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The Long-Term Effects of Fetal Anaemia

Alexandra Helen Wallace

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Paediatrics, the University of Auckland, 2014.
Abstract

Aim
Sheep made anaemic as fetuses have increased coronary conductance and susceptibility to ischaemic injury in adulthood. In humans, the effects of anaemia in utero, or ex utero at equivalent age in preterm infants, are unknown. The aim of our studies was to investigate cardiovascular and other health outcomes following intrauterine anaemia in adult humans, and following extrauterine anaemia after preterm birth in sheep.

Method
We measured anthropometry, socioeconomic variables, cardiovascular risk factors, cardiac structure and function, and myocardial blood flow in adults who received intrauterine transfusion between 1963–92 for treatment of fetal anaemia due to rhesus disease, and their unaffected sibling(s). In lambs born preterm, we induced anaemia prior to term equivalent age, and monitored growth, haematological and biochemical parameters to 12 months of age. We also piloted the experimental methodology to investigate adult cardiovascular outcome in these animals.

Findings
Compared to unaffected siblings, adult survivors of fetal anaemia had similar anthropometry, socioeconomic status and educational achievement, but an unfavourable lipid profile, augmented sympathetic tone, increased haemoglobin concentration, smaller, relatively thicker walled hearts, lower left ventricular function, and lower myocardial blood flow at rest and with cold pressor stress. In preterm sheep, anaemia was induced without compromising survival. Haemoglobin concentration was increased in adult sheep exposed to both moderate and severe preterm anaemia, but only severe anaemia impaired growth at 12 months. Cardiovascular outcome assessments were successfully piloted.

Conclusion
These findings provide the first evidence in humans that exposure to fetal anaemia may confer increased risk of cardiovascular disease in later life. Similar haematological findings in humans and sheep suggest that exposure to anaemia prior to term equivalent age, whether in or ex utero, may have similar long-term implications. The methodologies devised for induction of anaemia in lambs born preterm and assessment of cardiovascular outcome in adult sheep will allow further investigation of the cardiovascular implications of anaemia in the preterm period. The findings of these studies in humans and animals will help inform the management of anaemia in the fetus and preterm infant to optimise long-term outcomes.
This thesis is dedicated to the memory of two inspiring, courageous and clever men who provided the cornerstones upon which this work is built.

William Liley, 1929 – 1983
David Barker, 1938 – 2013
Acknowledgements

Undertaking a PhD was not a decision I made lightly. Having worked as a Paediatrician for several years, many friends, family members and colleagues could not understand why I wished to return to the obscurity and toil of student life. However, although I was passionate about Paediatrics and felt privileged to be allowed such a role in people’s lives, I knew it was not enough to sustain me in the long-term. It had also become increasingly apparent to me that acquiring research expertise was essential for the practice of excellent clinical medicine. And so my PhD journey began.

None of this work would have been possible without financial assistance. Throughout my PhD I have been generously supported by the Health Research Council of New Zealand, the New Zealand Lottery Grants Board, the Auckland Medical Research Foundation, the Maurice and Phyllis Paykel Trust and Gravida: National Centre for Growth and Development. Sincere thanks to all these organisations for their support.

The idea for the Fetal Anaemia Study evolved from a coffee break conversation at a conference between Kent Thornburg and my primary supervisor, Jane Harding. Kent and his team at Oregon Health and Sciences University had observed intriguing changes in coronary blood flow in adult sheep exposed to fetal anaemia, but they were unsure how to apply this knowledge to humans. Jane, wearing the hats of both a Neonatologist and Developmental Origins researcher, immediately saw that Rhesus Haemolytic Disease was the solution, and the Auckland-Portland collaboration was born. Throughout my PhD, Kent has provided much needed advice and practical assistance and I am indebted to him, not only for providing the impetus for this work, but also for his unfailing enthusiasm and support.

The research presented in this thesis was undertaken at the University of Auckland, within the Liggins Institute, Centre for Advanced MRI, Ngapouri Animal Laboratory, and Large Animal Facility. A number of individuals from these departments provided me with invaluable assistance. In particular, Coila Bevan worked tirelessly on many aspects of the Fetal Anaemia Study and Ruth Simons, Elba Escobar and Kate Sommers provided excellent administrative support. I am also immensely grateful to Chris McKinlay for taking over the supervision of Fetal Anaemia Study investigations while I was on parental leave, thus allowing the study to continue in my absence.
The team at the Centre for Advanced MRI undertook all MRI scans with great attention to detail and went out of their way to fit the Fetal Anaemia Study participants into their busy schedule. Brett Cowan and Alistair Young played a crucial role in the analysis of the MRI scans. I am especially grateful to them for undertaking the quantitative analysis of perfusion data. This proved to be a truly mammoth task, and I feel very fortunate that Brett, Alistair and their team at the Auckland MRI Research Group not only had the expertise required to do this, but were also willing to take it on.

Having never worked with animals, the Preterm Anaemia Study proved to be a very steep learning curve. Anne Jaquiery took me under her wing and showed me the ropes, sharing with me her love and respect for the animals we study. I am also grateful to Mark Oliver, Anita Wylie, Amanda van Zyl and the team at Ngapouri for their hard work and for sharing their knowledge. Thanks also to Eric Thorstensen from the Liggins Institute laboratory for teaching me how to analyse the blood samples from this study. The pilot experiments of cardiovascular outcome in adult sheep would not have been possible without the assistance of Ian LeGrice, Bruce Smaill, Greg Sands and Nigel Lever from the University of Auckland’s Bioengineering Institute and Linley Nisbet, technician at the Large Animal Facility. I am very grateful to them for their time, advice and expertise.

My supervisors, Stuart Dalziel and Jane Harding, have provided outstanding support, advice and encouragement throughout my PhD. Stuart, I have very much appreciated your wise counsel on matters related not just to this work but also to medicine, careers and the constant juggling act with family life. Jane, your patience has been remarkable, your advice always accurate, your guidance generously given and vision inspirational. I could not have wished for a better mentor and teacher. Thank you.

Undertaking a PhD with a small family in tow would not have been possible without the help of some incredibly special big people to care for my incredibly special small people. Particular thanks go to Lynne Rudman, Lynore Byers, and my parents for the loving care they have provided for our boys.

My dear friend Deb Harris is solely responsible for planting the seed of an idea to do a PhD in my mind. Deb, you have been a constant inspiration, your achievements are remarkable and I am so grateful for your friendship. I look forward to continuing our research journey together.
Finally, the most important people to thank are my boys, one big, two small. Lachie and Sam you are the only 2 and 4 year olds I know who pretend they are writing PhDs! Thank you for being so lovely and for putting up with my absences, I hope you will understand better when you are older. Gary, you are a champion husband and father. I am so very grateful for everything you have done to make this possible, for your love and your friendship.

