Thus, our specific objectives were to determine the effects of pre-term birth on cardiac ECM deposition, the final complement of cardiomyocytes, the volume of cardiomyocytes, and cardiomyocyte maturation in terms of nuclearity and ploidy.

We have used an ovine model of pre-term birth that replicates the majority of pre-term births in humans; importantly, the maturation of cardiomyocytes in sheep, in relation to birth, closely resembles that in the human. Cardiac structure was analysed at 9 weeks after term-equivalent age (TEA), at a time when cardiomyocyte proliferation and maturation have ceased.

Methods
Due to space requirements, some methodological details have been placed on line.

Ethical approval
All experimental procedures were approved by the Monash University Animal Ethics Committee and were in accordance with the National Health and Medical Research Council (Australia) guidelines.

Animal groups
Fifteen date-mated Border Leicester × Merino ewes carrying singleton foetuses were randomly assigned to deliver lambs either at term (147 days after mating) or 14 days prior to term. Pre-term lambs (n = 7; 6 female, 1 male) were born vaginally using an established technique. Briefly, at 131 days after mating, Epostane (50 mg, i.v.; Win-32729, Winthrop, UK) was administered to the ewes to induce delivery ~48 h later. The ewes were administered Betamethasonone (3.7–5 mg i.m., Celestone, Schering-Plough, Australia) to enhance the viability of pre-term lambs. The control group (n = 8; 5 female, 3 male) were born spontaneously at term. The age at which pre-term lambs were born is the earliest compatible with survival without respiratory support. Hence, the effects of being born pre-term can be observed in the absence of iatrogenic factors normally required to maintain life in more severe pre-term birth, such as mechanical ventilation with hypoxic gas mixtures.

The lambs were raised in individual pens with their mothers. For 1–3 days after birth, pre-term lambs were bottle-fed expressed milk from their mother. At 3 weeks post-TEA, lambs were anesthetized and underwent aseptic surgery for implantation of femoral arterial and venous catheters.

Physiologic measurements
Arterial pressure and heart rate were measured via the arterial catheter for 2 h on days 27 and 28 after TEA and again on days 55 and 56 after TEA, with an average being taken for each pair of successive days. Data were recorded and analysed using PowerLab 8/30 and Chart Version 5.01 (ADInstruments, Australia). We also monitored arterial pH, partial pressure of CO₂ (PaCO₂), partial pressure of oxygen (PaO₂), per cent oxygen saturation (SaO₂), haemoglobin concentration, haematocrit, and lactate and glucose concentrations (ABL 725, Radiometer, Denmark). At 9 weeks after TEA, lambs were euthanized and necropsy performed. No overt pathologies were found. Hearts were excised and weighed.

Perfusion fixation of the heart
Freshly excised hearts were retrogradely perfusion-fixed via the aorta. Prior to flushing the heart with saline, we infused heparin to prevent clotting, papaverine to dilate the cardiac vasculature, and KCl to arrest the heart in diastole. The saline infusion was followed by freshly prepared 4% formaldehyde. The fixed hearts were stored in 10% buffered formalin prior to tissue sampling. Each heart was assigned an arbitrary number to permit blinding to the experimental group in subsequent analyses.

Heart muscle sampling
The fixed hearts were weighed after removal of connective tissue and fat, and the atria were then removed. A transverse section, 5 mm thick, of ventricular tissue was removed ~10 mm below the plane of the valves. A digital photograph was taken for later image analysis of ventricular/septal wall thickness and ventricular lumen area. Due to the haemodynamic differences between the ventricles before and after birth, the LV and RV were analysed separately. The LV was sampled together with the adjacent septum (S) as the septum is structurally similar to the left ventricular wall. Sampling of heart tissue for morphologic and stereologic analyses was performed using a smooth fractionator approach. The selected samples of RV and LV + S were embedded in either glycolmethacrylate or paraffin.

Measurement of ventricular wall thickness and lumen area
Morphometric measurements were made on digital images of transverse sections of the heart (as described above) using image analysis (Image-Pro Plus Version 6.0, Media Cybernetics, USA). Images were used to determine ventricle wall thickness, septal thickness, and lumen area.

Cardiomyocyte number
Glycolmethacrylate blocks were sectioned at 20 μm and every 10th section was stained with haematoxylin in a 1000 W microwave oven at 50% power for 2–4 min. This ensured adequate nuclear staining throughout the sections. Cardiomyocyte number was estimated using an optical disector–fractonator approach. We used a light microscope (Olympus BX4, Japan) coupled with a motorized stage and a 2-axis sensor (Germany). Each second section was systematically sampled and projected onto a computer screen. An unbiased counting frame (329.6 μm²) was superimposed on the image using G.A.S.T. (Computer Aided Stereological Toolbox) software (Olympus, Denmark). Nuclei were counted when they came into clear focus in the disector area (so long as no part intersected the forbidden lines) within a 10 μm depth in the middle of the section. The total numbers of cardiomyocyte nuclei in the RV and LV + S were estimated by multiplying the number of nuclei counted stereologically by...
the inverse of all sampling fractions. Total cardiomyocyte numbers in the RV and LV + S were then determined following correction for binucleation (see below).

**Measurement of cardiomyocyte volume**

Paraffin-embedded sections of the RV and LV + S were cut at either 20 µm or 100 µm and stained with 10 µg/L of wheat germ agglutinin-Alexa Fluor 488 conjugate (Molecular Probes Invitrogen, USA) and 1:3000 YOYO-3 (Molecular Probes Invitrogen). Viable germ agglutinin-Alexa Fluor 488 stains the cell membranes and YOYO-3 stains the nucleus (see Supplementary material online for details).

To visualize the cardiomyocytes, we used a Leica SPS broadband multi-photon confocal microscope (Leica Microsystems, Germany) with a Spectra-Physics MaiTai Ti:Sapphire multi-photon source (Spectra-Physics, Newport Corporation, USA). Velocity Version 5 software (Perkin Elmer, UK) was used to visualize and measure cell volume. Each cell volume was manually assessed to ensure the entire cell was encompassed and no defects were present. At least 1000 cardiomyocytes were measured in the RV and LV + S for each animal.

**Quantification of interstitial and perivascular collagen**

Paraffin-embedded samples of RV and LV + S were sectioned at 5 µm and stained with picrorosirius red after pre-treatment with phosphomolybdic acid. To measure interstitial fibrosis, the sections were uniformly, systematically sampled and the percentage of collagen within the interstitium was quantified using image analysis (Image-Pro Plus Version 6.0, Media Cybernetics). To assess perivascular fibrosis, two intramyocardial arteries per section were selected according to Nyengaard and Marcussen. The area of collagen in the adventitia was measured using image analysis, normalized to the vessel lumen area and averaged for each animal.

**Collagen immunohistochemistry**

Collagens I and III were quantified in 5 µm paraffin sections using antibodies specific for collagen I (COL-I Clone, Abcam, USA) and collagen III (FH-7A Clone, Abcam, USA). The sections were uniformly, systematically sampled and the percentage of collagen in the tissue was quantified using image analysis (Image-Pro Plus Version 6.0, Media Cybernetics).

**Assessment of cardiomyocyte proliferation**

Cardiomyocyte proliferation was assessed in 5 µm paraffin sections using a Ki-67 antibody (MIB-1 Clone/Dako, USA). Antigen retrieval was achieved using 0.01 M Citrate Buffer (Ajax Finechem, Australia) with 0.1% Triton X-100 (Sigma-Aldrich, Australia) in a microwave oven for 20 min. Endogenous peroxidase activity was inhibited with a peroxidase blocker (Dako, Australia). The primary Ki-67 antibody was diluted 1:100 prior to use, and the tissue was incubated with the primary antibody overnight at 4°C in a humidified chamber. Detection was performed using the Dako EnVision+ Dual Link HRP/DA + immunohistochemistry kit (Dako). Positive controls were Zymed Laboratories Ki-67+ Control Slides (mouse tonsil, a known Ki-67 positive tissue) (Invitrogen, USA). Negative controls were tissues that were not incubated with the primary antibody.

**Cardiomyocyte nuclearity**

Cardiomyocyte nuclearity (i.e. the number of nuclei in cardiomyocytes) was determined using confocal microscopy. Thick paraffin sections (20 µm) were incubated with wheat germ agglutinin-Alexa Fluor 488 conjugate (to stain cell membranes) and TO-PRO-3 (to stain nuclei). Using a broadband multi-photon confocal microscope (Leica, Germany), we examined, on average, 250 cells in each LV + S and RV. Only cardiomyocytes with the entire cell membrane visible were examined. Using three-dimensional software (Imaris Version 6.16.2, Bitplane, Switzerland), the number of nuclei within the cells were counted.

**Cardiomyocyte ploidy**

To examine cardiomyocyte ploidy (i.e. the number of genome copies per nucleus), the sections were incubated with wheat germ agglutinin-Alexa Fluor 488 as described above and nuclei stained with YOYO-3 (Molecular Probes Invitrogen). The binding of YOYO-3 within a cell is proportional to its DNA content. Sections were incubated with a 1:2000 dilution of YOYO-3 for 20 min at room temperature and mounted in ProLong Gold (Molecular Probes Invitrogen). Using three-dimensional software (Imaris Version 6.16.2, Bitplane), images of whole nuclei were collected and relative fluorescence measured using Velocity Version 5 software (Perkin Elmer).

**Statistical analysis**

Statistical analyses were performed using SPSS version 17 (SPSS, USA). All data were analysed using an independent sample two-tailed t-test or two-way ANOVA as appropriate. Data are expressed as mean ± SEM. P < 0.05 was considered significant.

**Results**

**Physiologic status**

Pre-term lambs were born at 133 ± 1 days of gestational age and term lambs were born at 147 ± 0 days. At birth, pre-term lambs were lighter than controls (pre-term, 3.37 ± 0.24 vs. term, 4.39 ± 0.17 kg, P = 0.028); however, by nine weeks post-TEA, there was no difference in body weight (pre-term, 17.10 ± 0.59 kg vs. term, 17.14 ± 0.91 kg, P = 0.496). At 8 weeks post-TEA, there were no differences between term and pre-term lambs in arterial pH, PCO₂, and PO₂, or in plasma lactate, glucose, and cortisol concentrations. At 4 and 8 weeks post-TEA, there were no differences between groups in mean arterial pressure, systolic pressure, or diastolic pressure. At 8 weeks post-TEA, the mean arterial pressure in pre-term lambs was 77.1 ± 1.8 mmHg and in term lambs was 73.2 ± 2.6 mmHg (P = 0.215). There was a significantly lower heart rate in pre-term lambs (136.9 ± 9.0 b.p.m.) than in term lambs (167.3 ± 9.3 b.p.m.) at 4 weeks post-TEA (P = 0.026), but there was no difference at 8 weeks (pre-term 98.0 ± 6.9 b.p.m.; term, 89.6 ± 7.1 b.p.m., P = 0.376).

**Heart morphometry**

Data on gross heart morphometry are presented in Table 1. There were no significant differences between pre-term and term lambs in absolute or relative heart weight, RV weight, or LV + S weight. We found no significant differences between groups in wall thickness of the RV or LV + S, or in the luminal areas of the RV and LV.
Table 1  Heart weights and dimensions in lambs born at term and pre-term

<table>
<thead>
<tr>
<th></th>
<th>Term (n = 8)</th>
<th>Pre-term (n = 7)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Heart weight (g)</td>
<td>78.49 ± 3.93</td>
<td>86.18 ± 6.96</td>
<td>0.338</td>
</tr>
<tr>
<td>Heart weight/necropsy body weight (g/kg)</td>
<td>4.64 ± 0.25</td>
<td>5.05 ± 0.39</td>
<td>0.379</td>
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<tr>
<td>Left ventricle + septum weight (g)</td>
<td>48.81 ± 2.59</td>
<td>54.76 ± 4.63</td>
<td>0.264</td>
</tr>
<tr>
<td>Left ventricle + septum weight/necropsy body weight (g/kg)</td>
<td>2.87 ± 0.12</td>
<td>3.21 ± 0.27</td>
<td>0.244</td>
</tr>
<tr>
<td>Right ventricle weight (g)</td>
<td>16.12 ± 0.78</td>
<td>18.79 ± 1.45</td>
<td>0.116</td>
</tr>
<tr>
<td>Right ventricle weight/necropsy body weight (g/kg)</td>
<td>0.95 ± 0.06</td>
<td>1.10 ± 0.09</td>
<td>0.158</td>
</tr>
<tr>
<td>Left ventricle wall thickness (mm)</td>
<td>9.23 ± 0.51</td>
<td>9.36 ± 0.36</td>
<td>0.845</td>
</tr>
<tr>
<td>Right ventricle wall thickness (mm)</td>
<td>4.66 ± 0.41</td>
<td>4.60 ± 0.29</td>
<td>0.906</td>
</tr>
<tr>
<td>Septum thickness</td>
<td>10.08 ± 0.62</td>
<td>9.55 ± 0.40</td>
<td>0.498</td>
</tr>
<tr>
<td>Left ventricle lumen area (mm²)</td>
<td>376.42 ± 57.53</td>
<td>432.46 ± 89.28</td>
<td>0.597</td>
</tr>
<tr>
<td>Right ventricle lumen area (mm²)</td>
<td>379.91 ± 67.05</td>
<td>432.78 ± 41.92</td>
<td>0.601</td>
</tr>
</tbody>
</table>

Myocardial fibrosis

When assessed by picrosirius red staining, collagen deposition was significantly greater in the ventricular muscle of pre-term lambs than in term lambs (Figure 1A–C). In all pre-term lambs, but no term lambs, there were focal areas of severe collagen deposition (Figure 1C). The collagen content of the RV in pre-term lambs (2.90 ± 0.52%) was 6.6-fold greater than in term lambs (0.44 ± 0.11%, P = 0.025). Similarly, the collagen content of the LV + S of pre-term lambs (3.44 ± 0.79%) was 4.8-fold greater than in control lambs (0.71 ± 1.32%, P = 0.048).

Collagens I and III were localized to the myocardial interstitium and adventitia of the myocardial blood vessels. The relative proportion and ratio of collagen type I/type III fibres were not different between groups in the RV or LV + S.

There were no differences in perivascular fibrosis (adventitia area/luminal area) in either the LV + S (term, 0.81 ± 0.12 vs. pre-term, 0.88 ± 0.16; P = 0.885) or the RV (term, 0.71 ± 0.25 vs. pre-term, 0.76 ± 0.27; P = 0.509).

Cardiomyocyte proliferation

There were extremely low levels of cardiomyocyte proliferation in ventricular tissue of both term and pre-term lambs, as assessed morphologically while performing the stereology and by Ki-67 immunohistochemistry.

Lymphocytic infiltration

Focal areas of lymphocytic and mast cell infiltration were observed in five of the seven pre-term hearts, whereas these were not observed in any of the eight term hearts. In Ki-67-stained sections, these lymphocytes exhibited strong nuclear staining, indicating a proliferating phenotype.

Cardiomyocyte number

There was no significant difference between term and pre-term hearts in the total number of cardiomyocytes within the RV and LV + S (Table 2). After adjusting for ventricular weight (RV or LV + S), heart weight, or body weight, there remained no significant differences between term and pre-term hearts in the number of cardiomyocytes in the RV or LV + S (Table 2).

Cardiomyocyte volume

Cardiomyocytes in the pre-term hearts were generally larger than in term hearts. The mean volume of binucleated cardiomyocytes in the RV and LV + S was significantly greater in pre-term lambs than in term lambs (Table 3, Figure 2) (see Supplementary material online, Figure S1, cardiomyocyte volume distribution). The mean volume of mononucleated cardiomyocytes was also significantly greater in the RV of the pre-term lambs than in term lambs; a similar (near-significant) trend was observed in the LV + S (Table 3).

Cardiomyocyte nuclearity

In term lambs most cardiomyocytes were binucleated, both in the RV (98.6 ± 0.2% binucleated; 1.4 ± 0.2% mononucleated) and the LV + S (98.0 ± 0.3% binucleated; 2.0 ± 0.3% mononucleated). However, in pre-term lambs, the ventricles contained significantly fewer binucleated and more mononucleated cardiomyocytes, as well as some trinucleated cardiomyocytes (Figure 3). In the RV, 93.2 ± 0.5% were binucleated, 4.7 ± 0.4% were mononucleated, and 2.1 ± 0.3% were trinucleated; in the LV + S, 86.0 ± 1.1% were binucleated, 8.9 ± 0.9% were mononucleated, and 5.1 ± 0.7% were trinucleated. In pre-term lambs, the LV + S was more severely affected than the RV, having more mononucleated cardiomyocytes than the RV (P < 0.001), fewer binucleated (P < 0.001), and more trinucleated (P < 0.001) cardiomyocytes than the RV (Figure 3).

Cardiomyocyte ploidy

There was a marked increase in the ploidy of the mononucleated cardiomyocytes in the pre-term hearts; 94.1 ± 2.2% of these were tetraploid (4n DNA) in the LV + S and 86.4 ± 0.9% were tetraploid in the RV. In the relatively small number of mononucleated cardiomyocytes in the term hearts, 61.9 ± 5.4% were tetraploid in the LV + S and 47.7 ± 0.9% were tetraploid in the RV.

Discussion

Our study is the first to analyse the impact of pre-term birth on the growth response of the neonatal heart and the underlying mechanisms. The findings clearly demonstrate that, although
Figure 1 Photomicrographs of cardiac muscle of lambs at 9 weeks post-term-equivalent age. (A) Representative image of the left ventricle plus septum from a term lamb showing minimal collagen deposition. (B) Representative image of left ventricle plus septum from a pre-term lamb showing increased collagen deposition. (C) Severely affected portion of left ventricle plus septum from a pre-term lamb showing severe fibrosis. (D) Representative confocal image of left ventricle plus septum from a pre-term lamb showing mononucleated (white arrow), binucleated (yellow arrow) and trinucleated (red arrow) cardiomyocytes. Scale bars represent 100 μm.

Table 2 Cardiomyocyte numbers in the right ventricle and left ventricle plus septum in hearts of term and pre-term lambs

<table>
<thead>
<tr>
<th></th>
<th>Term (n = 8)</th>
<th>Pre-term (n = 7)</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>RV total cardiomyocyte number (× 10^6)</td>
<td>4.76 ± 0.54</td>
<td>6.90 ± 1.30</td>
<td>0.181</td>
</tr>
<tr>
<td>LV + S total cardiomyocyte number (× 10^6)</td>
<td>2.45 ± 0.43</td>
<td>2.41 ± 0.23</td>
<td>0.931</td>
</tr>
<tr>
<td>RV cardiomyocyte number/necropsy weight (number/kg × 10^3)</td>
<td>3.02 ± 0.52</td>
<td>4.21 ± 0.89</td>
<td>0.276</td>
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<tr>
<td>LV + S cardiomyocyte number/necropsy weight number/kg × 10^3</td>
<td>1.44 ± 0.27</td>
<td>1.42 ± 0.16</td>
<td>0.950</td>
</tr>
<tr>
<td>RV cardiomyocyte number/RV weight (number/g × 10^3)</td>
<td>3.20 ± 0.43</td>
<td>3.79 ± 0.59</td>
<td>0.432</td>
</tr>
<tr>
<td>LV + S cardiomyocyte number/LV + S weight (number/g × 10^3)</td>
<td>4.95 ± 0.76</td>
<td>4.68 ± 0.33</td>
<td>0.778</td>
</tr>
</tbody>
</table>

Pre-term birth does not alter the final number of cardiomyocytes. It can lead to marked alterations in the development of ventricular muscle, in particular, an increase in cardiomyocyte volume, increased collagen deposition, and an apparent alteration in cardiomyocyte maturation (including increases in ploidy). These changes are likely a result of the myocardium experiencing the obligatory birth-related haemodynamic transition before it has attained the level of maturity normally reached by full term. The observed alterations in myocardial structure in response to pre-term birth may be considered abnormal in that they may lead to persistent changes that adversely affect later function. Although there is no evidence of cardiac dysfunction in our lambs at 8 weeks of age, many of the cardiac changes that we observed are considered irreversible and thus have potential for long-term adverse implications for cardiac function, especially as cardiomyocyte proliferation is unlikely after the time point of our investigation.

Increased collagen deposition

There was no difference in the levels of perivascular fibrosis in the pre-term hearts relative to controls; however, there was a marked increase in interstitial fibrosis. We found a five- to seven-fold increase in ECM deposition within the myocardial interstitium in the pre-term hearts, but no apparent difference in the relative
Cardiac remodelling as a result of pre-term birth

Table 3  Volume of cardiomyocytes in the right ventricle and left ventricle plus septum of pre-term and term lamb

<table>
<thead>
<tr>
<th></th>
<th>Term (n = 8) (μm³)</th>
<th>Pre-term (n = 7) (μm³)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV mononucleated</td>
<td>10 051.07 ± 365.9</td>
<td>12 922.82 ± 968.59</td>
<td>0.04</td>
</tr>
<tr>
<td>RV binucleated</td>
<td>14 601.16 ± 381.86</td>
<td>18 435.89 ± 1038.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RV trinucleated</td>
<td>Not present</td>
<td>28 779.55 ± 2018.63</td>
<td>N/A</td>
</tr>
<tr>
<td>LV + S mononucleated</td>
<td>10 032.53 ± 339.41</td>
<td>11 032.16 ± 1403.53</td>
<td>0.09</td>
</tr>
<tr>
<td>LV + S binucleated</td>
<td>20 764.91 ± 709.88</td>
<td>24 138.85 ± 531.42</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LV + S trinucleated</td>
<td>Not present</td>
<td>27 790.72 ± 623.01</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Figure 2  Volumes (in cubic micrometres) of mononucleated, binucleated, and trinucleated cardiomyocytes in term and pre-term lamb hearts at 9 weeks post-term-equivalent age. *P < 0.05.

proportions of types I and III collagen. Increased myocardial collagen deposition in early life is important, as fibrosis is associated with impaired conductivity and contractility within the myocardium potentially leading to myocardial dysfunction.²⁵,²⁶ It is likely that the observed fibrosis will increase with age, and further studies are required to determine whether cardiac function is altered.

