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*†

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 cardiac 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

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1 M.J. Black and R. Harding are joint senior authors.
2 Corresponding author. Tel: +61 3 9902 9112; Fax: +61 3 9902 9223; Email: jane.black@med.monash.edu.au
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Cardiac remodelling as a result of pre-term birth

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 atra 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—fractionator approach; we used a light microscope (Olympus BX4, Japan) coupled with a motorized stage and a z-axis sensor (Germany). Every second section was systematically sampled and projected onto a computer screen. An unbiased counting frame (329.6 μm²) was superimposed on the image using C.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

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,11 in particular an increase in systemic arterial pressure,10–12 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.7 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,13 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.14,15 Cardiac structure was analysed at 9 weeks after term-equivalent age (TEA), at a time when cardiomyocytes have very limited proliferative potential after birth.7 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,13 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.

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 before term. Pre-term lambs (n = 7; 6 female, 1 male) were born vaginally using an established technique.16 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 Betamethasone (3.7–5 mg i.v.; Win-32729, Winthrop, UK) was administered to the ewes to induce delivery 48 h later. The ewes were administered Betamethasone (3.7–5 mg i.v.; 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 immature, pre-term heart. We reasoned that because pre-term

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.

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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 1:5000 YOYO-3 (Molecular Probes Invitrogen). Wheat germ agglutinin-Alexa Fluor 488 conjugate stains the cell membranes and YOYO-3 stains the nucleus (see Supplementary material online for details).

To visualize the cardiomyocytes, we used a Leica SP5 broadband multi-photon confocal microscope (Leica Microsystems, Germany) with a Spectra-Physics MaiTai Ti:Sapphire multi-photon source (Spectra-Physics, Newport Corporation, USA). Volocity 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 picrosirius red after pre-treatment with phosphomolybdic acid.19 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).20 To assess perivascular fibrosis, two intramyocardial arterioles per section were selected according to Nyengaard and Marcussen.21 The area of collagen in the adventitia was measured using image analysis, normalized to the vessel lumen area and averaged for each animal.22

**Collagen immunohistochemistry**

Collagens I and III were quantified in 5 μm paraffin sections using antibodies specific for collagen I (COL-1 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).20

**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-X100 (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/DAB+ 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 6.1/6.2, 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 above23 and nuclei stained with YOYO-3 (Molecular Probes Invitrogen). The binding of YOYO-3 within a cell is proportional to its DNA content.24 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 6.1/6.2, Bitplane), images of whole nuclei were collected and relative fluorescence measured using Volocity 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 kg 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>
</thead>
<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>
</tr>
<tr>
<td>Left ventricle + septum weight (g)</td>
<td>48.81 ± 2.59</td>
<td>54.78 ± 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 (mm)</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, 47 ± 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 (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
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

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**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>
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<tbody>
<tr>
<td>RV total cardiomyocyte number ($\times 10^8$)</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 ($\times 10^9$)</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 $\times 10^7$)</td>
<td>3.02 ± 0.52</td>
<td>4.21 ± 0.89</td>
<td>0.276</td>
</tr>
<tr>
<td>LV + S cardiomyocyte number/necropsy weight number/kg $\times 10^8$</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 $\times 10^7$)</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 $\times 10^7$)</td>
<td>4.95 ± 0.76</td>
<td>4.68 ± 0.33</td>
<td>0.778</td>
</tr>
</tbody>
</table>

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**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 $\mu$m.
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.\textsuperscript{20,26} 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 markedly\textsuperscript{9,11,27} such that the output and end-diastolic pressure of the LV increase, while the output of the RV decreases.\textsuperscript{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 hypertension\textsuperscript{28} and that the presence of mast cells induces fibrosis via recruitment of cardiac fibroblasts.\textsuperscript{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.\textsuperscript{30} 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).\textsuperscript{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.\textsuperscript{4,33} 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.\textsuperscript{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

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Volume of cardiomyocytes in the right ventricle and left ventricle plus septum of pre-term and term lamb</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Term (n = 8) (µm(^3))</td>
</tr>
<tr>
<td>RV mononucleated</td>
<td>10 051.07 ± 365.9</td>
</tr>
<tr>
<td>RV binucleated</td>
<td>14 601.16 ± 381.86</td>
</tr>
<tr>
<td>RV trinucleated</td>
<td>Not present</td>
</tr>
<tr>
<td>LV + S mononucleated</td>
<td>10 032.53 ± 339.41</td>
</tr>
<tr>
<td>LV + S binucleated</td>
<td>20 764.91 ± 709.88</td>
</tr>
<tr>
<td>LV + S trinucleated</td>
<td>Not present</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.
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. 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. 14,15,34 At the gestational age at which our pre-term lambs were born, ~50% of cardiomyocytes are binucleated, 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, 35 indicative of abnormal maturation. In addition, in both ventricles of pre-term lambs, we observed polyploid 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. 36 Of concern, however, polyploidy is considered to be irreversible and is linked to cardiac dysfunction; 37–39 for example, the incidence of polyploid cardiomyocytes is high in hearts with impaired function caused by pathological hypertrophy. 37 Importantly, it has been proposed that polyploidy of cardiomyocytes can be programmed in early development. 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, 13 which usually occurs during late gestation in humans and sheep. 7,15 Importantly, this is the developmental time point when pre-term birth was induced in

![Figure 3](https://academic.oup.com/eurheartj/article-abstract/31/16/2058/431785/2064)
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 lambs were exposed to a low dose of corticosteroids prior to birth. 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 maturational 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 as 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.

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