AHW

June 2014
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<th>Full Form</th>
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<tbody>
<tr>
<td>AMRG</td>
<td>Auckland Magnetic Resonance Research Group</td>
</tr>
<tr>
<td>AV</td>
<td>Atrioventricular</td>
</tr>
<tr>
<td>BPD</td>
<td>Biparietal diameter</td>
</tr>
<tr>
<td>BSA</td>
<td>Body surface area</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>CBF</td>
<td>Coronary blood flow</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>Circ</td>
<td>Circumference</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>CMR</td>
<td>Cardiovascular magnetic resonance</td>
</tr>
<tr>
<td>CRL</td>
<td>Crown rump length</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EDD</td>
<td>End diastolic diameter</td>
</tr>
<tr>
<td>EDV</td>
<td>End diastolic volume</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>ESD</td>
<td>End systolic diameter</td>
</tr>
<tr>
<td>ESV</td>
<td>End systolic volume</td>
</tr>
<tr>
<td>ffDNA</td>
<td>Free fetal DNA</td>
</tr>
<tr>
<td>FS</td>
<td>Fractional shortening</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GA</td>
<td>Gestational age</td>
</tr>
<tr>
<td>GGT</td>
<td>Gamma-glutamyl transferase</td>
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<tr>
<td>GV</td>
<td>Growth velocity</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>Hct</td>
<td>Haematocrit</td>
</tr>
<tr>
<td>HF</td>
<td>High frequency</td>
</tr>
<tr>
<td>HF&lt;sub&gt;nu&lt;/sub&gt;</td>
<td>High frequency power expressed in normalised units</td>
</tr>
<tr>
<td>hPV</td>
<td>Human parvovirus</td>
</tr>
<tr>
<td>HRV</td>
<td>Heart rate variability</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IVC</td>
<td>Inferior vena cava</td>
</tr>
<tr>
<td>IPT</td>
<td>Intraperitoneal transfusion</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
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<td>IUT</td>
<td>Intrauterine transfusion</td>
</tr>
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<td>IVIG</td>
<td>Intravenous immunoglobulin</td>
</tr>
<tr>
<td>IVT</td>
<td>Intravenous transfusion</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
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<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LF</td>
<td>Low frequency</td>
</tr>
<tr>
<td>LF&lt;sub&gt;nu&lt;/sub&gt;</td>
<td>Low frequency power expressed in normalised units</td>
</tr>
<tr>
<td>LGE</td>
<td>Late gadolinium enhancement</td>
</tr>
<tr>
<td>LMP</td>
<td>Last menstrual period</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricular</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>m</td>
<td>Metre</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MBF</td>
<td>Myocardial blood flow</td>
</tr>
<tr>
<td>MCA-PSV</td>
<td>Middle cerebral artery peak systolic velocity</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimetres of mercury</td>
</tr>
<tr>
<td>mmol</td>
<td>Millimole</td>
</tr>
<tr>
<td>MoM</td>
<td>Multiples of the median</td>
</tr>
<tr>
<td>MPRi</td>
<td>Myocardial perfusion reserve index</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
</tr>
<tr>
<td>mv</td>
<td>Millivolt</td>
</tr>
<tr>
<td>N</td>
<td>Newton</td>
</tr>
<tr>
<td>NICU</td>
<td>Neonatal intensive care unit</td>
</tr>
<tr>
<td>OCP</td>
<td>Oral contraceptive pill</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>pNN50</td>
<td>Percentage of NN intervals that differ by &gt;50 ms from the preceding interval</td>
</tr>
<tr>
<td>PC</td>
<td>Preterm control lamb group</td>
</tr>
<tr>
<td>PT30%</td>
<td>Preterm 30% anaemic lamb group</td>
</tr>
<tr>
<td>PT50%</td>
<td>Preterm 50% anaemic lamb group</td>
</tr>
<tr>
<td>RhHD</td>
<td>Rhesus haemolytic disease of the fetus and newborn</td>
</tr>
<tr>
<td>RMSSD</td>
<td>Square root of the mean of the squares of the interval between successive normal beats on electrocardiogram</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SD(\delta)NN</td>
<td>Standard deviation of difference between adjacent interval between normal beats on the electrocardiogram</td>
</tr>
<tr>
<td>SDNN</td>
<td>Standard deviation of the interval between normal beats on electrocardiogram</td>
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<td>SE</td>
<td>Standard error</td>
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<tr>
<td>SPECT</td>
<td>Single photon emission computed tomography</td>
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<tr>
<td>SR-FLASH</td>
<td>Saturation recovery fast low angle shot pulse sequence</td>
</tr>
<tr>
<td>SR-GRE/EPI</td>
<td>Saturation recovery gradient echo-echo planar pulse sequence</td>
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<td>SR-SSFP</td>
<td>Saturation recovery steady state free precession pulse sequence</td>
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<tr>
<td>SV</td>
<td>Stroke volume</td>
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<td>SVC</td>
<td>Superior vena cava</td>
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<td>T</td>
<td>Tesla</td>
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<tr>
<td>TC</td>
<td>Term control lamb group</td>
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<tr>
<td>TEA</td>
<td>Term equivalent age</td>
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<tr>
<td>TIBC</td>
<td>Total iron binding capacity</td>
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<tr>
<td>UIBC</td>
<td>Unsaturated iron binding capacity</td>
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<td>ULF</td>
<td>Ultra low frequency</td>
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<td>VLF</td>
<td>Very low frequency</td>
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<tr>
<td>WCC</td>
<td>White cell count</td>
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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Fetal Anaemia Study, chapters 3, 4 and 5

Nature of contribution by PhD candidate
Alexandra Wallace contributed to the study design, recruited all participants, supervised all investigations, contributed to statistical analysis, and completed the first draft and revisions of all manuscripts

Extent of contribution by PhD candidate (%)
85%

CO-AUTHORS

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<td>Chris McKinlay</td>
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The undersigned hereby certify that:
† the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
† in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

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Preterm Anaemia Pilot Study: Clinical features of the cohort, chapter 6

| Nature of contribution by PhD candidate | Alexandra Wallace contributed to the study design, performed most of the experiments, collected some of the growth data and blood samples, managed the clinical trial, contributed to the statistical analysis, and completed the first draft and revisions of the manuscript. |
| Extent of contribution by PhD candidate (%) | 75% |

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Chapter 1: Literature Review

1.1 Cardiovascular Disease: Epidemiology and Risk Factors
Cardiovascular disease is the leading cause of death worldwide (World Health Organisation 2011). Coronary heart disease, defined as disease of the vessels supplying blood to the heart muscle, accounts for over 40% of these deaths. The incidence of cardiovascular disease, in particular coronary heart disease, has reduced substantially in the developed world over the last three decades, although the overall burden of disease remains enormous (Ford, Ajani et al. 2007; Capewell, Ford et al. 2010). This reduction is accounted for in equal part by a reduction in risk factors and improvements in medical therapies for cardiovascular disease (Ford, Ajani et al. 2007; Wijeysundera, Machado et al. 2010). However, in the developing world there has been a dramatic increase in cardiovascular disease incidence and mortality over the same time period, with over 80% of global cardiovascular disease deaths occurring in low- and middle-income countries (Cheng, Zhao et al. 2009; World Health Organisation 2011). Increased exposure of individuals in developing countries to the major risk factors for cardiovascular disease, in addition to reduced access to primary prevention interventions and health services, account for the majority of this increase (Beaglehole 2001; Cheng, Zhao et al. 2009; Bandosz, O'Flaherty et al. 2012). Mitigation of this global cardiovascular disease epidemic in developing nations by the use of population-based risk factor reduction strategies is now a matter of considerable debate and urgency (Beaglehole 2001; Vedanthan and Fuster 2009; World Health Organisation 2011).