There are a number of potential explanations for the increased interstitial fibrosis seen in the pre-term lamb heart, including the premature haemodynamic transition at birth and/or myocardial inflammation. At birth, the function of the two ventricles changes markedly⁹,¹¹,²⁷ such that the output and end-diastolic pressure of the LV increase, while the output of the RV decreases.²⁷ The premature increase in haemodynamic load on the LV as a result of pre-term birth is likely to lead to an increase in myocardial ECM deposition to maintain ventricular wall strength in the presence of a high proportion of undifferentiated, immature cardiomyocytes. Alternatively (or perhaps concomitantly), an inflammatory response may arise within the myocardium as a result of pre-term birth, leading to induction of fibrosis. The observation of inflammatory infiltrates in most of the pre-term hearts that we studied supports this idea. Indeed, it has been shown previously that T lymphocytes are involved with the cardiac ECM remodelling seen in hypertension²⁸ and that the presence of mast cells induces fibrosis via recruitment of cardiac fibroblasts.²⁹

It was surprising that a significant increase in cardiac fibrosis was already evident in the hearts of pre-term lambs at only 11 weeks after birth. Hence, even moderately severe pre-term birth can adversely alter the architecture of the heart. If this fibrosis becomes further accentuated with age, it could impair the ability of the heart to respond to insults or hypertrophic challenges later in life.

Post-natal catch-up in growth

There is now substantial epidemiologic evidence demonstrating increased cardiovascular risk in individuals born of low birth weight followed by catch-up in body growth after birth.²⁰ Hence, the adverse effects on myocardial structure that we have observed following pre-term birth may contribute to the increased rates of cardiovascular disease among those born of low birth weight (of which pre-term birth is a frequent cause).³¹,³² Indeed, there is now evidence that pre-term birth per se is linked to risk factors associated with cardiovascular disease in adulthood such as hypertension.⁶,³³ However, at present there is no evidence linking pre-term birth to specific myocardial diseases in adulthood. To determine whether there is a causal relation between pre-term birth and adult cardiovascular disease, future studies could examine cardiac muscle architecture and function in the hearts of adults who were born pre-term.

Cardiomyocyte number

Contrary to our hypothesis, there was no effect of pre-term birth per se on the final number of ventricular cardiomyocytes. The number of cardiomyocytes in both ventricles was directly correlated with heart size. This is in accordance with a previous study in which we found that the number of cardiomyocytes in the LV was related to heart size.¹⁷ Given this relationship, we would expect that the pre-term lambs, which were much lighter at birth, would have fewer cardiomyocytes within the RV and LV compared with term lambs at the time of birth. However, as a high proportion of cardiomyocytes (~50%) would have still been dividing at the time of pre-term birth, it is likely that, with the catch-up in body growth after birth, there would be a concomitant increase in heart size and cardiomyocyte number. However, in more extremely pre-term offspring in whom post-natal growth is often retarded, cardiomyocyte endowment would likely be reduced.

As we found little evidence of cardiomyocyte proliferation at 9 weeks post-TEA, the complement of cardiomyocytes at this
age should be stable and subsequently affected only by cell death. The observed absence of cardiomyocyte proliferation was expected for lamb hearts at 9 weeks post-TEA, as previous studies have shown that cardiomyocytes have ceased proliferating by this age in sheep.\textsuperscript{14,15,17}

**Cardiomyocyte volume**

Cardiomyocyte volume was significantly greater in the pre-term lambs compared with controls, except for mononucleated cardiomyocytes in the LV + S (although the trend was still apparent). In the pre-term animals, binucleated cardiomyocytes were 16.7% larger in the LV + S than in term animals and 26.2% larger in the RV + S. This hypertrophy could be a result of the premature exposure of the heart to increased load following pre-term birth and could contribute to the trend for the hearts of pre-term lambs to be heavier than those of term lambs.

**Cardiomyocyte nuclearity and ploidy**

It is generally accepted that, in the developing ovine heart, cardiomyocytes that are mononucleated are immature and still capable of proliferation, whereas binucleated cardiomyocytes are mature and terminally differentiated and thus have ceased dividing\textsuperscript{14,15,34} At the gestational age at which our pre-term lambs were born, \( \sim 50\% \) of cardiomyocytes are binucleated,\textsuperscript{15} making this an ideal time point to observe an effect of pre-term birth on cardiomyocyte maturation. An unexpected finding was the apparent alteration of cardiomyocyte nuclearity in pre-term lamb hearts. This was shown by a significantly greater number, compared with controls, of mononucleated cardiomyocytes in both ventricles of pre-term lambs (from 1% to 5% in the RV and from 2% to 9% in the LV + S); conversely, we observed significantly fewer binucleated cardiomyocytes in both ventricles of pre-term lambs. Although, based on these findings, it is intuitive to consider that the ventricular muscle is more immature in the pre-term heart, our findings of increased ploidy in the mononucleated cardiomyocytes do not support this. Instead, in response to pre-term birth there appears to be endomitosis of the mononucleated cardiomyocytes without karyokinesis,\textsuperscript{35} indicative of abnormal maturation. In addition, in both ventricles of pre-term lambs, we observed polyplid cardiomyocytes with three nuclei (trinucleated), which were not observed in term lambs. Polyploidy of cardiomyocytes is well described in the human heart and in animal models.\textsuperscript{36} Of concern, however, polyplidploidy is considered to be irreversible and is linked to cardiac dysfunction;\textsuperscript{37–39} for example, the incidence of polyploid cardiomyocytes is high in hearts with impaired function caused by pathological hypertrophy.\textsuperscript{37} Importantly, it has been proposed that polyploidy of cardiomyocytes can be programmed in early development.\textsuperscript{13} Recent experimental evidence, whereby rats were exposed to a developmental insult at different time points in gestation, demonstrates that the critical window for the programming of cardiomyocyte polyploidy is during the cardiomyocyte maturational transition from proliferation to hypertrophy,\textsuperscript{13} which usually occurs during late gestation in humans and sheep.\textsuperscript{7,15} Importantly, this is the developmental time point when pre-term birth was induced in

**Figure 3** Proportions of mononucleated (A), binucleated (B), and trinucleated (C) cardiomyocytes within the left ventricle plus septum and right ventricle of term and pre-term lamb hearts. \( ^*P < 0.05. \)
Cardiac remodelling as a result of pre-term birth

the present study. Our findings support this concept, with the stresses related to the haemodynamic transition at birth in the pre-term lambs occurring during this critical window in maturation.

**Exposure to corticosteroids**

Women at risk of pre-term birth are commonly administered antenatal glucocorticoids to facilitate lung maturation. As our pre-term lambs were exposed to a low dose of corticosteroids prior to birth, it is conceivable that the observed effects on the pre-term heart could have been mediated by the actions of glucocorticoids. Although there is some evidence that cortisol acts as a growth hormone in the foetal heart, the role of cortisol on foetal cardiomyocyte growth remains controversial. For instance, the infusion of cortisol into late-gestation foetal sheep at subpressor doses had no effect on cardiomyocyte size or maturation state but instead showed a stimulation of cardiomyocyte proliferation. In contrast, recent evidence in rats demonstrates that the administration of post-natal glucocorticoids leads to an increase in cardiomyocyte size and increased ECM deposition in the heart.

**Conclusions**

We conclude that moderately pre-term birth alone leads to remodelling of the myocardium resulting in cardiomyocyte hypertrophy, increased collagen deposition, and an alteration of cardiomyocyte maturation. This is of major clinical significance given that the majority of human pre-term births occur between 32 and 36 weeks gestation. It is expected that the adverse alterations in cardiac muscle architecture would be accentuated at gestational age at birth decreases. It is likely that the remodelling of the pre-term heart in the neonatal period will programme for long-term cardiac vulnerability.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

**Acknowledgements**

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**Conflict of interest:** none declared.

**References**

Chapter 3

Preterm birth with antenatal corticosteroid administration has injurious and persistent effects on the structure and composition of the aorta and pulmonary artery.
Chapter 3 – Preterm birth with antenatal corticosteroid administration has injurious and persistent effects on the structure and composition of the aorta and pulmonary artery

Declaration by candidate

Chapter 3 was accepted on the 27th of August 2011 by Pediatric Research. Reprinted in this thesis is a copy of the final printed manuscript. Bensley, J. G., et al. (2012). "Preterm birth with antenatal corticosteroid administration has injurious and persistent effects on the structure and composition of the aorta and pulmonary artery." Pediatr Res 71(2): 150-155. The final version may be obtained at: http://www.nature.com/pr/journal/v71/n2/full/pr201129a.html

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
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<tbody>
<tr>
<td>Performed experimental analyses (including a small contribution to the animal studies), data analysis and wrote manuscript.</td>
<td>75%</td>
</tr>
</tbody>
</table>

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%) for student co-authors only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robert de Matteo, Richard Harding, M Jane Black</td>
<td>Conducted animal studies, designed the study, obtained funding, and assisted in writing the manuscript</td>
<td>25%</td>
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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work.

Candidate’s Signature

Main Supervisor’s Signature

Date 27/7/15

Date 27/7/15
Preterm birth with antenatal corticosteroid administration has injurious and persistent effects on the structure and composition of the aorta and pulmonary artery

Jonathan Guy Bensley¹, Robert De Matteo¹, Richard Harding¹ and Mary Jane Black¹

INTRODUCTION: Preterm birth affects 8–12% of live births and is associated with the development of elevated arterial blood pressure and aortic narrowing in later life; this suggests that preterm birth may alter the development of arteries. Our objective was to determine the effects of preterm birth, accompanied by antenatal corticosteroid administration, on the structure of the aorta and pulmonary artery, which experience different alterations in pressure flow at birth.

RESULTS: At 11 wk, preterm lambs had significantly thicker aortic walls and a smaller lumen, whereas the morphometry of the pulmonary artery was unaffected. Elastin deposition was markedly increased in the aorta and pulmonary artery and smooth muscle content was reduced in the aorta only. In preterm lambs we found injury in the aorta only; controls were unaffected.

DISCUSSION: We conclude that moderate preterm birth after antenatal betamethasone can cause injury and persistent alterations in the structure and composition of the aorta, with lesser effects in the pulmonary artery. Our findings suggest that preterm birth may increase the risk of atherosclerosis and aneurysms in later life.

METHODS: Using an established ovine model of preterm birth, lambs were born at 0.9 of gestation and underwent necropsy at 11 wk after birth; controls were born at term.

Preterm birth, defined as birth prior to 37 completed weeks of gestation, affects 8–12% of live births. As a result of advances in neonatal care, most preterm infants survive infancy, even those born as early as 23 wk (1). Epidemiological studies have linked preterm birth to hypertension and vascular abnormalities in children and young adults (2–3); the risk is increased with decreasing gestational age at birth. Suggestive of causality (2,3,6). For example, one study found a 0.31 mm Hg increase in adult blood pressure for every week of prematurity (2). Such increases are important because a 2 mm Hg increase in adult blood pressure is associated with a 6% increase in the risk of coronary artery disease and a 15% increase in the risk of stroke or transient ischemic attack (10).

Preterm birth has also been associated with increased vascular resistance and arterial wall thickness, which are both associated with hypertension and adverse cardiovascular outcomes (11). For instance, a study of adolescent girls born preterm found higher brachial artery and aortic pressures, a narrower abdominal aorta, and lower peripheral skin blood flow than in girls born at term (9). The cause of these vascular changes might relate to vascular adaptations that were set in place soon after preterm birth as a result of vascular immaturity.

In this regard, we have recently shown adverse structural remodeling of the myocardium in immature lambs as a result of being born preterm (12). The major components of the arterial wall are elastin, collagen, and smooth muscle, each of which is laid down during gestation and early postnatal life (13). Importantly, local hemodynamic conditions in the perinatal period play a major role in determining the structural composition of major arteries (14), which, in turn, directly influences their wall compliance. The human fetal aorta is exposed to a steadily increasing luminal pressure, rising from a mean of 28 mm Hg at 20 wk to 45 mm Hg at 40 wk (15); this increase in pressure provides the normal environment for aortic development. At birth, systemic arterial pressure and aortic flow increase substantially, largely as a result of the loss of the placental vascular bed, closure of vascular shunts, and a decrease in pulmonary vascular resistance (16). Preterm birth, including moderate preterm birth, occurs during this period of compositional change within the aorta (17), at a time when the aorta is immature, thus exposing it to increased shear stress and pressure before it is structurally mature.

In utero, the developing pulmonary artery is exposed to pressures identical to those of the fetal systemic circulation (18) and receives only a small fraction of cardiac output (16,19). At birth, pressure within the pulmonary artery falls (mean pressure ~15 mm Hg), and it receives all of the right ventricular output after closure of the ductus arteriosus (20). This change from high pressure/low flow to low pressure/high flow, when it occurs prematurely, may result in abnormal changes to the walls of the pulmonary artery.

¹Department of Anatomy and Developmental Biology, School of Biomedical Sciences, Monash University, Clayton, Australia. Correspondence: Mary Jane Black (jane.black@monash.edu)

The last two authors are joint senior authors on this study.

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A major recent advance in improving the survival of preterm infants is the administration of antenatal corticosteroids to women who show evidence of preterm labor. Our lamb model of preterm birth includes antenatal betamethasone administration to the ewe because the majority of preterm infants in developed countries will be exposed to antenatal corticosteroids (21).

Hence, we propose that, as a result of preterm birth, the immature aorta undergoes structural remodeling that differs from that occurring at term; we propose that these structural changes may lead to aortic narrowing, impaired aortic compliance, and subsequent development of hypertension. Second, in the pulmonary artery, as a result of the substantial rise in blood flow and the reduction in luminal pressure following preterm birth, we propose that there will be persistent changes in the morphometry and composition of the arterial wall, but these will differ from the changes in the aorta. We examined the proximal ascending aorta and pulmonary artery because these areas experience the highest blood flow velocity, shear stress, and dynamic pressures (22,23). Our objective was to determine and compare the effects of preterm birth, accompanied by antenatal corticosteroid exposure, on the structure and composition of the walls of the aorta and pulmonary artery.

RESULTS
Preterm lambs were born at 133 ± 1 d of gestational age and controls were born at term (147 ± 0 d). At birth, preterm lambs were significantly lighter than controls (preterm, 3.37 ± 0.26kg vs. term, 4.10 ± 0.18kg, P < 0.05); however, by 9 wk after term-equivalent age (TEA), there was no significant difference in body weight (preterm, 17.11 ± 0.62kg, vs. term, 16.83 ± 0.67kg). At 8 wk after TEA, there were no differences between groups, either in mean arterial pressure (preterm, 76.6 ± 2.0 mm Hg, vs. term, 76.1 ± 1.5 mm Hg) or in systolic and diastolic pressures and heart rate. At necropsy, no lamb had a patent foramen ovale or ductus arteriosus (dye or probe patent). No cardiac structural abnormalities (e.g., septal defects) were observed in term or preterm lambs.

Aortic Injury
Injury was observed histologically in the aortas of four of the seven preterm lambs. The injury was apparent in three of the four affected lambs in the second segment and in all four affected lambs in the third segment. The lesions occurred in one male and three female preterm lambs. Lesions were round at the intimal surface and were on average 2.5 mm in diameter and 800 μm deep. Lesions were located within the tunica intima. The lesions were observed to be composed primarily of collagen and smooth muscle, with low levels of elastin in an abnormal configuration (Figure 1).

Immunohistochemistry revealed minimal apoptosis (caspase 3) and proliferation (Ki-67) in the lesion or the surrounding tissue.

Aortic Morphometry
Aortic walls were significantly thicker in preterm lambs compared with controls, by an average of 22.6% across the three segments (Figure 2a). There was a statistically significant narrowing (24.7% reduction in lumen area) in the third segment of the aorta in the preterm lambs compared with controls (Figure 2b) and therefore an increased media/lumen ratio in the same segment (Figure 2f).

Aortic Wall Composition
Aortic elastin deposition was greater in all three segments in preterm lambs relative to controls (Figure 2c). Smooth muscle content was significantly lower in all segments of the aorta in preterm lambs compared with controls (Figure 2d); however, total collagen deposition did not differ among groups (Figure 2e).

Pulmonary Artery Morphometry
The mean thickness of the pulmonary arterial wall was 10% lower in preterm lambs compared with controls; however, the difference was not significant (P = 0.08, Figure 3a). After adjusting for lumen circumference, thickness between groups did not differ. No difference between groups was detected in pulmonary arterial lumen area (Figure 3b) or in the media/lumen ratio (Figure 3f).

Pulmonary Artery Composition
Pulmonary arterial elastin deposition was greater in all three segments from preterm lambs relative to term lambs (Figure 3c). Smooth muscle deposition did not differ between groups (Figure 3d), but there was a significantly lower amount of collagen compared with controls in the second segment of the pulmonary artery, with a nonsignificant trend for reduction in the first segment (Figure 3e).

DISCUSSION
Our findings clearly demonstrate evidence of aortic intimal injury and marked remodeling of the aortic wall, leading to a
Preterm birth and the arterial wall

Figure 2. Summary of morphometric data from the aorta of preterm and term lambs. Data from term lambs appear in blank bars and those from preterm lambs appear in solid bars. All plots show means ± SEM. *P < 0.05. (a) A marked increase in wall thickness was found in preterm lambs across all three segments. In the third segment, there was (b) a reduction in lumen area and (f) an increased media to lumen ratio. In all aortic segments, we observed (c) an increase in elastin, (d) reduced smooth muscle, and (e) no change in collagen deposition. seg. segment.

Figure 3. Summary of morphometric data from the pulmonary artery of preterm and term lambs. Data from term lambs appear in blank bars and those from preterm lambs appear in solid bars. All plots show means ± SEM. *P < 0.05. (a) Wall thickness was reduced by 10% in all three segments, but this was not statistically significant. There was no alteration in (b) lumen area or (f) media/lumen ratio. In all segments of pulmonary artery, there was (c) an increase in elastin (d) but no change in smooth muscle or (e) collagen deposition. seg. segment.

smaller luminal area, following preterm birth. These changes are likely irreversible and could contribute to long-term risk of cardiovascular disease, in particular, hypertension, aortic aneurysm, and atherosclerosis. Less dramatic changes were seen in the wall of the pulmonary artery, which probably reflects the difference in hemodynamic changes in these two arteries at the time of birth. The changes in vascular structure observed in the preterm lambs likely are an adaptive response of the immature arteries to the hemodynamic transition at birth, but we cannot exclude a possible influence of antenatal corticosteroids.

Aortic Injury
Surprisingly, we obtained striking morphological evidence demonstrating that injury can occur in the intima of the aortic wall following preterm birth. In four of the seven preterm aortas studied at 11 wk after birth, we observed focal areas of intimal thickening within the aorta, indicative of previous injury to the aortic wall; such intimal thickening was not observed in any of the term lambs at the same age.

Injury within the aortas of all preterm lambs is likely, but was only detected in four of the seven animals. Indeed, only a small segment of the aorta was analyzed, and aortic injury
may have occurred more distally in the other preterm lambs. Importantly, the areas of aortic injury were not associated with the remnants of the ductus arteriosus because this area was avoided during dissection.

In testing our hypothesis, we focused on the ascending aorta because it is the region of highest dynamic pressure. In comparison, there is relatively low pressure at the aortic valve (with some pressure being relieved by the branching of the coronary arteries at this level) (24). These differences in arterial pressure likely account for the most striking structural changes observed in our most distal segment because the first aortic segment analyzed was at the level of the aortic valves.

We propose that the large rise in aortic pressure and/or flow that occurs at birth (16,25–27) leads to intimal injury in the ascending aorta because the immature artery at the time of preterm birth is not sufficiently developed to accommodate the high postnatal levels of blood pressure and flow. Importantly, in this regard, we have found in preterm lambs that mean arterial pressure rises by more than 20 mm Hg at the time of birth (unpublished observations). Of particular concern, we found a substantial thinning of the media of the aortic wall at the site of injury. Indeed, the integrity of the aortic wall likely is substantially compromised at this location, rendering it vulnerable to atherosclerosis with aging, development of hypertension, and disease. In addition, intimal thickening acts as a precursor for atherosclerosis (28); hence, the preterm aorta appears to be predisposed to developing atherosclerosis at these sites.

We cannot exclude the possibility that the unavoidable use of antenatal betamethasone contributed to the development of the aortic injury, but we consider it unlikely owing to the low dose used.

Aortic Structure and Composition

In addition to aortic injury, we found marked remodeling of the aortic wall in response to preterm birth; there was an increase in aortic wall thickness that was associated with an increase in elastin deposition and reduced smooth muscle content but no change in collagen content. This is likely an adaptive response of the immature aorta to the high postnatal systemic arterial pressure, which is considerably higher than in utero (27). Indeed, local hemodynamic conditions in the perinatal period play a major role in the maturational changes in structural composition (elastin, smooth muscle, and collagen) of the aorta, which, in turn, directly influence aortic compliance (17). During the perinatal period, concentric lamellae of elastin rapidly form within the aortic wall and thereafter rates of elastin synthesis fall rapidly (29). These layers of elastin are essential for the normal compliance of the aorta, allowing it to accommodate the high volume of blood during systole and the elastic recoil during diastole, which maintains an even forward flow of blood to the body against a closed aortic valve. Elastin has a very low turnover because its half-life is ~40 y (30), and the number of elastin lamellar units in a particular vascular segment does not change after birth.

In contrast, the smooth muscle content within the aortic media of the preterm lambs was significantly less than in the controls. This implies that these aortas could be less responsive to vasostimulatory factors.

Significant narrowing of the aortic lumen accompanied the marked remodeling of the aortic wall, probably resulting from the thickened media. In accordance with our findings, a recent study showed that the aortas of adolescent girls born preterm were narrower and less stiff than those of girls born at term (9). In another study, aortic narrowing was reported in adolescents who were born preterm (7). Arterial narrowing is likely to be detrimental in the long term and may contribute to the now well-described elevation in arterial pressure in subjects born preterm (2–5,8,9,31).

Pulmonary Artery Less Vulnerable to Preterm Birth

In accordance with our hypothesis, we observed very few changes in the structure and composition of the pulmonary artery wall following preterm birth. Unlike the aorta, there appeared to be no deleterious effects of preterm birth on arterial wall structure, and there was no evidence of endothelial injury or luminal narrowing. The relative biochemical composition of the arterial wall was generally unaffected by preterm birth, except for a significant increase in elastin content. We propose that the differences observed in the aorta vs. the pulmonary artery result from differences in the mean luminal pressures to which these immature arteries are exposed in the period soon after birth.