The major risk factors for cardiovascular disease can be divided into behavioural factors and metabolic factors. Behavioural risk factors include smoking, excessive use of alcohol, physical inactivity and unhealthy diet. Collectively, these factors are implicated in up to 80% of coronary heart disease (World Health Organisation 2011). Metabolic risk factors include diabetes mellitus, hyperlipidaemia, hypertension and obesity. A plethora of evidence supports the hypothesis that some of these conditions are linked to adverse events before or around the time of birth, the so-called “Developmental Origins of Health and Disease”. For example, low birth weight is associated with hypertension, impaired glucose tolerance and impaired lipid metabolism in adolescence and adulthood (Barker, Osmond et al. 1989a; Hales, Barker et al. 1991; Whincup, Cook et al. 1992; Barker, Martyn et al. 1993; Hofman, Cutfield et al. 1997; Owen, Whincup et al. 2002). In addition, longitudinal studies have shown that elevated blood pressure and insulin resistance persist from childhood to adulthood, suggesting that an individual’s cardiovascular risk factor profile is partially
determined early in life (Bao, Threefoot et al. 1995; Srinivasan, Frontini et al. 2003; Nguyen, Srinivasan et al. 2008; Chen, Srinivasan et al. 2011). Furthermore, pathological evidence of asymptomatic cardiovascular disease has been found in children with a history of these risk factors (Berenson, Srinivasan et al. 1998). Taken together, these findings suggest that early risk factor identification and intervention are important components when considering strategies to halt the growing cardiovascular disease epidemic. Furthermore, it is clearly pertinent to consider the influence of the perinatal environment and early life events on the increasing global burden of cardiovascular disease.

1.2 Developmental Origins of Health and Disease

1.2.1 Origins of the Hypothesis: Geographical Studies

The concept of the “Developmental Origins of Health and Disease” originated over 30 years ago when a geographical association was identified between past living conditions and current mortality rates for coronary heart disease in Norway (Forsdahl 1977). Subsequently, the association between poor nutrition in fetal life and infancy and chronic disease in later life was explored extensively by David Barker and colleagues through a series of epidemiological studies in England and Wales (Barker 1992). Barker’s work indicated that an adverse environment in utero and/or during the early postnatal period could lead to increased disease risk in adult life through permanent deleterious effects on physiology and metabolism (De Boo and Harding 2006; Barker 2007). This concept became known as the “Developmental Origins” or “Barker” hypothesis (Lauren, Jarvelin et al. 2003).

Barker’s initial work involved detailed geographical studies comparing infant mortality rates from 1921 to 1925 with the leading causes of death in England and Wales from 1968 to 1978, using infant mortality as a marker of adverse maternal and perinatal conditions (Barker and Osmond 1986a). He observed that the areas of England and Wales with the highest rates of infant mortality also had the highest rates of death from ischaemic heart disease seventy years later. A similar geographical relationship was found between infant mortality and death from chronic bronchitis, rheumatic heart disease and stomach cancer. Barker concluded that the latter findings were to be expected, as these diseases were known to be associated with poorer living conditions and lower socioeconomic status. In addition, the rates of these diseases had declined throughout the twentieth century in concert with declining infant mortality rates. However, the observation that past infant mortality demonstrated a clear geographical relationship with death from ischaemic heart disease was paradoxical given the sharply increased rate of ischaemic heart disease with increasing
prosperity over the twentieth century (Barker and Osmond 1986a; Barker 2007). Barker’s group therefore hypothesised that adverse perinatal conditions directly increased an individual’s susceptibility to disease in later life, possibly through exposure to factors associated with affluence (Barker and Osmond 1986a; Barker, Osmond et al. 1989b; Barker 2007).

These findings were supported by a number of earlier, albeit smaller, studies from England, Norway, Finland and the United States of America, which consistently suggested a relationship between ischaemic heart disease and adverse conditions in early life (Forsdahl 1977; Buck and Simpson 1982; Marmot, Shipley et al. 1984; Barker 2007). Subsequent studies by Barker also demonstrated a geographical relationship between maternal mortality and death rates from stroke seventy-five years later (Barker and Osmond 1987).

At the start of the twentieth century, most neonatal deaths occurred in the first week of life and were most commonly associated with low birth weight. Therefore, neonatal death depended more upon adverse intrauterine factors leading to compromised growth than on later postnatal influences (De Boo and Harding 2006; Barker 2007). In conjunction with the findings from the geographical studies, these observations led Barker to hypothesise that undernutrition in utero, manifest by poor fetal growth and low birth weight, may permanently alter an individual’s physiology and metabolism, thereby placing them at greater risk of cardiovascular disease in later life (Barker 2004; Barker 2007).

1.2.2 Risk Factors for Cardiovascular Disease: Cohort Studies
Since Barker’s original geographical studies, a mass of further epidemiological work by Barker’s group and others around the world has demonstrated links between adverse perinatal events, death from coronary heart disease and some of the known risk factors for cardiovascular disease. Investigation of the association of low birth weight with coronary heart disease required identification of large cohorts of men and women for whom anthropometric data at birth and in early life had been recorded (Barker and Bagby 2005). Barker’s work focussed largely on a cohort of men and women from Hertfordshire due to the discovery of records containing detailed anthropometric data collected over the first year of life on every baby born in the county from 1911 onwards (Barker 2007).

Using these records, it was possible to trace individuals born 40 to 60 years earlier in order to relate body size in early life to cardiovascular mortality in later life. These investigations
confirmed a strong inverse relationship between both size at birth and one year of age, and mortality from ischaemic heart disease in later life, leading the authors to conclude that conditions affecting perinatal growth were strongly associated with risk of ischaemic heart disease (Barker, Osmond et al. 1989c). These findings have subsequently been replicated in other studies around the world (Osmond, Barker et al. 1993; Frankel, Elwood et al. 1996; Rich-Edwards, Stampfer et al. 1997; Leon, Lithell et al. 1998; Eriksson, Forsen et al. 2001). Furthermore, size at birth has been linked to a number of known risk factors for cardiovascular disease.

1.2.2.1 Blood pressure
Barker’s Hertfordshire studies demonstrated an inverse relationship between systolic blood pressure and birth weight in men and women aged 46 to 54 years. As birth weight increased from 5.5 pounds (2.5 kg) or less to 7.5 pounds (3.4 kg) or more, mean systolic blood pressure fell by 11 mmHg (Barker, Bull et al. 1990). The effect was shown to be continuous across the normal range of birth weight. These findings were replicated in a nation wide study of men and women at 36 years of age (Barker, Osmond et al. 1989a). In addition, in the Hertfordshire cohort, a relationship was found between placental size and mean systolic blood pressure, such that those with a placental weight greater than 1.5 pounds (680 g) had a mean systolic blood pressure 15 mmHg higher than those with a placental weight of 1 pound (450 g) or less. These relationships were independent of adult body mass index and alcohol consumption. Weight at one year of age was not related to blood pressure in adulthood, suggesting that the critical period for the programming of blood pressure is in fetal life (Barker and Martyn 1992).

The findings of these studies provided the first evidence that the intrauterine environment has an important effect on blood pressure in later life (Barker, Bull et al. 1990). Since then, a number of other investigations have consistently demonstrated a relationship between low birth weight and raised blood pressure in both childhood and adult life (Whincup, Cook et al. 1992; Law, de Swiet et al. 1993; Campbell, Hall et al. 1996; Curhan, Chertow et al. 1996; Curhan, Willett et al. 1996; Leon, Koupilova et al. 1996; Koupilova, Leon et al. 1997; Nilsson, Ostergren et al. 1997; Taylor, Whincup et al. 1997; Levitt, Steyn et al. 1999; Roseboom, van der Meulen et al. 1999; Whincup, Bredow et al. 1999). In addition, several studies have shown raised systolic blood pressure in growth restricted individuals who experienced accelerated postnatal catch-up growth (Whincup, Cook et al. 1989; Williams, St George et al. 1992; Donker, Labarthe et al. 1997; Nilsson, Ostergren et al. 1997; Labayen,
Ortega et al. 2009). Animal studies have also demonstrated that it is relatively easy to produce life long changes in the blood pressure of offspring by manipulation of maternal diet both before and during pregnancy (Kwong, Wild et al. 2000; Khan, Dekou et al. 2004).

Various mechanisms have been proposed to explain the observed relationship between low birth weight and hypertension. Alteration in the allocation of stem cells to different lineages following maternal dietary manipulation leading to hypertension in offspring has been demonstrated in rats (Kwong, Wild et al. 2000). Reduced nephron number with subsequent hypertension has been demonstrated in growth restricted animals, and similar effects on the kidney have been seen in humans who suffered fetal growth restriction (Hinchliffe, Lynch et al. 1992; Hughson, Farris et al. 2003; Luycrx 2005). Furthermore, a reduction in nephron number may not only predispose to hypertension but also increase the likelihood of renal damage due to higher glomerular capillary pressure and glomerular hyperfiltration (Brenner and Chertow 1993). Other mechanisms postulated to contribute to the relationship between hypertension and birth size include variations in homeostatic set points modulated through changes in hormonal and sympathetic nervous system responses and changes in vascular integrity, structure and reactivity leading to endothelial dysfunction (Barker, Bagby et al. 2006).

1.2.2.2 Glucose tolerance
Barker’s Hertfordshire studies also examined the relationship between glucose tolerance and birth size, demonstrating increased prevalence of impaired glucose tolerance and overt type 2 diabetes in individuals with lower birth weights, with a threefold difference between those in the highest and lowest birth weight groups (Hales, Barker et al. 1991). As for blood pressure, this effect was continuous across the normal range of birth weight. In addition, a similar relationship was found between weight at one year of age and impaired glucose tolerance, suggesting that the critical period for the programming of factors affecting glucose metabolism extends from fetal life to early infancy (Barker and Martyn 1992). A large number of studies have replicated these findings and also identified an inverse relationship between birth size and insulin resistance (Curhan, Willett et al. 1996; Lithell, McKeigue et al. 1996; Hofman, Cutfield et al. 1997; Carlsson, Persson et al. 1999; Rich-Edwards, Colditz et al. 1999; Egeland, Skjarven et al. 2000; Forsen, Eriksson et al. 2000; Levitt, Lambert et al. 2000). Again, as for blood pressure, those most at risk of impaired glucose and insulin metabolism in later life are those who are born small and experience accelerated catch up growth in childhood (Barker 2004).
There are several postulated mechanisms to explain these observations. Altered fetal and early postnatal nutrition may lead to insulin resistance in skeletal muscle, the main peripheral site of action of insulin (Osmond and Barker 2000). In addition, pancreatic development may be impaired, thereby limiting the eventual size or function of the adult pancreas (Barker and Martyn 1992). Furthermore, changes may be induced in the liver, leading to increased gluconeogenesis and altered lipid metabolism (De Boo and Harding 2006). Finally, alterations in hormone synthesis may occur in response to fetal undernutrition, leading to increased secretion of glucocorticoids, growth hormone and sex steroids which may persist in adult life and underlie the development of insulin resistance (Osmond and Barker 2000).

1.2.2.3 Lipid metabolism

It has been known for many years that serum cholesterol is directly associated with the development of atherosclerosis and risk of coronary heart disease (Lewis 1983). A study of a cohort of men and women from Sheffield aged 50 to 53 years examined the relationship between birth size and serum cholesterol levels in adult life (Barker, Martyn et al. 1993). This study revealed that specific body proportions at birth, rather than birth weight per se, were related to increased levels of total and low density lipoprotein (LDL) serum cholesterol and apolipoprotein B, a precursor of LDL cholesterol. Disproportionate birth measurements, particularly a short body and low birth weight in relation to head size, were associated with impaired cholesterol metabolism in adult life. In addition, as with fibrinogen concentrations, elevated LDL concentration was specifically associated with reduced abdominal circumference at birth but not reduced head circumference. The authors postulated that fetal undernutrition in late gestation could be responsible for the observed effects as diversion of oxygenated blood away from the trunk and abdomen to sustain growth of the brain, so-called “brain-sparing”, may result in impaired liver growth and consequent reprogramming of liver metabolism (Barker, Martyn et al. 1993; Osmond and Barker 2000).

Subsequent examination of the Sheffield cohort found increased mortality from ischaemic heart disease in those men with below average birth weight and small abdominal circumference. However, those men with above average birth weight and large abdominal circumference also had increased ischaemic heart disease mortality. These findings suggested that both reduced and accelerated liver growth in late gestation were risk factors for ischaemic heart disease in later life (Barker, Martyn et al. 1995). Haugen and colleagues
investigated the possibility of a “liver-sparing” effect induced by differences in maternal nutrition to explain the latter finding. By assessing the umbilical venous and ductus venosus blood flow of the fetuses of healthy women in late gestation, they found that slimmer mothers and those eating an unbalanced “imprudent” diet had fetuses with greater liver blood flow. They postulated that this may be due to the need for increased hepatic nutrient conversion and could result in deleterious effects on lipid metabolism and clotting factor haemostasis in a nutrient rich postnatal environment (Haugen, Hanson et al. 2005).

Research in experimental animals and humans suggests that serum cholesterol concentrations may also be influenced by infant feeding practice, as the critical period of plasticity of the liver extends into early postnatal life (Barker 2003). Rat pups born to protein restricted dams and/or fed by protein restricted lactating dams had lower cholesterol concentrations than controls, suggesting that cholesterol metabolism may be programmed by alterations to maternal nutrition during gestation or lactation (Lucas, Baker et al. 1996).