Potential Role of Antenatal Corticosteroids

Antenatal exposure to betamethasone could have contributed to the aortic injury and vascular structural remodeling observed in our preterm lambs. Elastic lamellae within arterial walls are predominantly laid down in the perinatal period, and betamethasone may have led to the increased deposition of elastin within the aorta and pulmonary artery because corticosteroids are potent regulators of elastin synthesis (29). Similarly, evidence exists that antenatal corticosteroids can induce permanent changes in endothelial and vascular smooth muscle function, ultimately leading to endothelial dysfunction (32,33). Given that exposure to corticosteroids can lead to endothelial dysfunction, their use may also lead to intimal injury; together with the hemodynamic changes at birth, it is likely that severe intimal injury will ensue. However, a study in which lambs born at term (~145 d) were administered 0.2 mg/kg dexamethasone twice a day, from birth to postnatal day 10, did not reveal aortic injury or increased elastin deposition in the aorta at necropsy on day 10 (29). In the present study, we have been unable to determine whether betamethasone exposure mediated any of the observed arterial injury and remodeling following preterm birth. Unfortunately, it was not possible for us to avoid the use of betamethasone because preterm lambs do not survive without it. However, our animal model closely reflects the human scenario in which it is routine practice for women at risk of preterm delivery to be administered antenatal steroids.
In our study, we have avoided many of the other confounding factors associated with preterm birth in humans, such as intrauterine factors thought to induce preterm delivery (for example, chorioamnionitis and intrauterine growth restriction) and factors associated with postnatal care (for example, mechanical ventilation, hypoxia, and postnatal medications) (34). Importantly, recent studies demonstrate that factors such as these can also influence the long-term vascular phenotype in subjects born preterm (8). Together with the present study, these studies highlight the vulnerability of the cardiovascular system of preterm infants and suggest a cumulative cardiovascular risk depending on the causes of preterm birth.

Conclusions

We have shown in a clinically relevant model of moderate preterm birth that there are injurious and persistent changes to the structure and composition of the aorta. These adverse effects appear to be the result of the rapid rise in systemic arterial pressure at the time of birth because they were not observed in the pulmonary artery, in which pressure falls after birth; it remains possible that exposure to antenatal corticosteroids could play a role in the observed effects. The aortic injury and luminal narrowing associated with preterm birth could predispose for long-term cardiovascular disease.

METHODS

Ethical Approval

This study was approved by the Monash University Animal Ethics Committee in accordance with the National Health and Medical Research Council (Australia) guidelines for the care and handling of animals for scientific purposes.

Experimental Protocol

Studies were performed using two groups of lambs: one group was born preterm (0.9 of term) following antenatal corticosteroid exposure and the other was born at term. Preterm birth was induced using an established protocol (35). Briefly, 19 date-mated crossed-bred ewes were randomly assigned to deliver their lambs either at term (147 d after mating) or 14 d prior to term (133 d after mating). All lambs were born vaginally. To induce preterm birth, Epostane (30 mg i.v., Win-32729; Winthrop, Guildford, UK) was administered to 7 ewes 131 d after mating to induce delivery ~48 h later. On the same day, the ewes were also administered a dose of betamethasone (3.7–5 mg i.m.; Celestone; Schering-Plough, North Ryde, Australia) to enhance the viability of preterm lambs. Control ewes did not receive betamethasone. The ewes gave birth to 1 male and 6 female preterm lambs. The control group (n = 12, 6 female, 6 male) were born spontaneously at term.

Lambs were raised in individual pens with their mothers. For 1–3 d after birth, preterm lambs were bottle-fed expressed milk from their mothers. Ewes had free access to feed and water. At 3 wk after TEA, lambs underwent aseptic surgery for implantation of femoral arterial and venous catheters; anesthesia was induced with Alfaxedone (3.5 mg/kg i.v. Alfaxon; Jurox, Rutherford, Australia) and maintained with 1%–1.5% isoflurane (Baxter Healthcare, Rutherford, Old Toongabbie, Australia).

Using the arterial catheter, mean arterial pressure and heart rate were measured for 2 h on days 55 and 56 after TEA. Data were recorded and analyzed using PowerLab 8/30 and Chart Version 5.01 (ADInstruments, Sydney, Australia).

Necropsy Protocol

Preterm lambs were euthanized 9 wk after TEA using sodium pentobarbital (325 mg/ml). The 12 control lambs were euthanized 9 wk after term birth; at this age, lambs are preadolescent. The thoracic aorta and pulmonary arteries were excised and placed in saline with papaverine hydrochloride (DBL Pharmaceuticals, Mulgrave North, Australia) to maximally dilate the vessels. Vessels were then immersion fixed in 4% buffered formaldehyde solution. In each animal, aorta and pulmonary arteries were sampled from the same anatomical site. One-millimeter segments of the aorta and pulmonary artery were cut from just above the level of the valve (first segment) and then another two segments (1-mm thick, second and third segments) were taken 2 cm apart from the level of the valve, giving a total of three segments of 1-mm thickness. Arbitrary numbers were assigned to the removed samples so as to blind the investigators to grouping.

Paraffin sections from each segment were cut at 6 μm and mounted on poly-L-lysine-coated slides. Slides from every segment were stained with picrosirius red to measure collagen content (12), Gomori’s alde- hyde fuchsin to measure wall thickness, and Verhoeff’s elastic stain to measure elastin content (36); other slides were prepared for immuno- histochemistry (see below). To minimize variation, for each segment at least five slides per stain were used and average values were taken. Slides were interleaved so that no section was followed by a section stained using the same technique.

Immunohistochemistry

Some slides were immunohistochemically stained for cellular proliferation utilizing Ki-67 (MIB-1 clone; Dako, Botany, Australia) and active caspase-3 (Polyclonal; R&D Systems, Minneapolis, MN) to detect cells undergoing apoptosis and α-smooth muscle actin (1A4 clone, Dako) to determine the amount of vascular smooth muscle. Detection was performed using Dako EnVision+ Dual Link HRP/ DAB+ kit (Dako). All negative controls were sections incubated with- out the primary antibody.

Sections were stained with the α-smooth muscle actin antibody (1:50 dilution) for 30 min. Positive controls were sections of adult ovine aorta.

Sections were stained with the Ki-67 (1:100 dilution) or active caspase-3 antibodies (1:250) for 18 h and 30 min, respectively. Positive controls were Zymed Ki-67+ control slides (mouse tonsil, a known Ki-67-positive tissue) (Invitrogen, Carlsbad, CA) and sections from the right ventricle of a lamb that suffered a myocardial infarction.

Image Acquisition

Slides were scanned at 20× magnification using the Olympus dotSlide system (Version 2.1; Olympus, Tokyo, Japan). This enabled the capture of very large vessels with excellent resolution. Images were batch exported from dotSlide as individual frames and reconstructed in PanaVue ImageAssembler Professional (PanaVue, Quebec, Quebec, Canada).

Image Analysis

Images were analyzed using Image Pro-Plus Version 6.2 (Media Cybernetics, Bethesda, MD). Measurements of wall thickness, media thickness, and intima thickness were measured continuously along the entire wall. Area measurements were reconstructed areas based on the perimeter length of the structure to eliminate any changes caused by vessel distortion during processing into paraffin. Percentages of collagen, elastin, and α-smooth muscle actin were expressed as percentages of the entire vessel wall area.

Statistical Analysis

Data were analyzed using an independent samples t test (two-tailed). Analysis was performed using PASW Statistics 19 (IBM SPSS, Chicago, IL). Significance was set at P ≤ 0.05. Data are presented as means ± SEM. Graphs were prepared in GraphPad Prism Version 5.04 (GraphPad Software, La Jolla, CA).

Chapter 3
Preterm birth and the arterial wall

Articles

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ACKNOWLEDGMENTS

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REFERENCES

Chapter 4

Exposure to intrauterine inflammation leads to impaired function and altered structure in the preterm heart of fetal sheep
Chapter 4 – Exposure to intrauterine inflammation leads to impaired function and altered structure in the preterm heart of fetal sheep

Declaration by candidate

Chapter 4 was accepted on the 12th of May 2014 by Clinical Science. Reprinted in this thesis is a copy of the final printed manuscript. Tare, M., Bensley, J.G., Moss, T.J., Lingwood, B.E., Kim, M.Y., Barton, S.K., Kluckow, M., Gill, A.W., De Matteo, R., Harding, R., Black, M.J., Parkington, H.C., Polglase, G.R. Exposure to intrauterine inflammation leads to impaired function and altered structure in the preterm heart of fetal sheep. Clin Sci (Lond) 127, 559-569, doi:10.1042/CS20140097 (2014). The final version may be obtained at: http://www.clinsci.org/cs/127/cs1270559.htm

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performed fibrosis, capillarisation, cardiomyocyte volume, nuclearity, ploidy and maturation component of the study. Performed some data analysis and co-authored manuscript.</td>
<td>40%</td>
</tr>
</tbody>
</table>

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%) for student co-authors only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tare, M</td>
<td>Conducted animal studies, physiological studies, designed the study, obtained funding, and co-authored the manuscript</td>
<td>60%</td>
</tr>
<tr>
<td>Moss, TJ</td>
<td></td>
<td></td>
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<tr>
<td>Lingwood, BE</td>
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<td>Kim, MY</td>
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<td>Polglase, GR</td>
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</table>

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work.

Candidate’s Signature [Redacted]  
Date 27/7/15

Main Supervisor’s Signature [Redacted]  
Date 27/7/15
Exposure to intrauterine inflammation leads to impaired function and altered structure in the preterm heart of fetal sheep

Marianne TARE\textsuperscript{a}, Jonathan G. BENSLEY\textsuperscript{a}, Timothy J. M. MOSS\textsuperscript{b}, Barbara E. LINGWOOD\textsuperscript{b}, Min Y. KIM\textsuperscript{c}, Samantha K. BARTON\textsuperscript{d}, Martin KLUCKOW\textsuperscript{d}, Andrew W. GILL\textsuperscript{e}, Robert DE MATTEO\textsuperscript{f}, Richard HARDING\textsuperscript{f}, M. Jane BLACK\textsuperscript{g}, Helena C. PARKINGTON\textsuperscript{h} and Graeme R. POLGLASE\textsuperscript{i}

\textsuperscript{a}Department of Physiology, Monash University, Clayton, Australia
\textsuperscript{b}Department of Anatomy and Developmental Biology, Monash University, Clayton, Australia
\textsuperscript{c}Department of Obstetrics and Gynaecology, Monash University, Clayton, Australia
\textsuperscript{d}Ritchie Centre, Monash Institute of Medical Research, Monash University, Clayton, Australia
\textsuperscript{e}University of Queensland Centre for Clinical Research, University of Queensland, Brisbane, Australia
\textsuperscript{f}Department of Neonatal Medicine, Royal North Shore Hospital and University of Sydney, Sydney, Australia
\textsuperscript{g}Centre for Neonatal Research and Education, University of Western Australia, Crawley, Australia

Abstract

Intrauterine inflammation is a major contributor to preterm birth and has adverse effects on preterm neonatal cardiovascular physiology. Cardiomyocyte maturation occurs in late gestation in species such as humans and sheep. We tested the hypothesis that intrauterine inflammation has deleterious effects on cardiac function in preterm sheep which might be explained by altered cardiomyocyte proliferation and maturation. Pregnant ewes received an ultrasound-guided intra-amniotic injection of lipopolysaccharide (LPS) or saline 7 days prior to delivery at day 127 of pregnancy (term 147 days). Cardiac contractility was recorded in spontaneously beating hearts of the offspring, perfused in a Langendorff apparatus. Saline-filled latex balloons were inserted into the left ventricle (LV) and right ventricle (RV). Responsiveness to isoprenaline and stop-flow/reperfusion was assessed. In other experiments, hearts were perfusion-fixed, and cardiomyocyte nuclei, volume, number and circumference determined. \(\beta\)-Adrenoceptor mRNA levels were determined in unfixed tissue. In hearts of LPS-exposed fetuses, contractility in the LV and RV was suppressed by \(
\sim 40\%\) and cardiomyocyte numbers were reduced by \(
\sim 25\%\). Immature mono-nucleated cardiomyocytes had lower volumes (\(\sim 18\%\)), whereas mature bi-nucleated cardiomyocyte volume was \(\sim 77\%\) greater. Although basal coronary flow was significantly increased by \(21 \pm 7\%\) in LPS-exposed hearts, following ischaemia/reperfusion (IR), end-diastolic pressure was increased \(2.4 \pm 0.3\)-fold and infarct area increased \(3.2 \pm 0.6\)-fold compared with those in controls. Maximum responsiveness to isoprenaline was enhanced by LPS, without an increase in \(\beta\)-adrenoceptor mRNA, suggesting altered second messenger signalling. Intrauterine inflammation altered cardiac growth, suppressed contractile function and enhanced responsiveness to stress. Although these effects may ensure immediate survival, they probably contribute to the increased vulnerability of organ perfusion in preterm neonates.

Key words: \(\beta\)-adrenoceptor, cardiomyocyte morphometry, chorioamnionitis, myocardial contraction, preterm birth

INTRODUCTION

Preterm birth occurs in 8–13\% of pregnancies in the Western world, and \(\sim 27\%\) of these are associated with inflammatory conditions, such as chorioamnionitis [1]. The incidence of preterm birth is increased by chorioamnionitis [2], with [3] or without [4] established infection, and by other pro-inflammatory conditions, such as growth restriction due to placental insufficiency [5], pre-eclampsia [6] and diabetes. Although the deleterious effects of inflammation on cardiac contractility in adults have been well...
studied, little is known of the consequences of intrauterine inflammation on heart structure and function in the fetus. Cardiac dysfunctions, particularly fetal heart rate anomalies, including tachycardia, absence of variability, arrhythmias and absence of accelerations, are extremely common in the fetuses/neonates of women with diagnosed chorioamnionitis [7–9]. Cardiac dysfunction and peripheral haemodynamic disturbances, measured 2–4 h after birth, have been described in human neonates exposed to chorioamnionitis [10]. These observations in preterm fetuses and infants suggest that prenatal inflammation adversely affects cardiovascular function over and above prematurity.

Intra-amniotic lipopolysaccharide (LPS, derived from the outer coat of Gram-negative bacteria) is used experimentally to induce intrauterine inflammation and, importantly, has been shown to lead to cardiac contractile dysfunction in preterm sheep [11] and mice [12]. The inflammatory response resulting from intra-amniotic LPS mimics acute chorioamnionitis such as that which occurs in women with premature preterm rupture of membranes, but does not reflect chronic inflammatory conditions such as pre-eclampsia and chronic chorioamnionitis, or acute infection that occurs in conjunction with sepsis. Using this model, it has been shown that in vivo exposure to LPS leads to an inflammatory response resulting in activation of Toll-like receptors and production of pro-inflammatory cytokines, activation of nuclear factor κB (NF-κB) and inducible nitric oxide synthase (iNOS) [13]. Maternal LPS administration increased cardiac afterload, reduced cardiac output, induced myocardial inflammation and impaired contractility and relaxation in fetal mice [14]. In adult monkeys, LPS increases P- and E-selectin in coronary microvessels resulting in leucocyte adhesion, endothelial damage and thrombus formation [15]. LPS also suppresses mitochondrial function in cardiomyocytes and increases cardiac vulnerability to brief oxygen deficit in monkeys [16]. These studies indicate that exposure to inflammation in utero not only impairs cardiac function, but may also impair development of the myocardium. The situation in the fetus and the preterm neonate is complicated by the immature nature of the organs, and hence the consequences of intrauterine inflammation may vary with gestational age [4]. Intra-amniotic injection of LPS results in a rapid inflammatory response in the fetus, causing a reduction in left ventricular output and an increase in systemic arterial pressure [17]. It may be that the development of cardiac muscle is affected. It is not known whether the cardiovascular remodelling is in response to increased afterload or to a direct effect of LPS on the heart.

Although the fundamental structure of the cardiovascular system is established early in pregnancy, cardiomyocytes generally cease dividing towards term when they undergo a process of maturation in preparation for the haemodynamic transition at birth. Importantly, 7 days after a single intra-amniotic injection of LPS, newborn preterm lambs have reduced left ventricular output, accompanied by an increase in the right ventricle (RV)-to-left ventricle-septum (LV + S) ratio [18]. We hypothesized that structural changes in the developing myocardium as a consequence of intrauterine exposure to inflammation underpin changes in cardiac function following LPS exposure. Hence, the objective of the present study was to determine the effects of a single intra-amniotic injection of LPS on cardiac function and cardiomyocyte morphometry and endowment 7 days later in preterm fetal sheep.

MATERIALS AND METHODS

Animals
Experimental procedures were approved by the Monash University Animal Ethics Committee A in accordance with the National Health and Medical Research Council (Australia) guidelines. Ultrasound-guided intra-amniotic injection of LPS (10 mg, *Escherichia coli*, O55:B5, Sigma–Aldrich) or saline (2 ml) was performed at 120 ± 1 days of gestation (term = 147 days) in singleton- or twin-bearing pregnant ewes, using established techniques [19]. At 7 days following LPS/saline treatment, maternal/fetal anaesthesia was induced using thiopentone [20 ml of Pentothal at 50 mg/ml, intravenously (i.v.); Boehringer Ingelheim], maintained with isoflurane (2–3%), and the fetuses were delivered by Caesarean section.

Heart function

**Contractility**

For these functional experiments, 12 ewes were studied: six LPS- and six saline-treated. At 7 days following LPS/saline treatment, anaesthesia was induced using thiopentone (20 ml of Pentothal at 50 mg/ml) followed by maintenance with isoflurane (Attane, Bovac Animal Health). The fetus was exteriorized and heparin (2500 international units in 0.5 ml) was injected into the umbilical vein. The chest was opened, the aorta clamped at the aortic arch and the heart rapidly removed into ice-cold Krebs solution without added calcium. The heart was mounted on a Langendorff apparatus [20]. Briefly, the heart was attached to the apparatus, at the base of a 27-cm column of Krebs solution, via the ascending aorta, permitting perfusion with Krebs solution via the coronary sinus in a physiological manner. Krebs solution contained 127 mM NaCl, 4 mM KCl, 2 mM MgSO4, 2 mM KH2PO4, 10 mM glucose, 10 mM Hepes and 1.5 mM CaCl2 and was prewarmed (35°C) and gassed with oxygen. A latex balloon filled with 0.9% saline was inserted into both the LVs and RVs, and diastolic pressure was set at 5 mmHg. The balloons were connected to a pressure transducer (MLT6099, ADInstruments) and bridge amplifier. Balloon pressures were recorded on LabChart 6.0 for later analysis. Perfusion pressure was maintained constant at 36 mmHg, the mean arterial pressure in fetal sheep at this stage of gestation [21], and a heated jacket was raised to enclose the beating heart, ensuring optimal temperature and humidity. The heart was allowed to equilibrate for 20 min. Coronary flow was determined by collecting the effluent dripping from a narrow exit in the base of the heating jacket over the final 10 min of the equilibration period.

Isoprenaline (0.006–0.6 μg/ml in 200 μl of warmed Krebs solution, with 200 μl of Krebs chase, 2 × 10 s) was applied through a fine tube (250-μm diameter) into the inflow immediately above the attachment of the aorta to the apparatus. Isoprenaline was delivered in increasing concentrations, allowing full recovery from the effects of each concentration before the
next was administered. The isolated Langendorff approach was chosen for assessment of heart function because LPS can alter baroreflex mechanisms [16].

Stop-flow reperfusion injury
Once the heart had returned to baseline contractility after the final dose of isoprenaline, solution flow through the Langendorff apparatus was stopped for 20 min. Reperfusion was then recommenced and continued for 60 min. The heart was removed from the apparatus and weighed, and the LV was sliced into 5-mm-thick sections and placed in PBS containing 1% 2,3,5-triphenyltetrazolium (TTZ) to develop infarcted areas. The sections were then fixed in 4% paraformaldehyde and photographed. Infarct area was calculated as follows:

\[
\text{Infarct area (\%) = \frac{I_{\text{sum}} \times 100}{C - L}}
\]

where \(I_{\text{sum}}\) is the sum of the total infarcted area, \(C\) is the circumference of the cross-section of the heart tissue and \(L\) is luminal area of the ventricle.

Heart sampling and analysis
In another cohort of animals, ewes and fetuses (five LPS- and five saline-exposed) were humanely killed using Lethabarb (sodium pentobarbitone, Virbac Animal Health), and the excised hearts were flushed retrogradely via the aorta with saline containing heparin to prevent blood coagulation, papaverine hydrochloride (1.2 mg, DBL Pharmaceuticals) to dilate the vasculature and KCl (100 mM) to arrest the heart in diastole. Hearts were then perfusion fixed using 4% buffered formaldehyde solution.

Hearts were weighed and then the RV and the LV+S were sampled separately. The LV+S and RV were sampled using a smooth fractionator approach, and the sampled pieces were processed into paraffin. Initially, 5-μm-thick paraffin sections were collected. Haematoxylin and eosin staining was used to determine cardiomyocyte orientation within the tissue. Toluidine Blue staining was used to identify mast cell infiltration.

Cardiomyocyte nuclearity, ploidy and volume
Samples of RV and LV+S were sectioned at 5, 20 and 100 μm. Cardiomyocyte cell membranes were delineated using Alexa Fluor® 488-conjugated wheat germ agglutinin (Invitrogen). Nuclei were stained using 10 mM DAPI. Sections were visualized using confocal microscopy to determine cardiomyocyte nuclearity (number of nuclei), ploidy (relative nuclear fluorescence), volume, mean cardiomyocyte length (the average length of the entire cardiomyocyte along the long axis of the cell) and mean cardiomyocyte diameter (the average diameter along the long axis of the cell). Cardiomyocyte endowment was estimated from ventricular volumes, adjusted for the proportion of myocardium composed of cardiomyocytes in relation to other cell types. At least 5000 cells were analysed per parameter, per ventricle, per animal.

Cardiac capillarization
Capillarization was assessed in Alexa Fluor® 488-conjugated wheat germ agglutinin-stained sections. Capillary density was determined in at least five randomly selected fields from each of the sampled pieces of each ventricle, per animal.

Cardiomyocyte proliferation and apoptosis
Cardiomyocyte proliferation was identified in 5-μm-thick paraffin sections using an anti-Ki-67 antibody. Proliferation was expressed as the percentage of Ki-67-positive cardiomyocyte nuclei relative to total cardiomyocyte nuclei. At least 10,000 nuclei were counted per section, per ventricle, per animal.

Apoptosis was measured using the ApopTag TUNEL system. At least 5000 cells were analysed per section, per ventricle, per animal.

Extracellular matrix deposition
Interstitial collagen deposition (% collagen) was assessed in 5-μm-thick paraffin sections stained with Picosirisius Red. Whole sections were scanned using Olympus dotSlide 2.4 and collagen (red staining area) was quantified in MetaMorph.

Adrenoceptor (AR) mRNA expression
Ewes and fetuses (five LPS- and six saline-exposed, used principally for other experiments, and not described in the present article) were humanely killed using Lethabarb, a small piece of ventricle was rapidly excised from the heart of each fetus and was snap-frozen for later study. Real-time PCR was performed in Mastercycler ep Realplex (Eppendorf South Pacific) using the primer sequences in Table 1.