In humans, investigation of the Hertfordshire cohort found that men aged 60 to 70 years old had higher serum LDL concentrations and higher ischaemic heart disease mortality if they had been breast fed beyond one year of age (Fall, Barker et al. 1992). However, a systematic review of 37 human studies found lower total cholesterol and LDL concentrations in adults who were breast fed in infancy compared to those who were bottle fed. Interestingly, this review also found that breast feeding was associated with higher total cholesterol and LDL concentrations in infancy compared to bottle feeding, suggesting that the effect of infant feeding practice on total cholesterol and LDL concentrations at different life stages may be due to nutritional programming, such that higher dietary cholesterol in infancy may reduce endogenous synthesis of cholesterol in later life (Owen, Whincup et al. 2002).

Two recent systematic reviews have questioned the existence of a relationship between birth weight and blood cholesterol concentration, with both concluding that the available evidence did not support a strong association (Lauren, Jarvelin et al. 2003; Huxley, Owen et al. 2004). However, in response to the first of these reviews, Barker himself highlighted that low birth weight is a heterogeneous condition, and that broad comparisons of all studies investigating low birth weight and lipid profile in later life will not untangle the myriad factors which may underpin such an association, in particular the influence of fetal and maternal nutrition (Barker 2003). For example, atherogenic lipid profiles have been observed in adults exposed to fetal undernutrition in the Dutch Famine and middle-aged Chinese men born to mothers with low body mass index in early pregnancy, but both these...
cohorts were of normal birth weight (Mi, Law et al. 2000; Roseboom, van der Meulen et al. 2000). These findings suggest that poor maternal nutrition during gestation may permanently affect adult health without manifesting as low birth weight (Roseboom, van der Meulen et al. 2000).

1.2.2.4 Haemostatic factors

The association between high plasma fibrinogen and factor VIII concentrations and the incidence of ischaemic heart disease was well described in the Northwick Park Heart Study (Meade, Brozovic et al. 1986). Elevations of one standard deviation (SD) of factor VIII and fibrinogen concentration were respectively associated with a 64% and 82% increased risk of myocardial infarction within 5 years in men, compared to a 43% increased risk for elevated cholesterol (Meade, Brozovic et al. 1986). In two different cohorts of men, Barker and colleagues (Barker, Meade et al. 1992) demonstrated an inverse relationship between plasma factor VIII and fibrinogen concentrations and weight at one year of age but not birth weight. They also found that lower fibrinogen levels were associated with decreasing placental to birth weight ratio. They concluded that reduced growth in utero and early infancy was strongly associated with increased levels of haemostatic factors and postulated that this may be due to impairment in hepatic development during a critical early period (Barker, Meade et al. 1992).

Subsequent work has supported this hypothesis. Martyn and colleagues confirmed that plasma fibrinogen concentration was related to both birth weight and abdominal circumference in men, but not in women, suggesting that reduced liver growth in fetal life in men may permanently influence fibrinogen levels (Martyn, Meade et al. 1995). More recently, investigation of a cohort of healthy Swedish children demonstrated an inverse relationship between fibrinogen levels and birth weight in childhood and adolescence, leading the authors to suggest that atherosclerosis begins in childhood (Labayen, Ortega et al. 2009).

1.2.3 Biological Basis

1.2.3.1 Thrifty phenotype hypothesis

Central to the developmental origins hypothesis is the concept of programming. This is the mechanism by which exposure to an environmental influence during a critical period in early life leads to permanent effects on the structure or function of an organism (Hales and Barker 1992; Gluckman, Hanson et al. 2005a; De Boo and Harding 2006). For most organ
systems, the “critical period” during which time exposure to an environmental factor can lead to phenotypic change occurs in utero. However, the exact critical period depends on the organ system involved, and there is growing evidence to suggest that long-term outcome can be influenced by events from conception, or even prior to conception, to the first few months after birth (Gluckman, Hanson et al. 2005a).

Over the last century, many populations have rapidly transitioned from a “life of thrift” in which nutrients are limited to a westernised “life of plenty”, thereby generating mismatch between the expected and actual environments (Newnham 2007). With this in mind, in 1992 Hales and Barker proposed the “thrifty phenotype” hypothesis to explain the epidemic of adult insulin resistance and type 2 diabetes seen in preceding decades (Hales and Barker 1992). They suggested that poor maternal nutrition in conditions of limited nutrient availability could lead to fetal hypoglycaemia, in turn resulting in glucose preserving adaptations by the fetus such as reduced insulin secretion and increased peripheral insulin resistance. However, if postnatal conditions provide an abundance of nutrients, the permanent metabolic adaptations programmed in fetal life could then lead to glucose intolerance and diabetes in later life (Hales and Barker 1992). The thrifty phenotype hypothesis therefore describes the defensive response of the developing fetus to an immediate in utero challenge, the consequences of which must be dealt with in postnatal life (Gluckman, Hanson et al. 2008).

1.2.3.2 Predictive adaptive response model
In recent years, the thrifty phenotype hypothesis has been modified and extended by Gluckman and Hanson to a model termed the predictive adaptive response (Gluckman and Hanson 2004). This model proposes that the fetus makes adaptive responses in anticipation of their future postnatal environment rather than for immediate advantage in utero. Key to this model is the concept of developmental plasticity, defined as “the phenomenon by which one genotype can give rise to a range of different physiological and morphological states in response to different environmental conditions during development” (West-Eberhard 1989). Plasticity of an organ system provides a means by which environmental influences in early life can “fine tune” the development of an individual to provide a better match for the anticipated environment in which the individual can expect to live. This process of fine tuning goes beyond that which can be achieved by natural selection, which determines the inherited genotype only (Gluckman, Hanson et al. 2005b).
Through the predictive adaptive response model, Gluckman and Hanson suggested that the risk of disease in later life is directly related to the degree of mismatch between the perinatal environment and the environment encountered in later life (Gluckman, Hanson et al. 2005a). Phenotypic modifications induced in the fetus by minor alterations in maternal environmental cues aim to physiologically prepare the fetus for the predicted adult environment. If this prediction proves accurate, the fetus will be well prepared to survive in its environment, even if it is outwardly unfavourable. For example a nutritionally poor intrauterine environment induces reduced skeletal muscle development and increased visceral fat deposition, factors which favour survival in a poor postnatal environment, a pattern described in some Indian babies (Yajnik, Fall et al. 2003). However if the predictions made in utero prove inaccurate for the environment encountered in later life, the individual may be more susceptible to disease. For example, an abundant postnatal environment places a nutritionally deprived fetus at greater risk of development of obesity, insulin resistance and development of the metabolic syndrome (Gluckman, Hanson et al. 2008).

Like the thrifty phenotype hypothesis, this model explains the increased disease risk seen when moving from adverse in utero conditions to an abundant postnatal environment. However, it also provides a basis for the possibility of increased disease risk in the reverse situation, that is a transition from in utero abundance to postnatal deprivation, a situation for which there is a growing body of evidence (Gluckman, Hanson et al. 2005a).