Every primer pair was subjected to a validation experiment in which the efficiency of the target amplification and the efficiency of the reference [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] amplification were tested for their equality. Each reaction contained 12.5 μl of Power SYBR green PCR master mix (Applied Biosystems), primers (GeneWorks, Table 1) and 5 ng of cDNA in a final volume of 25 μl per reaction. The data obtained were analysed using the comparative \(2^{-\Delta\Delta C_T}\) method. Briefly, the mRNA level for each gene expression assay was normalized to the internal standard and is expressed relative to control hearts. Dissociation curves for validation of homogeneity of amplification products were generated for all reactions, and no-template control samples were included in all assays to confirm the absence of non-specific amplification products due to primer interactions and minimal contamination of genomic DNA. The predicted sizes of the PCR products were verified by agarose gel electrophoresis (results not shown).

Statistical analysis
Data were analysed using GraphPad Prism 6 and GraphPad InStat (GraphPad Software). For all data sets, equality of S.D. and Gaussian distribution, using the Kolmogorov–Smirnov method, were tested. Data are expressed as means and S.E.M. Throughout, \(n\) represents the number of animals studied and \(P < 0.05\) was accepted as statistically significant.

Comparisons of ventricular pressure were determined using two-way ANOVA. Cardiomyocyte characteristics and adrenoceptor mRNA expression were compared using Student’s \(t\) tests (SPSS, IBM). Unless otherwise indicated, data are expressed as means ± S.E.M.
Chapter 4

Exposure to intrauterine inflammation leads to impaired function and altered structure in the preterm heart of fetal sheep.

**Table 1** Primer sequences used for AR qPCR and for the reference GAPDH

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Gene symbol</th>
<th>Primer sequence (5'→3')</th>
<th>GenBank® accession number</th>
<th>Amplicon size (bp)</th>
<th>Primer (nM)</th>
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<tr>
<td>β₁,AR</td>
<td>ADRB1</td>
<td>Fwd/S: CTGAGGAAAACTCTCTCTGCT</td>
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<td>β₂,AR</td>
<td>ADRB2</td>
<td>Fwd/S: TCTCACCAGACCCACCTGCTT</td>
<td>NM_00130354.1</td>
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<td>500</td>
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<td>α₁A,AR</td>
<td>ADRA1A</td>
<td>Fwd/S: ACGCACCCTCCTCGGACCTGTA</td>
<td>EU723257.1</td>
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<td>α₁D,AR</td>
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<tr>
<td>GAPDH</td>
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<td>NM_00130354.1</td>
<td>379</td>
<td>500</td>
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**Table 2** Baseline characteristics of the fetuses and their hearts at necropsy

P value denotes differences between saline and LPS exposure.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline (n = 10)</th>
<th>LPS (n = 10)</th>
<th>P</th>
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</thead>
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<tr>
<td>Baseline</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Gender (n) (female/male)</td>
<td>5/6</td>
<td>6/4</td>
<td>0.673</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>2.88 ± 0.17</td>
<td>3.16 ± 0.13</td>
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</tr>
<tr>
<td>Heart weight (g)</td>
<td>22.17 ± 1.18</td>
<td>25.03 ± 1.58</td>
<td>0.360</td>
</tr>
<tr>
<td>Heart weight/body weight (g/kg)</td>
<td>8.75 ± 0.52</td>
<td>8.01 ± 0.55</td>
<td>0.342</td>
</tr>
<tr>
<td>Heart characteristics (n = 5)</td>
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<tr>
<td>RV weight (g)</td>
<td>5.65 ± 0.36</td>
<td>6.68 ± 0.37</td>
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</tr>
<tr>
<td>RV weight/heart weight (g/g)</td>
<td>0.26 ± 0.02</td>
<td>0.29 ± 0.01</td>
<td>0.185</td>
</tr>
<tr>
<td>LV+S weight (g/g)</td>
<td>0.49 ± 0.02</td>
<td>0.59 ± 0.03</td>
<td>0.027*</td>
</tr>
<tr>
<td>LV+S weight/body weight (g/kg)</td>
<td>2.23 ± 0.21</td>
<td>2.08 ± 0.15</td>
<td>0.585</td>
</tr>
<tr>
<td>LV weight (g)</td>
<td>11.59 ± 0.66</td>
<td>11.37 ± 0.38</td>
<td>0.775</td>
</tr>
<tr>
<td>Atrial weight (g)</td>
<td>2.88 ± 0.43</td>
<td>3.73 ± 0.22</td>
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</tr>
<tr>
<td>Atrial weight/heart weight (g/g)</td>
<td>0.13 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.085</td>
</tr>
<tr>
<td>Atrial weight/body weight (g/kg)</td>
<td>1.13 ± 0.16</td>
<td>1.16 ± 0.06</td>
<td>0.882</td>
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**RESULTS**

**Baseline characteristics**

Successful injection of LPS into the amniotic sac was confirmed by electrolyte analysis of amniotic fluid aspirated at the time of injection. The presence of intrauterine inflammation was confirmed visually in all LPS-exposed fetuses by the presence of thickened and edematous fetal membranes characteristic of this experimental intervention [19]. Body weight, heart weight and heart weight relative to body weight were not different between groups (Table 2). None of the fetuses had any congenital abnormalities.

LV+S weights were not significantly different between LPS-exposed and control groups. There was a significant 20% increase in RV weight/LV+S weight in LPS-exposed fetuses compared with those in controls (Table 2). Intra-amniotic LPS injection had no effect on atrial weight.

**Heart contractility**

The rate of spontaneous beating in isolated hearts was not different between LPS-exposed and control groups (Figure 1B). However, basal coronary flow (corrected for heart mass) was 21 ± 7% greater in hearts of LPS-exposed fetuses than in controls (Figure 1A; P = 0.01).

Hearts of LPS-exposed animals had reduced baseline contractility; maximum pressure generation (P = 0.004) and maximum rates of contraction (P = 0.001) and relaxation (P = 0.001) were smaller than those of controls (Figures 1C–1E). There was no difference in baseline contractility between LVs and RVs for either group (Figures 1C–1E).

Left ventricular pressure irregularities (arrhythmias) were evident in the hearts of five of the six LPS-exposed fetuses (Figure 2), but these were not observed in any of the six hearts from saline controls.

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Chapter 4 – Exposure to intrauterine inflammation leads to impaired function and altered structure in the preterm heart of fetal sheep.

Figure 1  Effect of LPS exposure on heart contractility
LPS exposure had no effect on spontaneous heart rate in isolated hearts (B), whereas it caused a significant increase in coronary flow (A). Maximum pressure development (C) and rates of contraction (D) and relaxation (E) were similar in the RVs and LVs. LPS-exposed ventricles had significant reductions in these measures. Values are means ± S.E.M. for six saline- and six LPS-exposed animals.

Figure 2  Contractile activity of hearts in the Langendorff apparatus
Irregularities occurred in hearts from five out of the six LPS-exposed fetuses but not in hearts of saline-treated fetuses.

Response to isoprenaline
Isoprenaline induced concentration-dependent increases in ventricular pressure development and maximum rates of contraction and relaxation (Figure 3) and in heart rate (Figure 4). Peak ventricular pressure development and maximum rate of contraction in hearts from LPS-exposed fetuses were significantly greater than those in saline-exposed controls. In contrast, the maximum rate of relaxation in response to isoprenaline was not different between LPS-exposed and control hearts. There was no effect of LPS on the sensitivity to isoprenaline (defined as the concentration required to elicit a response that was 50% of maximal; Figure 3).

mRNA expression of AR sub-types \( \alpha_1A, \alpha_1B, \alpha_3C \) (results not shown) and \( \beta_1 \) and \( \beta_2 \) (Figure 4) were not different between hearts of LPS-exposed and saline-exposed fetuses. Stop-flow reperfusion
Ischaemia/reperfusion (IR) resulted in a significant increase in end-diastolic pressure development, which was significantly greater in LPS-exposed (244 ± 32%) than in control hearts (134 ± 41%) \( (P = 0.001; \) Figure 5A). At 15 min after re-establishment of perfusion, left ventricular developed pressure was similar in both groups. However, there was significant deterioration in left ventricular developed pressure in LPS-exposed hearts at 55 min but not in controls \( (P = 0.004; \) Figure 5B). Infarct area (at 60 min) was significantly increased in hearts from preterm fetuses exposed to LPS (3-fold) compared with those of controls \( (P = 0.03; \) Figure 5C).

Cardiomyocyte nuclearity and ploidy
In LV+S, cardiomyocyte nuclearity was not different between hearts of control (mono-nucleated 60 ± 1% and bi-nucleated 40 ± 1%) and LPS-exposed fetuses (mono-nucleated 60 ± 2%
and bi-nucleated 40 ± 2% (P = 0.992). In the RV, cardiomyocyte bi-nucleation was significantly greater in the LPS-exposed group (53 ± 1%) than in controls (36 ± 2%). Mono-nucleation was correspondingly lower in hearts of LPS-exposed (47 ± 1%) than in control fetuses (64 ± 2%) (P < 0.01).

There was no difference in ploidy in non-proliferating cardiomyocytes in either ventricle, with more than 97% of the cardiomyocyte nuclei containing 2n DNA in hearts of both control and LPS-exposed fetuses.

**Cardiomyocyte morphometry**

Mean volume of mono-nucleated cardiomyocytes was significantly reduced in hearts of LPS-exposed compared with control fetuses in the LV+S (P < 0.001) and RV (P < 0.0001) (Figure 6). In contrast, bi-nucleated cardiomyocytes from LPS-exposed fetuses were significantly larger than those of controls (P < 0.0001; Figure 6).

The mean total diameter of mono-nucleated cardiomyocytes in the RV of LPS-exposed fetuses was reduced compared with control fetuses (P < 0.0001), but mean length was increased (P < 0.0001; Table 3). The lengthening of these mono-nucleated cardiomyocytes in the RV was insufficient to make up for the reduction in cardiomyocyte volume in LPS-exposed animals. Bi-nucleated cardiomyocytes in the RV of LPS-exposed fetuses were longer than those in controls (P = 0.009), but the majority of the increase in volume was due to the large increase in mean total diameter.

There was no change in the mean total length of mono-nucleated cardiomyocytes in the LV+S from LPS-exposed fetuses compared with those from controls (P = 0.63), but almost all of the decrease in volume was due to a reduction in mean total diameter (Table 3). The increase in cardiomyocyte volume of bi-nucleated LV+S cardiomyocytes from the LPS-exposed fetuses compared with those from controls was due to an increase in both length and diameter.

In hearts of LPS-exposed fetuses, there were 29% fewer cardiomyocytes per mm² in the RV than those of controls (P = 0.0015) and 24% fewer cardiomyocytes per mm² in the LV+S than those of controls (F = 0.0005; Figure 7). The total number of cardiomyocytes in the RV (F = 0.0035) and LV+S (F = 0.009) was

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**Figure 3** Effect of LPS exposure on the contractile response of the heart to β-AR activation

Vagal (10 μM) application of the β-AR agonist isoproterenol evoked concentration-dependent increases in contractility in both ventricles. The maximum amplitude of pressure development and maximum rate of contraction (±dP/dt) were significantly greater in LPS-exposed hearts. Values are means ± S.E.M. for six saline- and six LPS-exposed animals.

**Figure 4** Effect of LPS exposure on heart rate and cardiac β-AR expression

The isoproterenol-induced increase in heart rate was enhanced following LPS exposure. There was no significant effect of LPS on β-AR mRNA.
Exposure to intrauterine inflammation leads to impaired function and altered structure in the preterm heart of fetal sheep. Figure 5 shows the effect of LPS exposure on left ventricular developed pressure and infarct area following I/R. Stop-flow followed by reperfusion resulted in (A) an increase in end-diastolic pressure (two-way ANOVA analysis), (B) a reduction in left ventricular pressure that was significantly greater at 55 min in LPS-exposed compared with saline-exposed hearts and (C) development of a markedly larger infarct area in hearts from LPS-exposed fetuses (at 1 h). Values are means ± S.E.M. for five saline- and five LPS-exposed animals.

Figure 6 shows the frequency distribution of cardiomyocytes volume in the LV+S and RV for mono- and bi-nucleated cardiomyocytes and the effects of LPS exposure. Values are means ± S.E.M. for tissues from five saline- and five LPS-exposed fetuses. Right: a confocal photomicrograph of an LV+S-exposed heart showing cardiomyocyte cell membranes stained with Alexa Fluor® 488-conjugated wheat germ agglutinin (green) and nuclei stained with DAPI (blue). Scale bar = 10 μm.

Significantly lower in LPS-exposed hearts than in controls (Figure 7).

Cardiomyocyte proliferation and apoptosis
Cardiomyocyte proliferation in control fetuses was low (RV: 1.8 ± 0.1%; and LV+S: 1.6 ± 0.5%) but was markedly increased following LPS exposure (RV: 12.5 ± 1.6%; and LV+S: 14.3 ± 2.1%) (P < 0.0001). Apoptosis levels in both ventricles were too low to quantify (<0.01%).

Cardiac capillarization and fibrosis
LPS-exposed fetuses had enhanced capillarization in the LV+S (0.83 ± 0.04 capillary profiles per cardiomyocyte in controls compared with 1.20 ± 0.08 in LPS-exposed hearts; P = 0.001) and in RV (0.72 ± 0.05 capillary profiles per cardiomyocytes...
Chapter 4

Exposure to intrauterine inflammation leads to impaired function and altered structure in the preterm heart of fetal sheep.

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Bi-nucleated</th>
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<tr>
<td></td>
<td>Saline</td>
<td>LPS</td>
</tr>
<tr>
<td>RV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (µm³)</td>
<td>4094 ± 24</td>
<td>3627 ± 44</td>
</tr>
<tr>
<td>Length (µm)</td>
<td>67.4 ± 0.8</td>
<td>92.5 ± 3.4</td>
</tr>
<tr>
<td>Diameter (µm)</td>
<td>8.8 ± 0.04</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>LV+S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (µm³)</td>
<td>3119 ± 159</td>
<td>2591 ± 107</td>
</tr>
<tr>
<td>Length (µm)</td>
<td>70.9 ± 1.6</td>
<td>71.8 ± 1.0</td>
</tr>
<tr>
<td>Diameter (µm)</td>
<td>7.5 ± 0.2</td>
<td>6.8 ± 0.2</td>
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Figure 7 Cardiomyocytes density (cardiomyocytes per cm³) and estimated cardiomyocyte endowment in the LV and RV

in controls compared with 1.01 ± 0.1 in LPS-exposed animals; \( P = 0.008 \).

There was a significant increase in interstitial collagen in the RV of hearts from LPS-exposed fetuses (control: 7.0 ± 0.4%; and LPS: 10.4 ± 0.6%; \( P = 0.002 \)), but collagen levels were not different in the LV+S (control: 9.3 ± 0.9%; and LPS: 8.9 ± 1.5%; \( P = 0.82 \)). Mast cells were not observed in either control or LPS-exposed hearts.

DISCUSSION

Our study demonstrates that fetal exposure to 7 days of intrauterine inflammation has major adverse repercussions on heart structure at preterm delivery. Contractile performance of the heart was markedly reduced following LPS exposure; this may relate to the reduced complement of cardiomyocytes in the LV+S and their relative immaturity. Arrhythmias occurred in five of the six LPS-exposed hearts, which did not occur in controls. The stop-flow reperfusion-induced infarct was 3-fold greater in hearts of LPS-exposed fetuses than those of controls, suggesting increased vulnerability to oxidative stress. The sensitivity of the heart to β-AR stimulation was not changed by LPS, and this was confirmed by a lack of effect of LPS on AR mRNA levels. However, maximal contractile response to isoprenaline was markedly enhanced in LPS-exposed hearts, suggesting changes in cardiomyocyte signal transduction.

Intra-cardiac pressure increases throughout gestation, with ventricular systolic and end-diastolic pressures increasing with advancing gestation [22]. We observed higher baseline ventricular pressures in the RVs and LVs in control hearts than in LPS-exposed hearts. Pressures in control hearts were comparable with those in ~32-week gestation human fetuses, whereas pressures in LPS-exposed hearts were analogous to those in 29–30-week human fetuses [22], indicative of a delay in pressure development following LPS exposure. Our results of adverse effects on cardiac contractility in LPS-exposed hearts support previous findings of fetal cardiac dysfunction in preterm mice after acute intra-amniotic LPS [12] and reduced cardiac output and increased systemic arterial pressure in LPS-exposed preterm ventilated lambs [18]. Also, cardiac dysfunction and peripheral haemodynamic disturbances have been demonstrated in preterm infants exposed to chorioamnionitis [10], and hypotension in such infants born preterm can jeopardize brain perfusion [23]. Although the cause of altered cardiac function after intrauterine inflammation is not known, it may be due to altered autonomic control. Autonomic control of the cardiovascular system matures in the last trimester of human pregnancy [24,25]. Exposure to chorioamnionitis has been shown to alter autonomic control in preterm newborn infants, which may underlie increased brain injury after chorioamnionitis [26].

Cardiomyocyte growth in the fetal heart was markedly affected by exposure to LPS, with evidence of accelerated maturation (increase in the proportion of bi-nucleated cardiomyocytes) exclusively in the RV; this may relate to the key role of the RV in fetal haemodynamics (providing 66% of combined ventricular output [27]), the greater arterial pressures and pulmonary vascular resistance seen by the RV in LPS-exposed fetuses [18]. The increased interstitial fibrosis in the RV and not in the LV+S also supports this concept. Hypertrophy was evident in all bi-nucleated cardiomyocytes of LPS-exposed hearts, whereas there was a decrease in size in mono-nucleated cells; this was observed in both the RV and LV. The stimulus for cardiomyocyte hypertrophy of bi-nucleated cardiomyocytes is unknown. LPS
acts on Toll-like receptor-4 to activate NF-κB, initiating early synthesis of inflammatory cytokines, interleukins and tumour necrosis factor-α (TNF-α) [28]. iNOS results in uncontrolled NO production and is stimulated by TNF-α. Cell dysfunction or death could place additional stress on remaining cardiomyocytes, stimulating the hypertrophy that we have observed in these cells. Oxidative stress can trigger both apoptosis and hypertrophy of cardiomyocytes [29] and could explain our observations. Although we did not detect apoptosis in the myocardium, it is possible that there may have been induction of cardiomyocyte apoptosis immediately following LPS exposure, which may have resolved in 1 week. Of major concern was the reduced density of cardiomyocytes in the RV and LV+S of LPS-exposed hearts and the concomitant reduction in the overall complement of cardiomyocytes in the LV+S. This is important because cardiomyocytes generally cease dividing when they become terminally differentiated (around the time of birth), with postnatal growth of the myocardium occurring predominantly by cardiomyocyte hypertrophy and deposition of extracellular matrix. Given that the LV is the predominant cardiac chamber after birth, the observed reduction in the complement of cardiomyocytes in the LV+S would lead to reduced cardiac functional reserve and is likely to be a cause of the dysfunction observed in the hearts at birth. In relation to this, it is important to note the markedly higher proliferation rate seen in LPS-exposed hearts, which may reflect a compensatory cardiac growth response leading to restoration of cardiomyocyte endowment to normal after exposure to insult. If the proliferation rate continues at the rate we observed, any reduction in cardiomyocyte endowment would be alleviated in 5–10 days. In future studies, it will be important to examine cardiomyocyte number at term (when cardiomyocytes have ceased proliferating) to determine whether the reactive cardiomyocyte hyperplasia consequently restores cardiomyocyte endowment to normal.

The large increase in the number of capillary profiles relative to cardiomyocytes that we observed in LPS-exposed lambs explains the 17% increase in coronary flow in the isolated hearts. The increase in capillary profiles may reflect an increase in angiogenesis within LPS-exposed hearts. Alternatively, inflammation may increase the synthesis of iNOS, increasing levels of the vasodilator NO in the heart. In iNOS-deficient adult mice, the hypertensive response to LPS administration was blunted and survival was improved. The dilation that persisted in these iNOS-knockout mice was due to LPS-induced hydrogen peroxide (H₂O₂) production [30]. H₂O₂ induces considerable vasodilation in human coronary arterioles [31] and, hence, could explain our results. In another study of iNOS-knockout mice, although nitrite production in response to LPS was reduced to 4% of control values, survival was not improved [32]. In studies in rats, LPS was injected into the uterus via the cervix on E15 (embryonic day 15) of pregnancy, and measurements were made in the offspring immediately after birth (on day 21). Blood flow velocity in the main cerebral artery was significantly reduced [33]. In contrast, although low-dose fetal intravenous LPS had no effect on carotid blood flow in fetal sheep [34], intra-amniotic LPS increased carotid blood flow after 4 days in this model of preterm fetal sheep [35]. Thus, regional and mechanistic variability exists in the role of iNOS in sepsis and inflammation [36].

We found that the sensitivity of the isolated heart to β-AR stimulation was not altered (similar EC₅₀ values in control and LPS-exposed animals) following exposure to LPS, consistent with the similar levels of β-AR mRNA in the hearts of these animals (Figure 4). However, maximum ventricular pressure development and rate of rise of contraction in response to isoprenaline were significantly enhanced in LPS hearts, consistent with the increase in cardiac output previously reported in neonates tested 2-4 h after delivery [10]. We did not measure protein levels of β-ARs, so it is possible that these were elevated, which could explain the increased pressure development.

The maximum rate of relaxation in response to isoprenaline remained unchanged, suggesting that alterations to signalling pathways probably underlie these findings. β-AR activation increases protein kinase A, which phosphorylates phospholamban, an endogenous inhibitor of endoplasmic, sarcoplasmic calcium ATPase, which facilitates removal of intracellular calcium between contractions, essential for diastolic relaxation [37]. Thus, although the delivery of calcium for contraction by β-AR activation is enhanced by exposure to LPS, relaxation mechanisms are not affected, at least at this stage of development.

Asphyxia at the time of delivery is a common complication of preterm birth. Our findings indicate that the heart of the preterm infant exposed to chorioamnionitis may be more vulnerable to ischaemia at the time of delivery. In the present study, there was a 3-fold greater area of infarction in hearts of fetuses previously exposed to LPS than those of controls. Reperfusion following a period of ischaemia induces a strong inflammatory response as well as release of cytokines and free radicals that cause rapid cell death in the immediate vicinity and vulnerability in more peripheral cardiomyocytes. Whether the preterm heart, exposed to IR injury, can repair the myocardium in the period immediately following birth is yet to be established; the immature cardiomyocytes are still capable of proliferation at this time. IR induces a robust inflammatory response involving TNF-α, xanthine oxidase and NO, as occurs in response to LPS [38]. Since both IR and LPS increase oxidative stress, the enhanced infarct area observed could be a consequence of oxidative stress, as discussed above.