1.2.3.3 Fetal insulin hypothesis
Proposed by Hattersley and Tooke in the late 1990’s, the Fetal Insulin Hypothesis suggests an alternative explanation for the association between low birth weight and insulin resistance in later life, which is not dependent on the intrauterine environment (Freeman 2010). Instead, they postulated that this relationship has a genetic basis, with the ability of an individual to secrete and/or respond to insulin being determined by inherited genes. As insulin is an important regulator of fetal growth, inheritance of genes favouring reduced insulin secretion or insulin resistance would result in low insulin-mediated fetal growth, with an ongoing propensity for insulin deficiency, resistance or even overt diabetes in adult life (Hattersley and Tooke 1999). They also hypothesised that these genetically inherited mediators of insulin synthesis or function would have influence over the development of hypertension and arteriosclerosis in later life through effects on endothelial development and later function. Therefore they argued that “low birth weight, measures of insulin
resistance in life and ultimately glucose tolerance, diabetes and hypertension would all be phenotypes of the same insulin-resistant genotype” (Hattersley and Tooke 1999).

Much of the evidence supporting the Fetal Insulin Hypothesis has come from the study of monogenic disorders of insulin secretion or function, in particular, mutations in the glucokinase gene (Hattersley, Beards et al. 1998). Glucokinase acts in the pancreas as a sensor of blood glucose concentration, and glucokinase mutations may result in mild hyperglycaemia due to impairment of pancreatic glucose sensing (Freeman 2010). When a mother has the glucokinase mutation but the fetus does not, the fetus is exposed to elevated maternal blood glucose concentrations, resulting in increased fetal insulin secretion and increased birth weight. However, when the fetus has the glucokinase mutation and the mother does not, less insulin is secreted by the fetus due to failure to sense the blood glucose concentration to which it is exposed, resulting in a lower birth weight (Hattersley, Beards et al. 1998). Thus, these findings suggest that insulin secretion and consequently fetal growth could be genetically determined (Hattersley and Tooke 1999).

Widespread support for the Fetal Insulin Hypothesis has not been forthcoming due to the relative rarity of these genetic polymorphisms compared to the frequency with which a relationship between birth weight and later insulin resistance is seen, including across the range of normal birth weights and a variety of different populations. Thus, given the widespread extent to which this relationship is observed, it seems likely that the in utero environment plays a key role in determining this health outcome in later life (De Boo and Harding 2006).

1.2.4 Molecular Mechanisms
1.2.4.1 Epigenetic modification
It is likely that changes in gene expression underpin the Developmental Origins hypothesis (De Boo and Harding 2006). The proposed mechanism, at least in part, for these changes in gene expression is epigenetic modification (Cutfield, Hofman et al. 2007). Epigenetics is the mechanism by which proteins and other molecules that bind to deoxyribose nucleic acid (DNA) and chromosomes can change gene expression without changing the DNA sequence (Simmons 2009). Epigenetic modification may be induced by changes in the intrauterine environment, thus leading to permanent alteration in the phenotype of offspring and potentially resulting in increased disease risk in adulthood (De Boo and Harding 2006).
The basis of epigenetic modification is the induction of conformational change in chromatin which in turn alters gene transcription (Cutfield, Hofman et al. 2007). For example, methylation of specific cytosine phosphate guanine dinucleotides in gene promoter regions can lead to “silencing” of the gene due to interference with transcription. Epigenetic modification may also occur via post-translational modification of histones by methylation, acetylation or phosphorylation (Wadhwa, Buss et al. 2009). Epigenetic modifications are usually stable throughout mitotic cell divisions and therefore continue throughout the lifetime of an organism (Gluckman, Hanson et al. 2008).

1.2.4.2 Role of epigenetic modification in the predictive adaptive response
Epigenetic changes act as a form of biological “memory” for environmental influences encountered during the phase of plasticity. As the plasticity of organ systems declines with increasing age, so does the opportunity for epigenetic modification. However, the effects of epigenetic modification may only become apparent in later life as an individual encounters an increasing array of environmental challenges (Godfrey, Lillycrop et al. 2007). As described by the predictive adaptive response model, the greater the degree of mismatch between the predicted and actual environment encountered in later life, the greater the risk of disease (Gluckman, Hanson et al. 2005a) (Figure 1.1).

Figure 1.1: Relationship between developmental plasticity, epigenetic modification, environmental influences and health in later life (redrawn from Godfrey et al, 2007)
Imprinted genes are the best understood example of epigenetic modification. Imprinting of genes occurs during early embryogenesis and involves a process of DNA demethylation and remethylation, in which some genes are labelled as being of maternal or paternal origin and thereby marked for subsequent inactivation (De Boo and Harding 2006; Godfrey, Lillycrop et al.). A substantial amount of experimental data from animals and observational data from humans now exists to suggest that imprinting may be disrupted by environmental influences in the periconceptional, late gestation or even early neonatal periods. It is likely that imprinted genes play key roles in directing placental growth, differentiation of specific cell lineages and the activities of various transporters, thereby influencing fetal growth through determination of placental nutrient delivery (Cutfield, Hofman et al. 2007). For example, methyl supplementation of the diet of pregnant mice has been shown to alter offspring phenotype through epigenetic modification of the imprinted agouti gene. The offspring produced are leaner, healthier and live longer than mice not exposed to a methyl rich intrauterine environment (Wolff, Kodell et al. 1998). More recently, Waterland and colleagues demonstrated transgenerational amplification of obesity in mice when the agouti gene is passed through successive generations of obese females. This process is prevented by hypermethylation of the maternal diet before and during pregnancy, providing evidence of epigenetic manipulation of body weight regulation. However, this effect was independent of coat colour, which is also controlled by the agouti gene, leading the authors to hypothesise that methyl supplementation induces epigenetic modification at other genetic loci affecting the regulation of body weight; for example, in the hypothalamus (Waterland, Travisano et al. 2008).

Epigenetic modification is also key to the control of non-imprinted genes important in the regulation of placental growth and function, maintenance of pregnancy and control of parturition (Cutfield, Hofman et al. 2007; Gluckman, Hanson et al. 2008). Several studies using human placental explants have shown effects of epigenetic modification on placental synthetic function, including prostaglandin biosynthesis and modulation of inflammatory markers such as IL-1β (Cutfield, Hofman et al. 2007).

Animal studies have demonstrated epigenetically mediated changes in gene expression in growth restricted offspring which may influence the development of chronic disease in later life. For example, in growth restricted mice, epigenetic modification of the glucose transporter GLUT 4, found in skeletal muscle, cardiac muscle and adipose tissue, results in reduced GLUT 4 expression and thus increased insulin resistance (Karnieli and Armoni
Epigenetic modification to Pdx-1, a pancreatic and duodenal homeobox transcription factor critical for pancreatic β-cell development and function, has also been demonstrated in growth restricted rats. These modifications resulted in increased susceptibility to type 2 diabetes in adulthood (Park, Stoffers et al. 2008).