We observed arrhythmias in five of the six LPS-exposed hearts but not in the six control hearts, similar to observations in mice [12]. Cardiac dysfunction of isolated hearts in a Langendorff preparation presents as variable heart beats, evidenced by a low amplitude contraction after a premature heart beat, followed by an amplified contraction. Such events can occur as a result of premature diastolic depolarizations, which are predominantly ventricular in origin and culminate in ectopic beats. Importantly, fetal heart rate anomalies, measured by ECG, are common in preterm infants with chorioamnionitis [7–9], but are not related to poor immediate outcome, and are of unknown aetiology. Calcium influx activates ryanodine receptors (RyR) in the sarcoplasmic reticulum (SR), and LPS suppresses calcium channel activity [39]. As mentioned above, SR calcium levels are replenished via the calcium pump. SR calcium levels critically determine the ability of RyRs to release calcium from the SR, and this relationship can depend on SR redox status and RyR phosphorylation [40]. Since LPS/inflammation influences redox status [41], it will be
important to investigate RyRs in future experiments, as RyR sensitivity to calcium gives rise to premature diastolic depolarizations [40]. A close spatially precise relationship between the calcium channels and RyRs is critical for normal heart contractile function [42], and the precision of this relationship is established late in the development of cardiomyocytes [43]. Although cardiomyocyte hypertrophy occurred in LPS-exposed hearts in our study, the consequences for the spatial relationships between calcium channels and RyRs are not known. LPS reduces the main calcium current in guinea pig hearts and disrupts pacing [39]. Disruption of these systems may be responsible for the arrhythmias observed.

Conclusions
Intrauterine inflammation gives rise to a suite of changes that result in compromised cardiac performance, vulnerability to damage and arrhythmias; this is of major clinical importance to the many infants born preterm and prematurely exposed to chorioamnionitis. Hence, the altered cardiac growth, suppressed contractile function, and enhanced responsiveness to stress probably contribute to the increased vulnerability in the neonatal period of preterm infants exposed to chorioamnionitis. The deleterious changes in the heart also have the potential to adversely affect cardiac function in the long term.

CLINICAL PERSPECTIVES

- Preterm birth occurs in 8–13% of pregnancies in the Western world, and ~27% of these are associated with inflammatory conditions, such as chorioamnionitis. Cardiac dysfunction and peripheral haemodynamic disturbances, particularly fetal heart rate anomalies, including tachycardia, absence of variability, arrhythmias and absence of accelerations, are common in neonates exposed to chorioamnionitis.
- We show that intrauterine inflammation altered cardiac growth, suppressed contractile function and enhanced vulnerability to reperfusion injury and stress.
- These deleterious changes in the heart probably contribute to the increased vulnerability for organ perfusion in preterm neonates and also have the potential to adversely affect cardiac function in the long term.

FUNDING

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REFERENCES


AUTHOR CONTRIBUTION

Helena Parkington, Marianne Tare, Graeme Polglase, Timothy Moss, Martin Kluckow, Andrew Gill, Richard Harding and Jane Black were responsible for study conception and design. Timothy Moss, Samantha Barton, Robert De Matteo and Graeme Polglase were responsible for animal preparation. Marianne Tare, Jonathan Bensley, Helena Parkington, Barbara Lingwood and Min Kim were responsible for data collection and analysis. All authors were responsible for interpretation of the data and manuscript drafting.

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Chapter 5

Preterm birth adversely impacts cardiomyocyte proliferation in the developing human heart
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Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
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<tr>
<td>Performed experimental analyses (except autopsies), data analysis and wrote manuscript.</td>
<td>90%</td>
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The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

<table>
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<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%) for student co-authors only</th>
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<tr>
<td>Lynette Moore, Robert de Matteo, Richard Harding, M Jane Black</td>
<td>Conducted autopsies, designed the study, obtained funding, and assisted in writing the manuscript</td>
<td>10%</td>
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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work.

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Chapter 5 – Preterm birth adversely impacts cardiomyocyte proliferation in the developing human heart

Preterm birth adversely impacts cardiomyocyte proliferation in the developing human heart

Jonathan Guy Bensley BBiomedSc(Hons)¹, Lynette Moore MBBS FRCPA²³, Robert De Matteo PhD¹, Richard Harding PhD DSc¹ and M Jane Black PhD¹*

¹Department of Anatomy and Developmental Biology, School of Biomedical Sciences, Monash University, Clayton, Victoria, Australia, 3800, ²Department of Surgical Pathology, South Australia Pathology, Women's and Children's Hospital, North Adelaide, South Australia, Australia, 5006, ³The University of Adelaide, Adelaide, South Australia, Australia, 5005

Corresponding Author*:
Associate Professor Mary Jane Black
Department of Anatomy and Developmental Biology
School of Biomedical Sciences
Monash University
Level 3, Building 76, Wellington Road
Clayton, Victoria, Australia 3800

Total word count (all inclusive): 4,267
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Abstract

Background: Preterm birth affects 8-12% of all live births in developed countries and exposes the immature cardiovascular system to the challenges of extra-uterine life before organ development is complete. The effect of preterm birth on the growth of the human heart in the early postnatal period is largely unknown. We hypothesised that preterm birth causes adaptive changes in the heart which may lead to long-term changes in cardiac structure and function.

Aim: To examine how preterm birth affects cardiac extra-cellular matrix deposition and cardiomyocyte proliferation, maturation and morphometry in the hearts of human infants.

Methods: Heart tissue was collected at perinatal autopsy from 13 infants born preterm between 22 and 36 weeks gestation, and who survived for between 1 and 42 days; 17 stillborn infants, born between 22 and 40 weeks gestation, were used for comparison. Cardiomyocyte maturation, ploidy and size were quantified in thick paraffin sections. Cardiomyocyte proliferation was identified using Ki-67 immunohistochemistry. Extra-cellular matrix deposition was measured using picrosirius red staining. Imaging was performed using confocal microscopy and whole-slide imaging, and quantified using image analysis.

Results: Preterm birth did not affect absolute or relative heart weight when compared to gestational age-matched controls. There was no effect of preterm birth on cardiomyocyte maturation, ploidy or size. Preterm birth led to a marked reduction in cardiac cell proliferation within 24 hours of birth (p<0.0001), and interstitial collagen within the myocardium was significantly increased (p=0.0081).
Chapter 5 – Preterm birth adversely impacts cardiomyocyte proliferation in the developing human heart

**Conclusion:** Preterm birth leads to cellular and extra-cellular changes in the myocardium in the neonatal period. Our findings of reduced cardiomyocyte proliferation suggest that these changes are likely to adversely impact upon cardiomyocyte endowment, cardiac functional reserve, and on cardiac reparative ability, rendering the preterm heart vulnerable to cardiovascular disease later in life.

Word Count: 291
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Introduction

Preterm birth, defined as birth before 37 completed weeks of gestation, affects 8-12% of all live births in developed countries\(^1\,^2\). Preterm infants are born at a time when their organs are structurally and functionally immature, potentially leading to structural alterations in key organs as a result of the altered physical and chemical environment secondary to preterm birth. We recently demonstrated in sheep that moderate preterm birth induced cardiomyocyte hypertrophy, abnormal cardiomyocyte maturation, and increased interstitial collagen deposition in the neonatal heart\(^3\). These effects were considered to be an adaptive response of the myocardium in response to the haemodynamic transition at birth (where there is a marked rise in blood pressure and heart rate)\(^4\).

More recently, cardiac imaging studies have been conducted in young adults and adults born preterm, aged between 20 and 39 years old; using Magnetic Resonance Imaging (MRI) it was shown that there was an increase in left ventricular free wall mass, abnormal left ventricular wall geometry and impaired left ventricular systolic/diastolic function in the subjects born preterm relative to subjects born at term\(^5\). In addition, adults born preterm had smaller right ventricles, but increased right ventricular wall mass, resulting in altered systolic function, and a reduction in right ventricular ejection fraction\(^6\).

During development of the heart, cardiomyocytes (in almost all mammalian species) withdraw from the cell cycle around the time of full term, whereby they become terminally differentiated which may be functionally beneficial during the haemodynamic transition at birth. After this time, cardiomyocytes are unable to re-enter the cell cycle in large numbers, even in the setting of severe injury\(^7\). Cardiomyocyte proliferation does continue to occur at very low rates postnatally\(^8\); however, this proliferation is inadequate in instances of cardiac
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repair after injury. In the human heart, approximately 5% of cardiomyocytes are replaced every year at age 15, falling to 0.5% per year at age 60\(^8\). This on-going proliferation results in a tripling of cardiomyocyte endowment between the ages of 1 year and approximately 20 years\(^9\). In the mouse, the ability of existing cardiomyocytes to re-enter the cell cycle and repair injury is diminished by post-natal day 2\(^9\), with approximately 0.7% of cardiomyocytes replaced every year. During prenatal and postnatal development, additional (new) and replacement cardiomyocytes rarely come from the c-kit\(^+\) cardiac progenitor cell pool; a recent study showed only 0.008% of cardiomyocytes in the mature mouse heart arose from a c-kit\(^+\) lineage (in the absence of cell fusion events)\(^10\), thus implying that additional and replacement cardiomyocytes either arise from existing cardiomyocytes or another, as yet unknown, progenitor cell population. Given the limited replicative capacity of cardiomyocytes soon after birth, a reduction in cardiomyocyte endowment in infancy, resulting from an adverse intra-uterine environment or perinatal environment, has the potential to adversely impact heart growth, cardiac function, functional reserve and reparative ability throughout postnatal life.

Preterm infants are born at time when the heart is still very immature; hence the haemodynamic changes imposed by birth may directly impact on cardiomyocyte growth and proliferation, and extra-cellular matrix deposition in the heart\(^11\). To date, no studies have investigated the impact of preterm birth on the cellular development and maturation of cardiomyocytes in the human heart. Therefore, the aim of this study was to examine, in the hearts of infants collected at autopsy, the effects of preterm birth on cardiac structure and, in particular, on the development and maturation of cardiomyocytes. In order to do this, we have initially characterised the growth of the cardiac muscle in the hearts of normally grown
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Infants during mid-to-late gestation and then compared them to infants who were born preterm at the same post-conceptional age.

Ethical approval, Patient Selection and Methods

Ethical approval: This study was approved by the Human Research Ethics Committee of the Women’s & Children’s Health Network (WCHN) (Adelaide, Australia) (Approval Number: REC2222/10/12) and the Human Research Ethics Committee of Monash University (Clayton, Australia) (Approval Number: 2011000929). Consent was obtained from all parents for the use of tissue for research purposes. During analysis the researchers were blinded to gestational age and grouping (preterm or control).

Subjects studied: Archived heart tissue samples were obtained from perinatal autopsies conducted at the Women’s and Children’s Hospital (North Adelaide, South Australia, Australia) between 1996 and 2009. Twelve preterm neonates, born before 37 completed weeks of gestation, with postnatal survival ≥ 2 days were included in this study. The heart of an additional preterm infant that survived for 24 hours was also analysed in order to establish how quickly cardiomyocyte proliferation is affected after preterm birth. The hearts from seventeen stillborn infants that died acutely in utero were used as gestational controls; stillborn infants ranged in age from 22 to 40 weeks of completed gestation. Details of preterm and control infants, stratified according to age at birth, are shown in Table 1.

Infants were excluded according to the following exclusion criteria: maternal smoking or substance abuse, presence of maternal infectious disease, neoplastic disease or autoimmune disease, chromosomal abnormality, congenital defect, two vessels in the umbilical cord, acute/chronic chorioamnionitis, viral infection, oligohydramnios,
Chapter 5 – Preterm birth adversely impacts cardiomyocyte proliferation in the developing human heart polyhydramnios, or intra-uterine growth restriction (body weight \( \leq 10^{th} \) percentile for gestational age at birth).

**Assessment of tissue morphology, identification of mast cells and interstitial collagen:**

Paraffin sections (5µm thick) were stained with haematoxylin and eosin to examine general tissue morphology and assess tissue quality; Toluidine Blue staining was used to determine the presence of mast cells\(^3\) and Picosirius red to stain collagen fibres within the myocardium\(^3\). Whole slide scanning was used to capture images of sections stained with Haematoxylin & Eosin, Toluidine Blue and Picrosirius Red, using an Olympus dotSlide 2.1 (Olympus Soft Imaging Systems, Germany). Whole slide images could not be analysed as a single image, and were therefore broken down into large non-overlapping areas prior to analysis. Mast cells were detected using machine learning software, ilastik 0.5 (University of Heidelberg, Germany), and then quantified using image analysis software, CellProfiler r2.0.11710 (Broad Institute, USA). Interstitial collagen was quantified with MetaMorph 6.1 (Molecular Devices, USA) and is expressed as the area occupied by collagen divided by total tissue area.

**Immunohistochemistry** The 5µm paraffin sections were de-waxed and underwent heat mediated antigen retrieval. Antigen retrieval was achieved using 0.01M Sodium citrate buffer (Sigma-Aldrich, USA) with 0.1% Triton X-100 (Sigma-Aldrich, USA) in a microwave oven for 20 minutes. Cell proliferation was detected using an antibody against Ki-67 (MIB-1 Clone, 1:100 dilution, 1 hour at room temperature, Dako, Denmark). A goat anti-mouse secondary antibody bound to Alexa Fluor 488 (1:1,000 dilution, 30 minutes at room temperature, Invitrogen, USA) was used for detection, along with DAPI as the nuclear stain. Sections were mounted using ProLong Gold anti-fade mounting medium (Invitrogen, USA).
Chapter 5 – Preterm birth adversely impacts cardiomyocyte proliferation in the developing human heart

Slides were scanned using an Olympus dotSlide 2.1 slide scanner equipped with an X-Cite 120Q (Lumen Dynamics, Canada) fluorescent light source. Images were exported in large non-overlapping areas, separated by colour. CellProfiler r2.0.11710 (Broad Institute, USA), was used to analyse the scanned images. Cell proliferation is expressed as the percentage of nuclei positive for Ki-67 across the entire slide.

**Cardiomyocyte ploidy, nuclearity and size analysis:** Cardiomyocyte ploidy, nuclearity and size were evaluated as previously described\(^3\). Briefly, thick paraffin sections (≥100µm) were stained using Wheat Germ Agglutinin – Alexa Fluor 488 (Invitrogen, USA) to permit visualisation of cardiomyocyte cell membranes and either DAPI hydrochloride or YOYO-3 (Invitrogen, USA) to stain nuclei. Z-stacks used for cardiomyocyte analysis (ploidy, nuclearity and size) were acquired using either a Leica SP5 confocal microscope (Leica Microsystems, Germany) or a Nikon C1 confocal microscope (Nikon, Japan). Cardiomyocyte ploidy, nuclearity and size were measured using Imaris software Versions 6.1.5 to 7.6.3 (Bitplane, Switzerland) and Volocity software Versions 5 to 6.2.1 (Improvion Perkin Elmer, UK) using a previously described technique\(^3\). For the analysis of cardiomyocyte ploidy, nuclearity and size, at least 5,000 cells were analysed per subject.

**Statistical analysis:** Data were statistically analysed using SPSS Statistics 22 (IBM SPSS, USA). Univariate analysis, with group (preterm or control), and gestational age grouping as factors, followed by Bonferroni post-hoc tests as appropriate. Linear regressions of body weight at birth, body weight at autopsy, heart weight, heart weight relative to body weight at autopsy, cardiomyocyte proliferation and cardiac collagen between controls and preterms were compared using an Analysis of Covariance (ANCOVA) to determine differences in the slope.
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and y-axis intercept. A p value < 0.05 was regarded as statistically significant. Graphs were prepared in GraphPad 6.04 (GraphPad Software, USA).

Results

Body weights and growth: Infants born preterm were on a significantly different growth trajectory at birth compared to in utero controls (p=0.0075) (Figure 1A). However, at autopsy, there was no difference between the preterm infants and controls at the gestational age at death (p=0.2) (Figure 1B). The amount of weight change per day of life (Figure 1C), and total weight change during life (Figure 1D) varied markedly between infants born preterm.

Survival: Survival for preterm infants varied from 1 day to 42 days, with a mean of 18±4 days and a 95% confidence interval of 9-26 days.

Heart characteristics: No differences were found in heart weight (Figure 1E), even after adjustment for body weight at autopsy (Figure 1F), between control infants and those born preterm. For the 17 control infants, all had probe patent ductus arteriosus and foramen ovale. Of the 13 infants born preterm, all had probe patent foramen ovale. Three preterm born infants had non-probe patent ductus arteriosus; these included two moderately preterm born babies (one who survived for 1 day and the other for 42 days) and one extremely preterm baby (alive for 28 days).

Inflammatory cell infiltrate: No inflammatory cell infiltrates were observed in any of the control or preterm hearts, and mast cells were rarely observed (<1 per 10^7 nuclei).
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**Interstitial collagen:** The amount of interstitial collagen within the myocardium declined with advancing gestational age. There was significantly more \((p=0.04)\) cardiac collagen in the preterm infants compared with controls (Figure 2A).

**Cardiomyocyte proliferation:** In control infants only, there was an inverse relationship between the percentage of proliferating cardiac cells and gestational age (Figure 2B) \((\text{Control } r^2=0.81 \ (p<0.0001), \text{ Preterm } r^2=0.02 \ (p=0.64))\). In contrast, cardiac cell proliferation was low in all preterm infants except in one preterm infant (Figure 2B) \((\text{Control vs preterm: } p<0.0001)\).

**Cardiomyocyte morphometry, nuclearity and ploidy:** Cardiomyocyte volume increased with advancing gestational age in all infants, and there was no effect of preterm birth (Figure 2C) \((\text{Control vs preterm: } p=0.36)\). In both control and preterm born hearts, the majority of cardiomyocytes were mononucleated and there were no differences in nuclearity between controls and preterm hearts at any time point \((\text{Control vs. preterm: } p=0.56)\) (Figure 2D). There was no difference between preterm and control hearts on the basis of ploidy at any gestational age, with >99% of non-dividing nuclei being 2n \((\text{Control vs preterm: } p=0.83)\).

**Discussion**

This is the first study to examine how preterm birth in human infants affects cardiac extracellular matrix deposition, cardiomyocyte morphometry, proliferation, growth, maturation and ploidy in the early post-natal period. Of major clinical importance, we have demonstrated that in the immediate period after preterm birth there is an almost complete cessation of cardiomyocyte proliferation, whereas cardiomyocytes of age-matched stillborn
infants were still undergoing proliferation. Furthermore, there was an increase in interstitial collagen in the hearts of preterm infants relative to the hearts of stillborn infants.

The results of this study clearly demonstrate that preterm birth, or some aspect of intervention surrounding preterm birth, leads to a very sudden and premature termination of cell proliferation within the heart. This abrupt cessation of proliferation contrasts with observations in age-matched control infants who were not born, and likely results from the sudden and increased haemodynamic demands that are placed on the immature heart at the time of birth (the cardiomyocytes need to become terminally differentiated to achieve and maintain function). Whether the termination of cardiomyocyte proliferation is a normal feature after birth, including term birth, is uncertain and we have not examined this issue in this study but it must be examined in future studies. Alternatively these effects may be due to the administration of corticosteroids\(^{12-15}\) (either to the mother prior to birth or in the immediate post-natal period) and/or other interventions relating to neonatal care, the control infants died from acute causes and were very unlikely to have received corticosteroids.

It is now common practice for all mothers with threatened premature delivery to be administrated antenatal corticosteroids. The doses given to mothers threatening preterm labour can be substantial (routinely 2 doses of 11.4 mg each) and additional doses are often given to the preterm infant after birth. Essentially all infants born preterm in developed countries will receive at least one dose of a corticosteroid\(^{16}\), with 80%+ of mothers threatening premature labour also receiving at least one dose of a corticosteroid\(^{17}\). The benefits of exposure to corticosteroids to the newborn preterm infant have been clearly demonstrated in terms of survival, lung maturation and the prevention of intra-ventricular
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haemorrhage\textsuperscript{18}. However, given that corticosteroids are linked to the accelerated maturation of some organs, it is likely that it is the exposure to corticosteroids in the preterm infants that has led to the premature termination of cardiomyocyte proliferation observed in this study. It must also be considered possible that corticosteroid administration led to accelerated cardiomyocyte maturation, followed by an earlier than usual transition into the slow, ongoing cardiomyocyte proliferation program that is usually observed after birth.

A significantly greater amount of myocardial collagen was observed in the hearts of preterm born babies compared with the unborn controls. This may represent a structural adaptation to the increase in systemic arterial blood pressure that occurs following birth\textsuperscript{4}. If this trend in collagen deposition were to continue, it may lead to excessive extra-cellular matrix deposition in preterm born hearts. These findings are in accordance with our previous studies where we showed an increase in myocardial interstitial collagen in sheep born moderately preterm at 9 weeks post-term equivalent age\textsuperscript{3}. Interestingly, however, we did not observe any differences in cardiomyocyte size, nuclearity or ploidy in the hearts of the preterm infants relative to gestational age-matched controls, which is in contrast to our previous findings in the preterm sheep. In the moderately preterm sheep heart we found a significant increase in cardiomyocyte size, differences in cardiomyocyte nuclearity (including the presence of trinucleated cardiomyocytes) and an increase in cardiomyocyte ploidy in the mononucleated cardiomyocytes. In the present study, although there were marked effects on cardiomyocyte proliferation, all the other parameters that we examined in relation to cardiomyocyte growth appeared within the normal range. The difference in findings in relation to cardiomyocyte growth likely relate to differences in species and stages of cardiac
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devlopment at the time of tissue analysis. Humans grow very slowly, taking 14-20 years to reach final adult size. In comparison, sheep grow quickly and begin puberty within 1-1.5 years. In our previous studies in sheep, we examined the preterm hearts at 9 weeks post-term equivalent age. This is equivalent to early adolescence in the human. Hence, it is possible that the induction of cardiomyocyte hypertrophy, hyper-nuclearity and polyploidy (observed in the preterm sheep heart) may not manifest until childhood/early adolescence. Further studies in preterm subjects of varying ages after birth up until adulthood would help to elucidate this. Certainly, the recent MRI studies conducted in preterm born young adults and adults have clearly shown differences in cardiac structure and how the heart grows postnatally, which would support this concept.

Further to this, in our lamb studies, the preterm lambs were healthy throughout their life, whereas the preterm infants in this study were critically ill during the postnatal period. As the preterm infants were critically ill, we cannot exclude the possibility of post-natal interventions affecting the results, such as mechanical ventilation, medication administration, hypoxia, and other unknown factors. The general effect of illness in exacting additional demands on the cardiovascular system could not be evaluated in this study, but it is likely to have had an effect.

Importantly, the abrupt cessation of cardiomyocyte proliferation as a result of preterm birth is likely to significantly impact on cardiomyocyte endowment throughout life, with the greatest impact likely to be in the earliest born preterm infants. An infant born extremely preterm that has cardiomyocytes that stop proliferating in great numbers much earlier in life will need to have more extensive cardiac remodelling (cardiomyocyte hypertrophy, extra-cellular matrix deposition) to achieve a normal cardiac mass than an infant born
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moderately preterm. It is also important to emphasise that the changes we observed were not confined to extremely and very preterm infants, but also included moderately preterm infants. The results of our study suggest that even moderately preterm babies have an abnormal cardiomyocyte proliferation trajectory and the cardiac growth of these individuals in the neonatal period must be therefore considered as quite different to the cardiac growth of individuals who were born at term. Hence, the findings of this study have major clinical implications and highlight the need for clinicians to consider preterm birth as a risk factor for adult cardiovascular disease. Indeed, a reduced complement of cardiomyocytes at the beginning of life will likely adversely impact on lifelong cardiac functional reserve and on the reparative and adaptive abilities of the myocardium. It may be prudent for clinicians to consider periodic echocardiographic examinations of adults born preterm to monitor cardiac performance and potentially intervene early, as this group may be at greater risk of cardiovascular disease at an earlier age than term born adults.

**Conclusion**

The results from the present study clearly show that human preterm birth can be detrimental to the normal development of the myocardium, in particular the final number of cardiomyocytes and extracellular collagen deposition. These findings are consistent with recent studies showing that the growth and structure of the heart in adults born preterm are markedly different to those born at term. Our findings indicate that individuals born preterm may be at heightened risk of cardiovascular disease and it is imperative to ascertain this before pathological sequelae ensues. Our study highlights the need for further investigations into how preterm birth and the interventions surrounding preterm impact on
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the long-term cellular structure of the heart; such information is especially important given that the first survivors of extremely preterm birth are now reaching adulthood.

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Declaration: No author reports any conflict of interest related to this study.

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References

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**Table 1**

<table>
<thead>
<tr>
<th>Gestational Age Group</th>
<th>Control Group</th>
<th>Preterm Group at Birth</th>
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<tbody>
<tr>
<td>Extremely Preterm (22-27 weeks)</td>
<td>3 Female/1 Male</td>
<td>3 Female/5 Male</td>
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<tr>
<td>Very Preterm (28-31 weeks)</td>
<td>1 Female/1 Male</td>
<td>1 Female/0 Male</td>
</tr>
<tr>
<td>Moderately Preterm (32-37 weeks)</td>
<td>6 Female/3 Male</td>
<td>1 Female/3 Male</td>
</tr>
<tr>
<td>Term (≥38 weeks)</td>
<td>1 Female/1 Male</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>11 Female/6 Male</td>
<td>5 Female/8 Male</td>
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<tr>
<td></td>
<td>17 in total</td>
<td>13 in total</td>
</tr>
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</table>

Table 1: Summary of gestational age group by patient group (control/preterm) and gender
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Figure 1

A) Body weight at birth (in grams) versus gestational age at birth (in weeks)

B) Body weight at autopsy (in grams) versus gestational age at autopsy (in weeks)

C) Body weight change (average) per day of life (in grams/day), for preterm born infants only
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D) Body weight change (total) between birth and autopsy by number of days of survival, for preterm born infants only

E) Heart weight at autopsy (in grams) versus gestational age at autopsy (in weeks)

F) Heart weight corrected for body weight (grams/grams) compared to gestational age at autopsy (in weeks)

$P(\text{Group})$ refers to in utero compared to preterm. $P(\text{Age})$ refers to the gestational age at birth in weeks. $P(\text{Group} \times \text{Age})$ refers to the interaction effect between group (Control vs. preterm) and gestational age. $P(\text{Slope})$ refers to the difference in the slope of the two lines of best fit, as calculated by an ANCOVA. $P(\text{Intercept})$ refers to the difference in the elevations (intercept with axes), as calculated by an ANCOVA.
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Figure 2

A) Collagen (as a % of tissue area in the heart) versus gestational age at autopsy (in weeks)

B) Proliferating cells (% of total nuclei that were Ki-67+) versus gestational age at autopsy (in weeks)

C) Mean cardiomyocyte volume per patient (x100 um³) grouped by gestational age category. Control (Extremely preterm n=4, Very preterm n=2, Moderately preterm n=9 and Term =2). Preterm (Extremely preterm n=5, Very preterm n=3, Moderately preterm n=5).
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D) Percentage of cardiomyocytes that were mononucleated in each patient’s heart.

Control (Extremely preterm n=4, Very preterm n=2, Moderately preterm n=9 and Term =2). Preterm (Extremely preterm n=5, Very preterm n=3, Moderately preterm n=5).

P(Group) refers to in utero compared to preterm. P(Age) refers to the gestational age at birth in weeks. P(Group*Age) refers to the interaction effect between group (Control vs. preterm) and gestational age. P(Slope) refers to the difference in the slope of the two lines of best fit, as calculated by an ANCOVA. P(Intercept) refers to the difference in the elevations (intercept with axes), as calculated by an ANCOVA.
Chapter 6

An improved method for 3D measurement of cardiomyocyte volume, nuclearity and ploidy in thick histological sections
Chapter 6 – An improved method for 3D measurement of cardiomyocyte volume, nuclearity and ploidy in thick histological sections

Declaration by candidate

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performed experimental analyses and wrote manuscript.</td>
<td>90%</td>
</tr>
</tbody>
</table>

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

<table>
<thead>
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<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%) for student co-authors only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robert de Matteo, Richard Harding, M Jane Black</td>
<td>Obtained funding, and assisted in writing the manuscript</td>
<td>10%</td>
</tr>
</tbody>
</table>

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work.

Candidate’s Signature: [Signature]
Date: 27/7/15

Main Supervisor’s Signature: [Signature]
Date: 27/7/15
Chapter 6 – An improved method for 3D measurement of cardiomyocyte volume, nuclearity and ploidy in thick histological sections

An improved method for 3D measurement of cardiomyocyte volume, nuclearity and ploidy in thick histological sections

Short title: Cardiomyocyte characterisation in thick sections

Jonathan Guy Bensley, Robert De Matteo, Richard Harding and Mary Jane Black

Department of Anatomy and Developmental Biology, School of Biomedical Sciences, Monash University, Clayton, Victoria, Australia, 3800

Corresponding Author:
Associate Professor Mary Jane Black
Department of Anatomy and Developmental Biology
School of Biomedical Sciences
Monash University
Level 3, Building 76, Wellington Road
Clayton, Victoria, Australia 3800
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Word Count: 3,326

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Disclosure: No author reports any relevant disclosures.
Abstract

Background: Quantitative analysis of myocardial development, and cardiac disease, requires accurate measurements of cardiomyocyte volume, nuclearity (number of nuclei per cell) and ploidy (number of genome copies within the cell). Conventional histochemical methods are unable to visualise cardiomyocyte membranes. Current methods often require the enzymatic isolation of cardiomyocytes to measure volume directly, which requires fresh unfixed tissue. Here we describe an improved method of cardiomyocyte analysis that permits the simultaneous measurement of cardiomyocyte volume, nuclearity and ploidy in thick histological sections; myocardial capillarisation can also be assessed.

Methods: Heart tissue was obtained from 4 species (rat, mouse, rabbit and sheep) at 3 life stages (in utero, weaning and adulthood). Sections were stained with wheat germ agglutinin-Alexa Fluor 488 to visualise cell membranes and a nucleic acid dye (e.g. DAPI, YOYO-3) to visualise nuclei. Using confocal microscopy and 3-dimensional image analysis, cardiomyocyte volume, nuclearity and ploidy were measured.

Results: The combination of wheat germ agglutinin-Alexa Fluor 488 and a nuclear dye gave excellent visualisation of cardiomyocytes in 40µm thick paraffin sections. There was a wide range of cardiomyocyte volumes within all species and time-points. Nuclearity and ploidy increased with age in mice and sheep.

Conclusions: This improved method of staining and analysis allows the full range of cardiomyocyte analyses (volume, nuclearity and ploidy) to be performed in thick sections, in a time and cost efficient manner. This method, confirmed to be effective in a number of different species, provides a valuable method for accurate assessment of cardiomyocyte
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size, nuclearity and ploidy. Our approach permits the entire cell to be visualised in 3D directly; re-alignment of serial sections is avoided entirely.

Abstract word count: 278
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Introduction

Cardiomyocytes are the individual functional units of the cardiac muscle, providing the contractile power of the heart. Postnatal growth of the heart is a far more dynamic process than previously realised; humans triple their cardiomyocyte endowment between birth and 20 years of age\(^1\) and replace their cardiomyocytes at a rate of 5% per year at age 15 and 0.5% at age 60\(^2\). However, these additional and replacement cardiomyocytes do not have the capacity to repair the heart after injury\(^3\). Given that abnormal growth of the myocardium, particularly ventricular hypertrophy, is strongly linked to adverse cardiovascular events\(^4\), it is important to understand how the cardiac muscle grows over time; this is also of critical importance in understanding the myocardial response to disease and injury.

In order to characterise the growth and properties of individual cardiomyocytes within the myocardium there are two methodological options that can be employed; one is to fix and embed tissue samples in paraffin, OCT or resin and the other is to enzymatically isolate cardiomyocytes from fresh unfixed heart tissue using a collagenase enzyme\(^5\).

Enzymatically isolated cardiomyocytes can be used for the measurement of cell volume, nuclearity (number of nuclei per cell) and ploidy (number of genome copies per nucleus). Enzymatic isolation is also essential for studies employing cell culture, flow cytometry or ex-vivo analysis of calcium signalling by confocal microscopy. Obtaining enzymatically isolated cells usually requires fresh cardiac tissue and this approach prevents the examination of the cardiomyocytes in situ and there is the possibility that cell dimensions and volume are influenced by the isolation process. Cellular isolation from fixed, archival tissue is difficult. It is possible to isolate cells from fixed cardiac tissue, using 12.5 mol/L aqueous potassium hydroxide\(^6\); however, the yield is low and the data generated does not match that obtained
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from enzymatically isolated cardiomyocytes from unfixed fresh tissue\(^7\). This method can also lead to the destruction of smaller mononucleated cardiomyocytes\(^7\).

Cardiac tissue is usually archived as tissue samples embedded in paraffin. Typically the analysis of heart tissue from paraffin blocks has been restricted to thin sections (4-7\(\mu\)m), which only presents a restricted view of cardiomyocytes. As cardiomyocytes are differentially oriented, depending on where they are in the heart, analysis of thin sections can result in inaccurate morphometric measurements. To date, the most common approach to assessing cardiomyocyte dimensions or volume in paraffin sections has been to measure the cross-sectional area of the cardiomyocyte; however, there are limitations with using cross-sectional area as a surrogate measure of cardiomyocyte volume. Cross sectional area around the short axis of the cardiomyocyte presents a 2-dimensional picture of a 3 dimensional cell; importantly it ignores the length of the cardiomyocyte, which can vary considerably between cells, and is unable to account for cardiomyocytes that branch (a common characteristic of differentiated cardiomyocytes)\(^8-10\). Other researchers have utilised serial sections of thin sections (4-7\(\mu\)m), followed by immunohistochemistry to identify cell boundaries, and then re-constructed the cell volume\(^11\). This method, whilst accurate, is time consuming, technically challenging and expensive.

Measuring cardiomyocyte nuclearity is also important in studies of cardiac development and structure, before and after birth. In many species, binuclearity is a mark of cardiomyocyte maturity\(^12\) (but not in humans or other primates\(^13\)). Another important measure is ploidy, especially in humans\(^2\) and other primates in which the majority of cardiomyocytes remain mononucleated throughout life, with these mononucleated cardiomyocytes usually
becoming tetraploid (4n) during adolescence\(^1\). Cardiomyocyte polyploidy is often an indicator of stress or abnormal cardiomyocyte development and maturation\(^12\).

Recent studies suggest that abnormalities in cardiac growth may have their origins in early development. Much of our knowledge in this regard has been derived from animal studies\(^10,14-18\). The applicability of these findings to humans is dependent on how closely the growth of cardiomyocytes in the animal models resembles that in the human heart. In order to interpret the findings from animal models it is therefore imperative to fully characterise the normal growth of cardiomyocytes in animal models and determine how prenatal, perinatal and postnatal growth of cardiomyocytes resembles and/or differs to that in the human.

Owing to shortcomings with existing methods of cardiomyocyte analysis, our aim was to develop an accurate, time-efficient and cost-efficient method to analyse cardiomyocyte growth (size, nuclearity and ploidy) in paraffin-embedded samples from different species at different life stages; the improved technique that we describe is robust and can be used by researchers to advance our knowledge of cardiomyocyte growth.

**Methods**

**Ethical approval:** This experiment utilised archival fixed heart tissue from previously conducted experiments, all of which were approved by the Monash University Animal Ethics Committee.

**Cardiac samples:** Heart tissue used for analysis included formalin-fixed paraffin embedded hearts from: 10 weanling (post-natal day 2-3) and 3 adult mice (> 1 year old), 4 weanling rabbits (post-natal day 5-7), 3 adult rats (1 year old), 7 near-term fetal sheep (~143 days out
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of 147 days of gestation), 5 nine-week-old lambs and 2 adult sheep (> 1 year old). Weanling mouse and rabbit hearts had been immersion fixed in formaldehyde solution. All sheep hearts, adult mouse and adult rat hearts had been retrogradely perfusion fixed with formaldehyde solution via the aorta.

**Tissue sectioning and staining:** The improved method we describe here is based on the ability of wheat germ agglutinin (WGA), conjugated to a fluorescent dye such as Alexa Fluor 488 (AF488), to selectively stain cell membranes and blood capillaries. Additionally, a stoichiometric nuclear dye was utilised to display DNA present in the nucleus. As the amount of fluorescence is proportional to the amount of DNA present in the nucleus\(^{19}\), the brightness of the nuclei is proportional to the amount of DNA present within the nucleus. In conventionally stained sections using haematoxylin and eosin sections, it is impossible to identify cardiomyocyte cell boundaries. In contrast, our improved method using fluorescently labelled wheat germ agglutinin (WGA-AF488), in combination with a nuclear dye (e.g. DAPI, YOYO-3, Hoechst 33342), enables the determination of cardiomyocyte volume (the volume enclosed by the cell membrane stain), nuclearity (the number of nuclei enclosed in the cell membrane), and ploidy (the brightness of the nuclei), as discussed below in greater detail. Capillaries are clearly visible within the sections and hence myocardial capillarisation can also be assessed using this method; however, this was not quantified in the heart samples reported here. Controls for ploidy depend on the expected ploidy level. As a general principle, semen from the same species as the heart makes an excellent control; semen contains spermatozoa with 1N DNA. We placed a single spot of thawed semen on one end of the slide; this was stained, and acquired, identically to the heart tissue. If semen is not available, paraffin sections of testis from an adult animal are
acceptable, as testis contains both 1N and 2N cells. Alternatively, endothelial cells within the myocardial section could be used; endothelial cells are almost always 2N when not proliferating and their nuclei are morphologically different to that of the cardiomyocytes.

**Formalin-fixed paraffin embedded sections:** The optimised staining protocols for formalin-fixed paraffin-embedded heart sections (4-8 µm and 40 µm) are described in Table 1. The recommended section thickness for measuring cardiomyocyte volume is 40µm, as this provides good scope for capturing whole cardiomyocytes. Prior to sectioning at 40µms, we cut a 4-5um paraffin section and stained it with haematoxylin and eosin, in order to determine the orientation of cardiomyocytes within the tissue. The desired orientation is with the long axis of the cardiomyocytes in the cutting plane; if only the short axis is present, we re-embedded the specimen at 90 degrees to the original cutting plane to align the cardiomyocytes in the longitudinal plane (Figure 1). When staining the sections, we used a 5 slide Coplin jar to minimise the amount of reagent required. This method also has the advantage of catching any sections that become detached from the slides. The sections were protected from light at all times during the staining procedure. The sections should be protected from light because, although Alexa Fluor 488 and the nuclear dyes used (e.g. DAPI) are very bright and photostable, exposure to room light will cause some degree of photobleaching, and prolonged z-stack imaging such as performed here will generally result in moderate to severe photobleaching of the sections. All sections should be stored in the dark, and should be imaged as soon as possible after staining (within 7 days of staining). ProLong Gold and similar anti-fade mounting media greatly reduce photobleaching during confocal microscopy, and reduce the rate of fluorescence loss during prolonged storage; however, no anti-fade medium provides complete protection from photobleaching.
Confocal microscopic acquisition of cardiomyocyte z stack images: In order to undertake morphometric analyses of cardiomyocytes, confocal z-stacks were captured using either a Leica SP5 confocal microscope (Leica Microsystems, Germany) or a Nikon C1 confocal microscope (Nikon, Japan). The optimisation of the confocal acquisition of images is described below.

Signal intensity is crucial to measuring ploidy reliably and accurately: a signal that is too dim will be indistinguishable from background noise, whereas a signal at the limit of detection eliminates dynamic range (a signal 4 times the detector upper limit will appear the same as a signal 20 times the detector upper limit). The appearance of cells with the desired intensity of DAPI intensity is shown in Figure 2A. The mean intensity through the z-stack is flat, with a ‘drop-off’ noted as the section ends (on the right-hand side of the chart). When this was not achieved, nuclei deeper in the tissue appeared dimmer than in reality and nuclei closer to the objective lens appeared brighter than they actually were. For the image shown in Figure 2A, at no point does the signal intensity reach zero and is not near the intensity limit. The intensity profile for WGA-AF488 appears in Figure 2B; intensity linearity was not required in this channel as is not being quantified and is optimised for image analysis performance. When using a Leica confocal microscope equipped with LAS AF (Leica Application Software – Advanced Fluorescence) software, we performed compensation on a z-stack acquisition using the Photomultiplier Tube (PMT) method and/or the Acousto-Optical Tuneable Filter (AOTF) method. The PMT method regulates the voltage within the PMT; the voltage is the acceleration energy (the gain) applied to the electrons within the PMT. AOTF works by applying a filter to regulate the amount of laser light delivered to the specimen, without changing the power of the laser itself (which is usually constant).
setting the AOTF method, the amount of laser light in percentages was rated against 100% of maximum rated power of the laser that is allowed to pass through the filter. When using a Nikon confocal microscope, we utilised the Z intensity correction feature when performing the acquisition in NIS-Elements (Nikon, Japan). When performing ploidy analysis, we found it essential that the brightest pixels in the acquisition were well away from the saturation limit of the PMT and above the noise floor (usually zero).

The optical design and software in other confocal microscopes (e.g. Bio-Rad, Zeiss) are different from the Leica and Nikon and it would be necessary to contact the microscope manufacturer to determine how to perform an intensity correction if using these other microscopes.

**Image Analysis:** Images were analysed using 3 dimensional image analysis software programs, Imaris 7.7 (Bitplane, Switzerland) and ilastik 1.1.3 (University of Heidelberg, Germany). Alternative 3-dimensional image analysis software programs include: Amira (FEI, France), Volocity (Perkin Elmer Improvision, United Kingdom) or Huygens (Scientific Volume Imaging, The Netherlands).

**Measurement of cardiomyocyte volume:** With the cardiomyocyte membranes stained with WGA-AF488, cardiomyocyte volume was measured by collation of the Z-stack images. Cardiomyocytes within the sections were easily recognisable by their cytoplasmic striations (Figure 3A). The approach to measuring cardiomyocyte volume differed depending on the 3-dimensional software employed. To define the cardiomyocyte volume, using ilastik (University of Heidelberg, Germany), a single “seed” (starting point for propagation) was placed in the centre of each cell (Figure 3B) and bright lines used to determine the cell boundary during propagation. The seed forms the starting point within the notional
cytoplasm, and the software will propagate in every direction until the cell membrane is reached (the brightest lines will be the cell membrane staining) and stop at that point. If necessary, such as when there is high background fluorescence, we encircled the cell with a background seed to reject the background from the cell measurement. We ensured that each cardiomyocyte lay completely within the z-stack. Manual segmentation was occasionally required to separate inappropriately joined cells.

As an alternative to analysis with Imaris, other 3-dimensional software (e.g. Amira, Volocity, Huygens) can be utilised and this requires some modifications in the analysis procedures. When analysing z-stacks using Volocity, Amira or Huygens, we found it preferable to perform a brightness inversion on the WGA-AF488 channel to produce a bright cytoplasm with dark membranes (or dark cytoplasm and bright membranes) (Figure 3D). The brightness inversion required some manual ‘clean-up’ to produce clear membranes and enable easy analysis of the cardiomyocyte volume. It was necessary that the ‘fill holes’ option was enabled when creating the final cardiomyocyte volumes, to create a single object without internal holes. This is detailed below.

**Measurement of cardiomyocyte nuclearity and ploidy:** In the present analyses, cardiomyocyte confocal z-stack images were imported into Imaris, along with the objects created in ilastik via FIJI. FIJI is a build of ImageJ that allows Imaris to understand the file format produced by ilastik. The typical appearance of the nuclei when analysing using Imaris is shown in Figure 3C. Cardiomyocyte nuclei were clearly recognisable by their distinctive ovoid-shape, and strong nucleoli staining.

Using this approach, each object created in ilastik forms the basis for each individual cardiomyocyte cell volume to be measured in Imaris. To analyse nuclearity and ploidy in
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Imaris, the nuclei are selected in the DAPI (or other nuclear dye) channel. For the definition of the cell cytoplasm, we chose the cardiomyocyte volume objects created in ilastik. Imaris can define the parent/child relationship between the cardiomyocyte volume (parent object) and the nuclei (child objects). Imaris will not accept a nucleus that does not reside in a cell, or a cell without a nucleus. After the analysis is complete, Imaris displayed per-cell cytoplasmic volume, number of nuclei per cell, integrated intensity of the nuclei within the cell (measure of overall cardiomyocyte ploidy) and integrated intensity per-nuclei (measure of individual nuclear ploidy). Mitotic/proliferating cardiomyocytes did not have their ploidy measured (Figure 4). Ploidy is measured relative to the control nuclei integrated intensity (semen, testis or endothelial cell). If spermatozoa are used as the control cell, 2N nuclei will have approximately double the integrated intensity of the spermatozoa; if using a 2N endothelial cell within the heart section as the control, the 2N cardiomyocyte nucleus will have approximately the same integrated intensity as the endothelial cell. For a tetraploid (4N) nucleus, the integrated intensity will be 4 times that of the spermatozoa or twice that of the endothelial cell.