1.2.4.3 Intergenerational effects
There is increasing evidence in both human and animal studies that adverse environmental influences in pregnancy can affect not only the offspring of that pregnancy but also subsequent generations (De Boo and Harding 2006). For example, rats exposed to glucocorticoids during pregnancy (F₀ generation) have offspring (F₁ generation) with altered glucose homeostasis and lower birth weights. These abnormalities are transmitted to the subsequent (F₂) generation without exposure of the F₁ generation to the same challenge (Drake, Walker et al. 2005). In an earlier study, Stewart and colleagues investigated the effect of mild protein malnutrition on rats over several generations. When refed with a normal diet it took three generations for growth and development to return to normal (Stewart, Sheppard et al. 1980). Evidence of similar intergenerational effects on metabolism and growth has been seen in humans. For example, Kaati and colleagues demonstrated a link between grandparental nutrition and the risk of diabetes in the F₂ generation in epidemiological studies in Sweden (Kaati, Bygren et al. 2002). In addition, women exposed to intrauterine undernutrition in the first and second trimester of pregnancy during the Dutch Winter Famine of 1944 to 1945 have offspring of lower birth weight than mothers who were not malnourished in utero (Lumey 1992), and mothers who were in utero during Ramadan have smaller and thinner babies than mothers who were not in utero during Ramadan (Alwasel, Harrath et al. 2013).

Several mechanisms may underlie these observed intergenerational effects. Epigenetic modifications may be passed on directly to subsequent generations (Reik, Santos et al. 2003). For example, exposure of F₀ pregnant mice to diethylstilbestrol results in increased susceptibility to tumour formation in male and female F₂ generation offspring (Newbold, Padilla-Banks et al. 2006). This effect is passed through the maternal line and is very similar to an intergenerational effect of diethylstilbestrol in humans (Blatt, Van Le et al. 2003). Alternatively, intrauterine undernutrition may mediate non-epigenetic changes in the development of the reproductive tract in the F₁ generation, thereby affecting subsequent generations. For example, young women born small for gestational age have reduced uterine size and ovarian volume and increased gonadotrophin levels compared to women born of
appropriate size for gestational age (Ibanez, Potau et al. 2000). Environmental factors affecting F₀ pregnancies may also be transferred non-epigenetically through the F₁ female offspring, including effects of maternal size, behaviour or body composition. For example, in rats, maternal behaviour towards pups in the first week after birth can influence the stress response of offspring as adults, even if the pups were not the genetic progeny of the mother (Francis, Diorio et al. 1999). Therefore, familial clusters of metabolic disease may be based on environmental and epigenetic factors rather than multigenetic inheritance alone (Gluckman, Hanson et al. 2008).

1.2.5 Physiological Factors Implicated in the Developmental Origins Hypothesis

1.2.5.1 Altered fetal nutrition

There is now a substantial amount of evidence from both animals and humans that fetal nutrition plays an important role in the programming of susceptibility to disease in later life (Harding 2001; Bloomfield, Oliver et al. 2006; De Boo and Harding 2006; Gluckman, Hanson et al. 2008; Victora, Adair et al. 2008). Fetal nutrition is a key determinant of fetal growth, and both fetal under- and over-nutrition influence later health outcomes. However, when considering the influence of fetal nutrition on later disease susceptibility, several factors must be considered, as discussed below.

1.2.5.1.1 Maternal nutrition versus fetal nutrition

Experimental manipulation of maternal diet in animal studies has proved to be a successful way of emulating the permanent changes in postnatal physiology seen in observational human studies (De Boo and Harding 2006). However, the “supply line” of nutrients from mother to fetus is complex and comprises several components other than just maternal diet, including metabolic and endocrine status, uterine and umbilical blood flows and placental function (Harding 2001). Perturbations in any of these components may interrupt the delivery of adequate nutrients to the fetus, which may become undernourished even if maternal nutrition, the first step in the “supply line”, is adequate. For example, maternal hypertension can interfere with placental function thereby limiting fetal nutrient supply and causing fetal undernutrition. Conversely, fluctuations in maternal diet may not translate into alterations in fetal nutrition if there is sufficient capacity within the nutrient supply line to compensate for this. Thus, although fetal nutrition is the key determinant of fetal growth, maternal nutrition is much easier to manipulate and quantify experimentally and is therefore the usual proxy upon which animal and human data on fetal growth and subsequent disease are based (Harding 2001).
1.2.5.1.2 Birth weight versus fetal growth
Traditionally, birth weight has been used in both animal and human studies when assessing the relationship between fetal growth and health outcomes in later life. However, birth weight is an isolated measure and may not accurately reflect the growth trajectory and thus, the nutrition, of the fetus throughout gestation. Different growth trajectories may occur as a result of altered in utero conditions at various stages of gestation, although final birth weight may be unaffected and even fall within the normal range. In this situation, normal birth weight does not exclude the possibility that the fetus has made physiological adaptations in response to a period of undernutrition in utero. For example, Bloomfield and colleagues demonstrated that fetal lambs undernourished for 20 days in late gestation were of lower birth weight than controls, but lambs undernourished for 10 days in late gestation were of similar birth weight to controls. Despite no difference in birth weight, sheep undernourished for 10 days in utero displayed altered hypothalamic-pituitary-adrenal axis function at 3 years of age, with accentuated responses to corticotrophin releasing hormone and arginine vasopressin compared to controls (Bloomfield, Oliver et al. 2003a). These findings clearly demonstrate that intrauterine undernutrition can affect postnatal physiology without affecting birth weight.

1.2.5.1.3 Relevance of altered body proportions
Given the limited relationship between birth weight and fetal growth, the possibility that alterations in body proportions may represent a more accurate assessment of fetal growth has been explored. The traditional hypothesis that symmetrical growth restriction indicates undernutrition of the fetus in early gestation and asymmetrical growth restriction indicates undernutrition later in gestation has not been supported by review of large human data sets (Kramer, McLean et al. 1989), although animal and human studies have demonstrated that the fetus does make adaptations resulting in differential organ growth in response to adverse intrauterine conditions. For example, redistribution of blood flow away from non-essential organs such as the gut and skin in favour of essential organs such as the brain and heart occurs in fetal sheep exposed to intrauterine hypoxia (Bocking, Gagnon et al. 1988). Similar changes have been demonstrated ultrasonographically in the human fetus in response to hypoxia (Al-Ghazali, Chita et al. 1989). These observations help explain the findings of brain and liver-sparing discussed previously. However, it is likely that other endocrine and metabolic factors also contribute to the alterations in fetal body proportions that occur in response to adverse intrauterine conditions, as brain sparing is also found in fetuses exposed to nutrient limitation but not hypoxia in utero (Harding 1997a). Therefore, changes in the
distribution of cardiac output together with hormonal and metabolic adaptations may all contribute to alterations in body proportions at birth (Harding 2001).