As an alternative to analysis with Imaris, other 3-dimensional software was tested but this required some modifications in the analysis procedures. For example, when using Volocity, we defined the cardiomyocyte volumes using the brightness inverted WGA-AF488 channel, being careful to ensure the cardiomyocytes were separated properly. If necessary, we used the cutting tool to manually separate out cells that were inappropriately joined together. As the images are binarised (either black or white), no thresholding was necessary as the voxels are either part of the membrane or cytoplasm, but never both. In the nuclear stain channel (e.g. DAPI), the cardiomyocyte nuclei were selected with the thresholding tool to capture
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the full volume of the nuclei; any background staining and the segmenting/de-clustering of nuclei were rejected. The nuclei objects were manually reconciled with the cardiomyocyte volumes created and the data per cell and per nucleus was manually extracted from the statistics panel.

In Amira and Huygens, the procedure was slightly different, in that the cardiomyocyte objects become the parent objects, with the nuclei as the child objects.

When using 3-dimensional software programs such as those described above it was necessary to measure nuclear ploidy as the integrated intensity of the nucleus (the sum of brightness values within an enclosed object), with the amount of DNA within the nucleus proportional to the nuclear fluorescence20.

**Assessment of myocardial capillarisation:** In addition to staining cellular membranes, the fluorescently labelled WGA-AF488 also clearly binds to blood vessels because endothelial cells have a large sialic acid content, particularly on their apical surface (Figure 5). Hence, using the described staining approach, we were able to accurately measure capillarisation (such as capillary density, capillary length and surface area, and diffusion radius)21,22. We have recently demonstrated the ability of wheat germ agglutinin to selectively stain capillaries; in that study we assessed capillarisation by measuring the number of capillary profiles relative to the number of cardiomyocyte profiles in the fetal lamb heart23.

**Graphing and analysis parameters:** In the present study cardiomyocyte assessments were performed in at least 500 cardiomyocytes from each of the ventricles (left ventricle with adjoining septum and right ventricle) from each animal. Graphs were prepared in GraphPad
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6.01 (GraphPad Software, USA). Given that the number of hearts analysed was low, statistical analysis between species, ages or ventricles were not undertaken.

Results

Cardiomyocyte volume, nuclearity and ploidy: To demonstrate the utility of this technique, cardiomyocytes were analysed from 34 animals, in four species at different ages. Table 2 describes the results from each species and age, stratified by ventricle (left ventricle with septum, right ventricle) and nuclearity (mononucleated, binucleated).

Mouse cardiomyocytes: The majority of cardiomyocytes in weanling mice were binucleated (LV+S: 80±1.4%, RV: 85.1±1.4%). Binucleated cardiomyocytes had larger average volumes than mononucleated cardiomyocytes. In the LV+S, binucleated cardiomyocytes were 42.1% larger in volume than mononucleated cardiomyocytes, whereas in the RV the binucleated cardiomyocytes were 122% larger in volume than mononucleated cardiomyocytes. Right ventricular cardiomyocytes were substantially larger in volume than those from the LV+S (both mononucleated and binucleated), which may reflect the right ventricular dominance of the heart in utero. No tetraploid (4n) cardiomyocyte nuclei were observed.

Cardiomyocytes from adult mice were predominantly binucleated in both ventricles (LV+S 95.6±0.4%, RV: 96.2±0.9%). In the LV+S, the binucleated cardiomyocytes were 41.5% larger in volume than mononucleated cardiomyocytes, whereas in the RV the binucleated cardiomyocytes were 15.5% larger in volume than mononucleated cardiomyocytes. Tetraploid cardiomyocyte nuclei were present in both ventricles, with mononucleated cardiomyocytes (1 nucleus of 4n) and binucleated cardiomyocytes (2 nuclei of 4n) detected, albeit at relatively low rates and with high intra-mouse variability.
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**Rabbit cardiomyocytes:** The majority of the cardiomyocytes in the weanling rabbits were binucleated in both ventricles (LV+S: 86.4±1.1%, RV: 86±3.7%), with no evidence of tetraploidy. In the LV+S, binucleated cardiomyocytes were 48.6% larger in volume than mononucleated cells, whereas in the RV, binucleated cardiomyocytes were 69.4% larger in volume than mononucleated cardiomyocytes. Overall right ventricular cardiomyocytes (mononucleated and binucleated) were larger in volume than those from the LV+S.

**Rat cardiomyocytes:** The majority of cardiomyocytes in the adult rat heart were binucleated (LV+S: 96.5±0.3%, RV: 96.8±1.1%). However, 7 - 10% of the cardiomyocyte nuclei, from both mononucleated and binucleated cardiomyocytes in both ventricles were tetraploid, with low variability between rats. In the LV+S, binucleated cardiomyocytes were 41.4% larger in volume than mononucleates, whereas the RV binucleated cardiomyocytes were 34.5% larger in volume than mononucleated cells.

**Sheep cardiomyocytes:** In the fetal sheep hearts at ~143 days of gestation the majority of cardiomyocytes were binucleated (LV+S: 87.1±1.6%, RV: 91.1±1.2%). RV binucleated cardiomyocytes were 92.3% larger in volume than mononucleated cells and LV+S binucleated cardiomyocytes were 61.6% larger in volume than mononucleated cells. All fetal cardiomyocyte nuclei that were analysed were diploid. Mitosis was also clearly visible in some cardiomyocyte nuclei as shown in Figure 4.

At 9 weeks of age (adolescence), binucleated cardiomyocytes predominated (LV+S: 97.6±0.9%, RV: 98.4±0.7%), and the proportion of these did not increase in adulthood (LV+S: 98.5±1.5%, RV: 98.5±0.3%). At 9 weeks a small proportion of RV cardiomyocytes were tetraploid (0.1±0.1%). In adult sheep, nuclear tetraploidy was variably present in
mononucleated cells (one of the two adult sheep had no tetraploidy), and was present in 2-10% of nuclei in binucleated cardiomyocytes in both adult sheep.

**Discussion**

In this study we have developed and optimised an improved technique allowing analysis of cardiomyocyte volume, nuclearity and ploidy simultaneously in 3-dimensional z-stack images utilising thick histological sections. Using this method, approximately 100-200 cardiomyocytes can readily be analysed per day, even by inexperienced researchers. When starting, we would recommend staining one heart section, completing the analysis and repeating the analysis again. This should demonstrate the degree of repeatability, with <5-10% being generally acceptable.

The findings relating to cardiomyocyte morphometry have not been quantitated in the present report, owing to the low numbers of animals analysed. Our aim was to undertake the analyses in a number of different species and life stages to illustrate the utility of the technique and the qualitative findings provide valuable reference data for subsequent analyses. Overall, cardiomyocyte volumes were much smaller in the fetus and juvenile animals compared to adults. It is interesting to note, when comparing cardiomyocyte volume between species, there were no observable differences in cardiomyocyte volume between adult mice, rats and sheep.

In an earlier publication, examining the effect of preterm birth on the development and maturation of cardiomyocytes in an ovine model, we utilised an earlier version of the method described in this paper to measure cardiomyocyte volume, nuclearity and ploidy¹². To our knowledge, this was the first published method to measure cardiomyocyte volume,
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nuclearity and ploidy in 3 dimensions directly from thick paraffin sections, without serial sections or reconstructions. This earlier version utilised carboxymethylcellulose in the staining solution to inhibit over-staining, ≥100µm paraffin sections to capture more cells in one section and a multi-photon laser to obtain sufficient light penetration into the tissue\textsuperscript{12}. Cutting paraffin sections at ≥100µm is challenging and obtaining a good section is very difficult. The improved method described here eliminates these restrictions and enables this method to be undertaken in a cost and time efficient manner; it has the added advantage that it does not require a multi-photon laser.

**Conclusion:** Our improved technique of analysis of cardiomyocytes in thick sections can be readily used to comprehensively analyse the physical properties of individual cardiomyocytes, and is particularly useful when analysing archived samples of heart tissue.
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References

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Table 1: Recommended staining protocols

<table>
<thead>
<tr>
<th></th>
<th>4-8µm (Slide) Paraffin Sections</th>
<th>40µm (Slide) Paraffin Sections</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dewax and rehydration</strong></td>
<td>3 changes of 2 minutes each - Xylene; 3 changes of 2 minutes each - Ethanol</td>
<td>3 changes of 5 minutes each - Xylene; 3 changes of 5 minutes each - Ethanol</td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>2 minutes in running water*</td>
<td>10 minutes in running water*</td>
</tr>
<tr>
<td><strong>Stain</strong></td>
<td>10µg/mL WGA-AF488 and 1µg/mL DAPI in PBS or HBSS; 15 minutes</td>
<td>10µg/mL WGA-AF488 and 1µg/mL DAPI in PBS or HBSS; 4 hours to overnight</td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>2 changes of 2 minutes each in PBS or HBSS</td>
<td>3 changes of 5 minutes each in PBS or HBSS</td>
</tr>
<tr>
<td><strong>Mount</strong></td>
<td>ProLong Gold or similar</td>
<td>ProLong Gold or similar</td>
</tr>
</tbody>
</table>

Table 1: Recommended staining protocols for heart tissue on glass slides (4 to 40µm). WGA-AF488=Wheat Germ Agglutinin - Alexa Fluor 488 (Invitrogen, USA). * It is acceptable to go for longer without adverse effects.
## Table 2: Summary of cardiomyocyte statistics from each species and time-point. Segregated by Left Ventricle with Septum, and Right Ventricle, and further divided by mononucleated and binucleated cardiomyocytes. N= refers to the number of animals, and below appears the total number of cells analysed.

<table>
<thead>
<tr>
<th>Species</th>
<th>Left Ventricle with Septum</th>
<th>Right Ventricle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mononucleated Percentage</td>
<td>Mononucleated Ploidy (n)</td>
</tr>
<tr>
<td>Weanling Mouse (n=10)</td>
<td>20±1.4%</td>
<td>5,634±1,035</td>
</tr>
<tr>
<td>Adult Mouse (n=3)</td>
<td>4.4±0.4%</td>
<td>16,851±403</td>
</tr>
<tr>
<td>Weanling Rabbit (n=4)</td>
<td>13.6±1.1%</td>
<td>8,063±529.5</td>
</tr>
<tr>
<td>Adult Rat (n=3)</td>
<td>3.5±0.3%</td>
<td>18,006±541</td>
</tr>
<tr>
<td>Fetal Sheep (n=7)</td>
<td>12.9±1.6%</td>
<td>3,417±1,169</td>
</tr>
<tr>
<td>10 week old Lamb (n=5)</td>
<td>2.4±0.9%</td>
<td>9,998±648</td>
</tr>
<tr>
<td>Adult Sheep (n=2)</td>
<td>1.5±1.5%</td>
<td>19,942±2,732</td>
</tr>
</tbody>
</table>
Figure 1: (A) Cardiomyocytes in long section, DAPI appears in blue, WGA-AF488 appears in green. (B) Cardiomyocytes in cross-section, DAPI appears in blue, WGA-AF488 appears in grey.
Figure 2: Representative mean intensity chart. A represents the mean intensity through the z-stack for DAPI. B represents the mean intensity through the z-stack for WGA-AF488. The X axis represents the depth through the section (z-stack), the Y axis is the mean intensity per channel at each depth (frame of the z-stack).
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Figure 3A: Cardiomyocyte example. 3.5µm pitch across 12 sections (42µm coverage). DAPI appears in fire colours, WGA-AF488 appears in green. * denotes the example cell analysed in Figure 3B. Scale bar represents 25µm.
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Figure 3B:

Figure 3B: Cardiomyocyte delineation example. The selected cardiomyocyte is in green. In the top left hand corner is the Z-stack position, in the top right hand corner is the X axis projection. In the bottom left hand corner is the Y axis projection. DAPI appears in fire colours, WGA-AF488 appears in green. Seed was laid down at the cross hair point.
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Figure 3C:

Figure 3C: 3D volumetric rendering of nuclei. DAPI appears in blue on a black background.
Figure 3D: Alternative method for cardiomyocyte volume measurement. In the left panel, WGA-AF488 staining appears in green. The middle panel is a brightness inversion of the left panel. In the right panel, the image was binarised and manual clean-up performed to produce dark cytoplasm and bright membranes.
Figure 4:

Figure 4: Z-stack through the left ventricle of a fetal lamb left ventricle (with septum). 333nm pitch across 4µm of total coverage. DAPI appears in red, WGA-AF488 appears in green. Mitotic nucleus appears in the white circles.
Figure 5: Identification of capillaries by WGA-AF488 enhancing staining. On the left, DAPI appears in fire, WGA-AF488 appears in green. On the right, DAPI appears in blue, WGA-AF488 appears in green. Arrows identify capillaries.
Chapter 7

General Discussion
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7 General Discussion

This thesis presents a series of experiments that have examined how preterm birth affects cardiac structure, cardiomyocyte growth, and the structure of the ascending aorta and pulmonary arteries in the neonatal period. In addition, the effect of exposure to late gestational chorioamnionitis, the most commonly identified antecedent of preterm birth\(^{1,2}\), on the growth of the fetal heart was investigated. Whilst conducting the studies in this thesis, I developed an improved method for the simultaneous measurement of cardiomyocyte volume, nuclearity and ploidy in histological sections. This improved method has the potential for wide use in cardiovascular research, as it allows for a full spectrum of cardiomyocyte investigations to be performed in thick paraffin-embedded sections.

Importantly, the findings of this thesis strongly suggest that preterm birth will lead to an elevated risk of cardiovascular disease later in life. The findings clearly show maladaptive changes in the heart and great vessels in response to being born preterm which are likely to predispose to long-term cardiovascular disease. In the hearts of moderately preterm born sheep there was evidence of cardiomyocyte hypertrophy, abnormal maturation, ongoing inflammation and an increase in extra-cellular matrix deposition in the heart at 9 weeks post-term equivalent age\(^3\). In addition, there was altered structure of the aorta and pulmonary arteries and evidence of injury to the ascending aorta\(^4\). In the hearts of preterm infants there appeared to be an abrupt cessation of cardiomyocyte proliferation and an increase in extra-cellular matrix deposition in the immediate period following preterm birth, which is likely to adversely impact on lifelong cardiac structure and functional reserve.

Moreover, fetal exposure to LPS-induced intrauterine inflammation (chorioamnionitis), led to altered cardiomyocyte growth in the fetal heart, which may in turn render the
myocardium more vulnerable to preterm birth. Exposure to intrauterine inflammation led to a reduction in cardiomyocyte density, a reduction in the size of mononucleated cardiomyocytes, but conversely, an increase in the size of binucleated cardiomyocytes. There was a 6-8 fold increase in cardiomyocyte proliferation in LPS-exposed lambs.

7.1 Preterm birth leads to maladaptive changes in an immature heart

At the time of birth there is a major hemodynamic transition, whereby the infant commences breathing and is no longer dependent on the placenta for the delivery of oxygen and nutrients, and the removal of waste products. At this time, the fetal cardiovascular system changes to the post-natal configuration whereby the foramen ovale is closed, the ductus arteriosus constricts and the left ventricle becomes the dominant ventricle. This is accompanied by a marked increase in systemic arterial blood pressure and heart rate. Prior to the commencement of studies for this thesis, there was very little information as to how the immature preterm heart responds and adapts to the increased functional demands associated with the hemodynamic transition that occurs at birth.

The findings in this thesis (Chapters 2 and 4) were the first studies to be published worldwide examining how the immature heart adapts to preterm birth. In order to address this clinically important issue, I initially used a clinically relevant model of preterm birth in sheep and subsequently conducted studies in the autopsied hearts from preterm human infants.

Importantly, the growth and maturation of cardiomyocytes in sheep resembles that in the human heart, with the majority of cardiomyocytes being mature and terminally differentiated at the time of term birth. Hence, it was considered an excellent animal model to conduct my initial analyses. In addition, in the case of the sheep heart, the immature and mature cardiomyocytes are usually easy to identify, as immature
cardiomyocytes are mononuclear whereas the mature cardiomyocytes become binucleated\textsuperscript{17}. Interestingly, however, my findings suggest that this may not be the case in the preterm heart.

The results of this thesis clearly demonstrated that induction of moderate preterm birth in sheep leads to significant effects on cardiomyocyte maturation, size, ploidy and extracellular matrix deposition at 9 weeks after term-equivalent age\textsuperscript{3}. In my studies, at the time when premature birth was induced (at 133 days of 147 days gestation) it is likely that about half of the cardiomyocytes in the hearts of the preterm lambs were immature (mononucleated) based on previously published studies by Burrell et al\textsuperscript{17}. Interestingly, in my analyses of the preterm lamb hearts at 9 weeks after term-equivalent age, there remained a high proportion of mononucleated cardiomyocytes (right ventricle \textasciitilde 4.5\%, left ventricle with septum \textasciitilde 8.4\%), implying that immaturity of the myocardium persists for many weeks after birth in the preterm heart. An alternative explanation to my findings is that the mononucleated cardiomyocytes in the preterm heart at 9 weeks after term-equivalent age have undergone a maturation process without becoming binucleated. Indeed, I showed that many of these mononucleated cardiomyocytes were no longer diploid (2N DNA) and instead were tetraploid (4N DNA); hence, the mononucleated cardiomyocytes had undergone DNA replication but there was no nuclear division. It is therefore possible that these tetraploid cardiomyocytes do, in fact, exhibit a mature cardiomyocyte phenotype. In order to verify this, future studies employing electron microscopy should examine the ultrastructure of the mononucleated cardiomyocytes to confirm whether the cytoplasm is filled with actin and myosin filaments arranged in sarcomeres (indicative of a differentiated mature phenotype). Tetraploid cardiomyocytes have not been previously identified in the
sheep and their gene program, functional characteristics and significance to future disease risk of cardiomyocyte polyploidy in the sheep are unknown. Trinucleated cardiomyocytes (which are also polyploid) were observed only in the hearts of preterm lambs. Again, the significance of these unusual cardiomyocytes, at the present time, can only be speculated upon, but may they may indicate a more stressed heart. Indeed, induction of polyploidy in cardiomyocytes is generally associated with stress of cardiomyocytes\textsuperscript{18-20}. Heart failure, cardiac hypertrophy, and advanced age in humans are frequently characterised by increases in nuclear number and high ploidy (>8n)\textsuperscript{21-23}. The presence of cardiomyocyte hypertrophy and polyploidy in the preterm sheep hearts may represent pathological hypertrophy or an acceleration of normal maturation. It is possible that the observed changes in ploidy, hypertrophy and nuclearity may be partially or completely reversible. Indeed, in the human heart, high polyploidy in cardiomyocytes is reversible\textsuperscript{20}, as was demonstrated in patients with heart failure who were treated with a left ventricular assist device (LVAD) which partially unloads the left ventricle. Interestingly, however, cryptosporidial gastroenteritis in the early postnatal period in rats (at a time when the cardiomyocytes were undergoing maturation) led to an induction in cardiomyocyte hypertrophy and polyploidy, and these changes in ploidy were shown to persist into adulthood\textsuperscript{24}.

One of the more concerning aspects of the changes I observed in the hearts of preterm born lambs was the increase in interstitial collagen. Collagen is continuously turned over within all organs (albeit at different rates), but once produced in the heart, collagen is not significantly reabsorbed or destroyed\textsuperscript{25-28}, hence, increased deposition of collagen very early in life is likely to have long-term deleterious implications to the contractility of the cardiac muscle \textsuperscript{26,28}. In addition, I observed numerous lymphocytes and mast cells within the
myocardium of the preterm lambs many weeks after preterm delivery suggesting that the increase in interstitial collagen may be an ongoing subclinical inflammatory process; thus, the deposition of collagen may worsen with time. My initial studies in sheep (Chapter 2) allowed me to examine how the developing heart responds to preterm birth in the absence of confounding factors that usually accompany preterm birth in humans. Subsequently, however, given the maladaptive changes I observed in the preterm lamb hearts, it was important to determine whether the changes I had observed in cardiomyocyte growth and extra-cellular matrix deposition in the preterm sheep also occurs in the heart of preterm human infants. To do this, I compared heart tissue taken at perinatal autopsies from preterm babies and compared the cardiac structure to appropriately grown babies that had died acutely in utero (Chapter 5).

Of major concern, I found that in the hearts of preterm infants, cardiomyocyte proliferation essentially ceases within 24 hours of birth. This occurred in the hearts of all preterm infants studied, regardless of the degree of prematurity. Given the low replicative capacity of cardiomyocytes in postnatal life, premature cessation of cardiomyocyte proliferation, as a result of preterm birth, is likely to adversely impact on the final number of cardiomyocytes formed within the heart and life-long functional reserve, thus rendering the heart vulnerable to disease processes in adulthood. Certainly, it is well established that the proliferative capacity of cardiomyocytes is markedly diminished throughout postnatal life, so if a preterm heart commences life with a reduced complement of cardiomyocytes, it is unlikely to have sufficient proliferative capacity to restore the complement of cardiomyocytes to normal levels; this is likely to be especially important when there is no proliferation of cardiomyocytes in the immediate period following birth (as observed in my
studies). Previous studies report that, in the human heart, the total number of cardiomyocytes needs to triple between birth and adolescence in order to achieve the normal cardiomyocyte endowment of the adult heart\textsuperscript{16}. In general, the majority of these additional and replacement cardiomyocytes are thought to arise from existing cardiomyocytes and only very rarely arise from c-kit\textsuperscript{*} progenitor cells\textsuperscript{34}. Hence, it is likely that if there are fewer cardiomyocytes at the beginning of life, this will impact on adult cardiomyocyte endowment and therefore cardiac function.

It is important to emphasise that in my preterm sheep studies at 9 weeks after term-equivalent age there was no difference in the number of cardiomyocytes in the left ventricle plus septum of lambs born moderately preterm compared to those born at term; this finding does not fit with my predictions (of long-term reduction in functional reserve) in the human preterm heart. Whether cardiomyocyte proliferation ceased immediately after birth in our preterm lambs (as observed in the preterm human infants) is currently unknown. There are two possible scenarios: 1) in the preterm sheep heart the cardiomyocytes continue to proliferate in the immediate period after birth, or 2) cardiomyocytes regain the ability to proliferate in the neonatal period and undergo catch-up hyperplasia. Certainly, the second scenario is a likely possibility, given that there appeared to be induction of cardiac hypertrophy in the preterm lambs. Although there were no statistically significant increases in heart weight, heart weight corrected for body weight, left ventricular weight or right ventricular weight in the animals I studied; in the larger cohort of animals (82 preterm born animals, 31 control lambs), the heart weight at autopsy was increased by 25\% in preterm lambs compared to controls (p<0.05), and heart weight corrected for body weight was increased by 20\% in preterm lambs (p<0.05)\textsuperscript{35}. In future studies it is imperative to
investigate whether cardiomyocyte proliferation also ceases in the preterm sheep in the immediate period after birth, to verify whether it is a direct effect of preterm birth that has led to the cessation of cardiomyocyte proliferation.

It is interesting to note that overall, my findings show a number of differences relating to the effects of preterm birth on the developing heart between the sheep and human. In the hearts of the human infants there was no effect of preterm birth on cardiomyocyte volume, nuclearity or ploidy, a significant reduction in cardiomyocyte proliferation and a small but significant increase in interstitial collagen deposition. In contrast, the hearts of the preterm lamb showed cardiomyocyte hypertrophy, derangements of cardiomyocyte maturation (tetraploid mononucleated cardiomyocytes and trinucleated cardiomyocytes), increased interstitial collagen deposition, and mast cell infiltration. These differences in the cardiac responses between the preterm lambs and infants likely relate to species differences between humans and sheep, the degree of severity of the premature delivery (moderately preterm in the sheep and a range from extremely to moderately preterm birth in the human infants), the relative timing of analysis (childhood in sheep versus neonatal period in human infants), the overall health of the newborns and/or the many confounding factors associated with the neonatal care of the human preterm infants but not applicable in the sheep studies. Indeed, the hearts from preterm infants were derived from babies that were extremely ill throughout life; it is possible that the effects we observed on cardiomyocyte proliferation may relate to underlying disease processes in these infants. In addition, there are many factors associated with the causes of preterm delivery and in the management of the mother at-risk of premature delivery and of the preterm infant in the neonatal care unit which have the potential to adversely impact on cardiomyocyte growth. Antenatal or
postnatal use of corticosteroid is an example of such a factor, as will be detailed later in this general discussion.

Certainly, my important findings, both in the sheep and human heart form the basis for many future avenues of research.

7.2 Altered long-term growth of the heart following preterm birth

Since the commencement of studies described in this thesis there have been two important studies published that have compared cardiac structure and function in preterm and term-born young adults. Following on from my findings of the effects of preterm birth in the sheep heart (Chapter 2), which were published in The European Heart Journal in 2010\(^3\), Lewandowski and colleagues conducted two studies that firstly examined the growth and function of the left ventricle and subsequently the growth and function of the right ventricle of young adults who were born preterm, using cardiac magnetic resonance imaging (MRI).

In their first study, they showed an increase in left ventricular wall mass, abnormal left ventricular wall geometry, and systolic and diastolic dysfunction\(^36\); however, ejection fraction was preserved. In their subsequent studies of the right ventricle in these same subjects the findings were even more pronounced\(^37\). Young adults born preterm exhibited a reduced right ventricular wall volume and this was accompanied by a 20% increase in right ventricular wall mass. Importantly, 6% of the preterm-born young adults had a right ventricular ejection fraction of less than 45%, signifying mild systolic dysfunction. Overall, the findings of these studies showed that the level of prematurity at birth directly influenced the growth of the heart. For each week of prematurity there was an increase in left ventricular mass of 1.5%, an increase in right ventricular mass of 2.7% and a reduction in right ventricle ejection fraction of 2.5%; there was no change in left ventricular ejection
fraction. The findings of the MRI studies by Lewandowski et al, as mentioned above, demonstrate a strong inverse correlation between the severity of cardiac dysfunction and gestational age at birth\textsuperscript{36,37}. It is important to emphasise that the people examined in this study were all under 35 years of age; hence, it is likely that the adverse effects on the cardiovascular system will be exacerbated as these individuals age.

Collectively, taking the findings of my studies (in the preterm sheep and human heart early in life) and the studies of Lewandowski and colleagues (in young adults born preterm) it would be beneficial to conduct a long-term prospective study in preterm subjects (commencing in infancy), where cardiac growth and function is monitored throughout life. This could be achieved by 4D ultrasound, which allows for the detailed examination of cardiac function and structure\textsuperscript{38-40}. Additionally, MRI combined with simultaneous magnetic resonance spectroscopy, would permit the analysis of the composition of the myocardium and the degree of capillarisation within the myocardial wall\textsuperscript{41-51}. Magnetic resonance spectroscopy allows for the identification of fat, iron, protein and other cell components in living patients. Moreover, the examination of hearts from deceased adolescents and adults who were born preterm, but subsequently died due to traumatic causes, would facilitate our understanding of how the preterm heart adapts and grows in adolescence and adulthood. These studies would provide insight into the mechanisms leading to the altered cardiac geometry described by Lewandowski and colleagues\textsuperscript{36,37}.

Long-term studies in sheep born preterm would also be beneficial; induction of preterm birth in an animal model eliminates many of the confounding factors (maternal and neonatal) associated with preterm birth as well as lifelong postnatal lifestyle factors. As a direct follow-on from my studies, it would be beneficial in future to examine lambs born
moderately preterm (~133 days) and studied immediately after birth, and then followed out to adulthood. These studies would determine if the cardiac effects observed at 9 weeks after term-equivalent age persist into adulthood and would also inform of the long-term effects of preterm birth on cardiovascular function (e.g. ejection fraction, arterial pressure) and cardiomyocyte structure, including cardiomyocyte endowment. It would also be beneficial to repeat these studies, but decrease the gestational age at delivery. Experimentally, these studies would be much more difficult to conduct as they would require neonatal intensive care of the lambs and purpose built facilities.

7.3  **Preterm birth alters the structure of the aorta and pulmonary artery and leads to injury in the ascending aorta**

Previous studies have examined the vasculature of infants born preterm, using non-invasive physiological methods such as sphygmomanometry to measure peripheral systolic/diastolic blood pressure\(^{52,53}\), ultrasound to examine blood vessel morphometry and motion\(^{54}\), steady-state free procession (SSFP) MRI to examine the aorta throughout the cardiac cycle\(^{55}\), laser Doppler to measure blood flow in skin capillaries and intra-vital microscopy to directly measure capillary density in the skin\(^{56}\). Whilst the mechanical properties and composition of blood vessels can be inferred from physiological measurements, it cannot replace direct histological examination.

Late in gestation there are major changes to the biochemical composition of the great vessels (aorta and pulmonary artery), whereby the structure of the blood vessel wall becomes adequately prepared by term for the haemodynamic transition at birth\(^{57-60}\). The mechanical qualities of blood vessels are related to their commitment to an anatomical location, and are also affected by the demands placed upon them in utero\(^{59,61}\). Development of the aortic and pulmonary arterial walls are directly related to advancing gestation,
particularly in relation to the increase in blood pressure, and blood flow with advancing gestational age\textsuperscript{62}. By adulthood, there is an almost perfect relationship between body size, and aortic diameter and wall thickness in most adult animals\textsuperscript{63}.

A blood vessel, whether it is a vein or artery, is a tube through which a fluid must flow. The innermost surface of the blood vessel is lined with a single layer of endothelial cells, originating from the mesodermal germ layer. Smooth muscle, also derived from the mesodermal germ layer, is laid down in layers in a circumferential direction; the larger the artery, the greater the amount of smooth muscle that is required. The amount of smooth muscle in the great vessels (aorta and pulmonary artery) increases with advancing gestational age and its formation is mediated by an increase in blood flow; the same is true for all blood vessels. In the fetus, although the pulmonary artery is not supplying blood in great quantity to the developing lungs, around 25\% of cardiac output will pass through the pulmonary artery, to facilitate lung growth\textsuperscript{64}. The constant mechanical stretch imposed on the blood vessel supports the normal development of the vessel\textsuperscript{59,61,65}. After birth, the pulmonary artery becomes a high flow, low resistance and low pressure vessel; the mean arterial pressure in the pulmonary artery in a healthy adult is 10-20mmHg\textsuperscript{66}. The pulmonary artery does not become a thinner walled vessel after birth, but maintains a thick wall with plentiful smooth muscle. The aorta, particularly in the aortic arch, also receives a steadily increasing quantity of blood flow throughout gestation that supports the normal development of the vessel and prepares the vessel to withstand the entire output of the left ventricle after birth.

In addition to growth of the vascular smooth muscle, the other major contributor to blood vessel development is the laying down of elastin and collagen in the arterial wall. Elastin
within blood vessel walls, as well as in almost all tissues, is produced within fibroblasts as tropoelastin, converted to elastin and then cross-linked with other elastin proteins by the formation of desmosine cross-links\textsuperscript{67,68}. Cross linking of elastin occurs mostly by the action of lysyl oxidase which converts lysine residues to aldehyde groups, thereby forming desmosine linkages between adjacent elastin proteins\textsuperscript{67,68}. Elastin, as the name suggests, possesses elasticity and recoil ability when laid down in tissue and has formed cross-links normally\textsuperscript{69}. Focusing on the great vessels in particular, elastin is laid down in concentric layers (termed lamellae). In the adult human thoracic aorta, there are approximately 30 lamellar layers, and 28 in the abdominal aorta\textsuperscript{63}. These lamellar units are what provide the elastic recoil of the arteries, thereby maintaining continuous blood flow throughout the cardiac cycle, resisting the deformation of the vessel under high pressure and maintaining the arteries’ patency in a passive manner\textsuperscript{62,63,69,74}. Elastin has an extremely long half-life of >40 years in humans\textsuperscript{75}. The unique aspect of arterial elastin production is that it is mostly accomplished during intra-uterine life and in the early post-natal period\textsuperscript{70}; relatively little is produced thereafter\textsuperscript{70}. Hence, a change in elastin production in early life can have life-long consequences to the individual and this is of particular importance to large elastic arteries.

Collagen is interspersed throughout the vessel wall, both as a basement membrane for vascular smooth muscle, endothelial and fibroblast cells and in larger collagen bundles. Collagen provides strength to the vessel wall, and increases the passive stability of the vessel wall to pressure\textsuperscript{62,63,74,76}. Collagen is synthesised and degraded continuously; however the overall amount of collagen remains fairly static in most organs\textsuperscript{77-82}. The rate of turnover is higher in the fetus and young animals compared to adults, and collagen turnover is deranged in conditions such as hypertension\textsuperscript{83}. 

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Importantly, the findings of my thesis have shown that moderate preterm birth, leads to structural adaptations of the wall of the great vessels in the early postnatal period, with both the composition and morphometry of the aorta and pulmonary artery affected. The major effect observed in the preterm pulmonary artery at 9 weeks post-term equivalent age was an increase in elastin deposition. In the aorta, the effects were more pronounced. In the ascending aorta, preterm lambs had aortic walls that were 25% thicker than term lambs; there was an increase in elastin deposition, a reduction in smooth muscle area and a reduced lumen area in the most distal segment examined. It is likely that the exaggerated deposition of elastin, both in the aorta and pulmonary artery of the preterm lambs is an adaptive response of the immature blood vessels to the marked rise in blood pressure that they are prematurely exposed to following preterm birth. Given that elastin is such a long-lived molecule, this increase in elastin content will probably persist throughout life; whether it confers improved compliance of the aorta is yet to be elucidated. Of major concern, in 4 of the 7 preterm born lambs there was evidence of injury to the ascending aorta and subsequent repair. These aortic lesions were roughly round (approximately 2.5mm in diameter and 800µm deep) and extended deep into the media. The repair tissue was composed of collagen and smooth muscle, with very low levels of elastin. Immunohistochemistry showed there to be minimal proliferation or apoptosis, suggesting that this injury occurred a significant period of time prior to necropsy. Indeed, it is most likely that the injury occurred in the immediate period following birth when the immature aorta was ill-prepared for the postnatal haemodynamics. Given that the aortic injury occurred in the ascending aorta (in the 2nd and 3rd segments from the aortic valve) the most likely cause of the lesions was the jet of flow through the aortic valve when combined with the sudden rise in systemic arterial pressure following birth. No injury was detected in the
area of the 1\textsuperscript{st} segment; it is conceivable that some of the dynamic pressure was relieved in this area by outflow into the two coronary arteries. Certainly, it is well established that dynamic pressure and wall shear stress is greatest in the anatomical region of segments 2 and 3, even in normal aortae\textsuperscript{74,84,85}. The significance of the structural changes in the ascending aorta has far-reaching clinical ramifications. The areas of injury will likely weaken the aortic wall, leading to an increased risk of aneurysm formation and the lesions themselves may act as initiating sites for the development of atherosclerotic plaques. Additionally, the thickening of the aortic wall, (particularly in the media) with encroachment on the lumen may affect afterload on the left ventricle. If the narrowing of the arterial lumen also occurs in other blood vessels, this has the potential to elevate arterial blood pressure. As a follow up of my findings it is now imperative to undertake a comprehensive examination of the vasculature throughout the arterial tree following preterm birth. It is likely that the structural changes and arterial injury I have observed in the great vessels will be reproduced throughout the vasculature, and if so, it is imperative to disseminate the findings to clinicians. As the first survivors of very and extremely preterm birth are now reaching adulthood their risk of cardiovascular disease is likely to be greatly increased. If their cardiovascular risk can be managed from an early age, this may help to prevent the onset of overt cardiovascular disease in later life.

To date there is limited information relating to the effect of preterm birth on vascular structure in human subjects. In accordance with my findings, MRI studies of adolescent preterm born girls showed significantly narrower thoracic and abdominal aortae when examined\textsuperscript{55}, but with greater elasticity when examined by ultrasound\textsuperscript{54}. Hence, the changes in aortic structure in the preterm lambs in my study are consistent with the vascular
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changes previously observed in humans born preterm. In particular, the ovine model of moderate preterm birth appears to reliably replicate aspects of vascular changes associated with preterm birth seen in humans, such as a narrower aorta\textsuperscript{54}, and provide an explanation for the higher aortic elasticity observed in adolescents born preterm\textsuperscript{54}.

In future studies, prospective life-course studies (from birth to adulthood) of preterm and term born subjects should be conducted in order to gain an understanding of the temporal and structural changes to blood vessels of subjects born preterm and the subsequent effects on vascular function and the propensity for hypertension and vascular disease. Additionally, it will be important to establish whether particular blood vessels and/or vascular beds are at greater risk in people born preterm and at what life stage pathologies manifest.

7.4 Factors that lead to preterm birth can adversely impact cardiac development prior to preterm delivery

In addition to preterm birth leading to adverse effects in the heart it is conceivable that factors that are linked to preterm delivery may deleteriously affect cardiac heart development whilst the baby is still \textit{in utero}. The findings of this thesis and those of others support this concept. If so, these babies would be exposed to two major insults to their developing immature heart; one occurring \textit{in utero} and the second occurring following premature delivery. There are many factors in the \textit{in utero} environment, linked to preterm delivery that could adversely impact on the developing fetal heart including: exposure to chorioamnionitis, intrauterine growth restriction, high maternal alcohol consumption and exposure to antenatal steroids.
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7.4.1 Chorioamnionitis

Exposure to chorioamnionitis is the most common risk factor for preterm birth\(^2,86\). In this thesis, using a model of chorioamnionitis in sheep I showed that exposure to LPS-induced intrauterine inflammation leads to marked alterations in cardiomyocyte growth\(^5\) (Chapter 4). In particular there was a reduction in cardiomyocyte density, coupled with a reduction in the volume of mononucleated cardiomyocytes and an increase in extracellular matrix deposition. As well there was hypertrophy of binucleated cardiomyocytes, and a significant increase in cardiomyocyte proliferation.

Given that intrauterine inflammation is directly linked to the induction of preterm birth, the negative effects on the growth of the developing fetal heart are likely to be further adversely impacted upon when the infant is delivered preterm. In future studies, it is imperative to investigate the combination of prenatal exposure to chorioamnionitis followed by preterm delivery, as this is a very common clinical sequence.

7.4.2 Intrauterine growth restriction

Restricted growth in utero is a common co-morbidity of preterm birth and is often a key factor in preterm deliveries\(^87-90\). Importantly, intrauterine growth restriction, as evidenced by reduced birth weight, is independently linked to the development of cardiovascular disease in humans\(^91-93\). It is particularly hazardous when coupled with postnatal catch-up growth, which is when an under-weight individual at birth catches up in body growth after birth. Previously in our laboratory, it has been shown that IUGR leads to a reduced complement of cardiomyocytes in the heart at the time of birth\(^94,95\); the cardiomyocytes also appear to be more immature (increased proportion of mononucleated cells) and the
coronary arteries are more responsive to vasoconstrictors and are more mechanically compliant\textsuperscript{96}. Given that IUGR is a common co-morbidity of preterm birth it is imperative in future studies to examine growth of the neonatal heart when IUGR is combined with preterm birth. It is likely that these very vulnerable preterm individuals will have multiple insults to the developing heart; hence, it is imperative that the effects of preterm birth compounded with IUGR and chorioamnionitis are also investigated.

7.4.3  \textit{Maternal alcohol consumption}

Maternal alcohol consumption during pregnancy is another factor linked to induction of preterm delivery. Studies in our laboratory have shown altered cardiac growth in fetal lambs as a result of exposure to high levels of alcohol late in gestation; there was an increase in heart weight relative to body weight, increased relative left ventricular volume, cardiomyocyte hypertrophy and an acceleration of cardiomyocyte maturation\textsuperscript{97}; however, there was no effect on collagen deposition or cardiomyocyte endowment.

7.4.4  \textit{Antenatal corticosteroids}

It is now routine practice for women at risk of delivering prematurely to be administered corticosteroids; this facilitates maturation of the fetal lungs and leads to a marked improvement in survival if the baby delivers preterm\textsuperscript{98-100}. Betamethasone is usually administered as either two doses of Betamethasone (11.4 mg, i.m.) given 24 hours apart, or 4 doses of Dexamethasone (6mg, i.m.) given 12 hours apart\textsuperscript{98-100}. Importantly, a number of studies have shown that corticosteroids can accelerate maturation in other organ systems including the heart. In the rat, neonatal treatment with Dexamethasone on postnatal days 1, 2 and 3 led to systolic dysfunction, cardiomyocyte hypertrophy, increased interstitial
collagen deposition and mast cell infiltration at 50 weeks of age\textsuperscript{101,102}. The lifespan of the male rats was reduced by \textasciitilde25\%, with female rats having an 18\% reduction in lifespan\textsuperscript{103}. In fetal sheep, Betamethasone exposure during mid to late gestation on a weekly basis (on days 104, 111, 118, and 124 of gestation, with term at \textasciitilde147 days) resulted in a 20\% reduction in brain weight and reduced birth weight at term birth\textsuperscript{104,105}. High dose cortisol (72 mg, per day, for 2 to 3 days) given to fetal sheep (129 days gestation) did not affect cardiomyocyte binuclearity, but did increase the size of binucleated cardiomyocytes in the left ventricular free wall\textsuperscript{106}.

In a 30 year follow-up study of subjects enrolled into a randomised controlled trial of antenatal corticosteroids (placebo vs Betamethasone), it was found that people born preterm who received Betamethasone demonstrated insulin resistance compared to those who received placebo, but no changes in blood pressure, lipid profile or body composition were found\textsuperscript{107}.

The efficacy of antenatal corticosteroids is unquestionable in reducing the probability of death, increasing the rate of survival in the absence of developmental abnormalities and decreasing the incidence of intra-ventricular haemorrhage following preterm birth\textsuperscript{98,99}. The development of other agents that promote lung maturation in the absence of corticosteroids’ off-target effects could, in theory, be a solution to this problem.

In the studies of preterm sheep conducted in my thesis, the ewes were administered a low dose of Betamethasone; this was necessary to facilitate the survival of the preterm lambs in the immediate period after birth, particularly the males. Although the dose of Betamethasone was much smaller than that administered to women clinically at risk of delivering preterm, it remains a confounder in my studies. Given that only ewes in the
preterm groups were administered Betamethasone, there remains the possibility that my findings in relation to the cardiovascular system were the result (in full or in part) of exposure to Betamethasone rather than preterm birth *per se*.

In summary, there are many factors associated with the *in utero* environment that have the potential to adversely impact on cardiac development and importantly many of these factors occur in combination. These initial insults (singularly or in combination) to the developing fetal heart likely render the immature heart more vulnerable to the effects of preterm birth and this is likely to be further impacted upon by the degree of prematurity. It will be important in future studies to systematically examine the effects on an immature cardiovascular system of the many other factors associated with preterm birth and when they are coupled with premature delivery.

### 7.5 Moderate preterm babies form a large group with potentially increased cardiovascular disease risk

The majority of preterm infants are born moderately preterm (between 32 and 37 completed weeks of gestation), comprising ~ 80% of all preterm births and 6-8% of all live births\(^\text{108}\). Moderately preterm human babies have a survival rate equivalent to babies born at term, are generally not admitted to neonatal intensive care, and usually receive no special follow-up care or attention\(^\text{109}\). Although the majority of babies born moderately preterm are in good health in the neonatal period, the findings of this thesis clearly demonstrate the potential for deleterious effects to the cardiovascular system in the neonatal period; my findings also provide strong experimental support for long-term cardiovascular follow-up of all individuals born preterm, including those born only a few weeks early (moderately preterm).
Indeed, in the first two experimental chapters (Chapters 2 and 3), the adverse consequences of moderately preterm birth (0.9 of term) to the cardiovascular system of the lamb, examined at 9 weeks of post-term equivalent age, were striking. The results from the study of human preterm heart tissue (Chapter 5) were even more alarming, in terms of cardiomyocyte development and maturation. An almost complete cessation of cardiomyocyte proliferation within 24 hours of preterm birth occurred independent of gestational age at birth.

7.6 Conclusion

The findings of this thesis demonstrate that preterm birth (even when birth is only a few weeks early), leads to maladaptive structural changes to the heart and to altered structure of the great arteries, including injury to the wall of the ascending aorta. In addition, exposure to inflammation in utero, as commonly seen during chorioamnionitis (the most commonly identified antecedent of preterm birth), was shown to lead to altered development of the fetal heart. My findings highlight the importance of long-term cardiovascular follow-up of subjects who were born preterm, even those born moderately preterm. Given that between 8 and 12% of all live births are preterm, it is essential that patients born preterm receive appropriate care and follow-up throughout life, to enable early intervention and prevent overt cardiovascular disease.
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7.7 References


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