1.2.5.1.4 Applicability of animal data to human conditions
When interpreting data from animal studies, it is important to be mindful of the differences in anatomy and metabolism between species that may influence responses to nutrient restriction. For example, the commonly studied sheep, which is a ruminant, produces circulating blood glucose endogenously via gluconeogenesis in the liver. As a consequence, maternal undernutrition in sheep rapidly leads to a large decrease in maternal blood glucose concentration due to reduced availability of gluconeogenic substrates (Harding 1997a). By comparison, maternal blood glucose concentration in humans is relatively well preserved in conditions of maternal undernutrition, with 3 to 4 days of complete starvation required to produce a reduction in blood glucose concentration similar to that seen in sheep (Kim and Felig 1972; Saleh, al-Muhtaseb et al. 1989). Therefore, since glucose is the major substrate for the fetus, maternal undernutrition in sheep is likely to have a more immediate effect on fetal nutrition than in humans (Harding 2001). Furthermore, placental structure and function differs between humans and other commonly studied animal models. For example, the ovine placenta is relatively impermeable to ketones compared to the human placenta, thus the human fetus can utilise ketones as a substitute for glucose in conditions of maternal fasting but the fetal sheep cannot (Adam, Raiha et al. 1975). Such interspecies differences will impact upon the relative influence that variation in maternal nutrition has upon ultimate fetal nutrition.

Despite these concerns, the sheep has become one of the most widely adopted animals for investigating the effects of altered maternal nutrition on fetal development due to its long gestational period, frequent production of a singleton fetus, maturity of hypothalamic-pituitary-adrenal axis in the newborn period and rate of protein accretion throughout gestation, all of which are similar to that found in human pregnancies (Symonds, Budge et al. 2005a). Rodents are also commonly used for manipulating fetal nutrition via manipulation of maternal nutrition.

1.2.5.1.5 Global maternal undernutrition
Global maternal undernutrition results in reduced birth weight, increased systolic blood pressure and impaired glucose tolerance in offspring in several animals including rats, guinea pigs and sheep, with the effects observed depending in large part on the timing of maternal undernutrition (Vickers, Breier et al. 2000; Kind, Simonetta et al. 2002; Kind,

The longer gestational period of sheep has allowed investigation of the short and long-term effects of global maternal undernutrition at various stages of gestation (Buckley, Jaquiery et al. 2005). Nutrition in the periconceptional period is thought to be particularly important, with periconceptional undernutrition resulting in reduced fetal growth in late gestation but normal birth weight in sheep (Harding 1997b). In addition, modest periconceptional undernutrition in sheep can lead to premature activation of the fetal hypothalamic-pituitary-adrenal axis, resulting in an early fetal cortisol surge and premature birth (Bloomfield, Oliver et al. 2003b). Furthermore, periconceptional undernutrition has been shown to abolish relationships between growth-regulating hormones, such as cortisol, insulin and insulin-like growth factor-1, and postnatal growth and nutrient intake to at least 12 weeks of age, suggesting that dissociation of growth from key endocrine regulators may contribute to adverse metabolic outcomes following periconceptional undernutrition (Jaquiery, Oliver et al. 2011).

In late gestation, maternal undernutrition results in decreased birth weight in sheep and rats (Lesage, Blondeau et al. 2001; Borwick, Rae et al. 2003). However, the response of the fetal sheep to undernutrition in late gestation may depend on maternal nutritional status in the periconceptional period (Harding 1997a). Furthermore, if the duration of undernutrition of the pregnant ewe is extended beyond a critical timeframe, fetal growth restriction may become irreversible despite restoration of a normal maternal diet (Oliver, Harding et al. 2001).

Evidence that global maternal undernutrition can have long-term health implications for offspring has also emerged from animal studies. For example, in sheep, periconceptional undernutrition results in an increase in perirenal fat mass in offspring that persists into adulthood (Symonds, Budge et al. 2005b). These offspring also demonstrate altered baroreceptor responses and hypertension relative to controls in adulthood (Gardner, Pearce et al. 2004; Gopalakrishnan, Gardner et al. 2004). In addition, impaired glucose tolerance has been demonstrated in the adult offspring of ewes undernourished from 61 days prior to 30 days after mating (Todd, Oliver et al. 2009), as has impaired cortisol secretion and behavioural responses to stress induced by isolation. (Hernandez, Matthews et al.) Adult rats exposed to intrauterine undernutrition demonstrate hyperphagia, elevated systolic blood
pressure (Vickers, Breier et al. 2000), increased adiposity and decreased locomotor activity (Vickers, Breier et al. 2003).

Evidence of the long-term effects of global maternal undernutrition also exists in humans. Most of these data have been extracted retrospectively, particularly from studies of the offspring of pregnant women exposed to the Dutch winter famine of 1944 to 1945. As in animal studies, long-term health outcomes depend upon the timing of the intrauterine nutritional insult. Those exposed to undernutrition in early to mid gestation had normal birth weight but increased risk of cardiovascular disease and lipid abnormalities in later life, while undernutrition in late gestation resulted in lower birth weight and glucose intolerance in adulthood (Roseboom, van der Meulen et al. 1999; Roseboom, van der Meulen et al. 2000; Roseboom, van der Meulen et al. 2001). In addition, exposure to famine in early gestation was associated with an increased body mass index in women at age 50 years, as well as a greater likelihood of consumption of a high fat diet in both men and women, suggesting that intrauterine nutrition may affect dietary choices in later life (Ravelli, van der Meulen et al. 1999; Lussana, Painter et al. 2008). Evidence from human studies in developing countries also suggests that maternal undernutrition in early pregnancy may effect gestational length (Yajnik, Fall et al. 2003; Rayco-Solon, Fulford et al. 2005). These findings are in keeping with cardiovascular and metabolic outcomes from animal studies following maternal undernutrition (Symonds, Budge et al. 2005b). However, a prospective study of a well nourished cohort of pregnant women in the United Kingdom failed to demonstrate a relationship between nutritional intake in the first and third trimesters and offspring birth weight, head circumference or placental weight (Langley-Evans and Langley-Evans 2003).

1.2.5.1.6 Maternal macronutrient imbalance
An imbalance of carbohydrate, protein and fat in the maternal diet may also impact upon the later health outcomes of offspring. This has been extensively investigated using maternal protein restriction in rats, mimicking a maternal low protein diet frequently seen in developing countries (Warner and Ozanne 2010). For example, a low protein diet throughout gestation and lactation results in low birth weight rat pups that remain permanently growth restricted, even if weaned onto a normal diet (Desai, Crowther et al. 1996). These animals also have impaired glucose tolerance in adulthood with development of overt type 2 diabetes in males (Petry, Dorling et al. 2001). In addition, several studies have demonstrated elevated blood pressure in the offspring of rat dams fed a low protein
diet during throughout pregnancy, which is thought to result from increased exposure of the fetus to maternal glucocorticoids in mid to late gestation due to down-regulation of the placental hormone 11-β hydroxysteroid dehydrogenase type 2 (Langley and Jackson 1994; Langley-Evans, Welham et al. 1996; Langley-Evans, Welham et al. 1999). This hormone converts active forms of glucocorticoid to inactive forms in the placenta, thus protecting the fetus from exposure to excessive levels of maternal glucocorticoids (Langley-Evans 2001). Although raised glucocorticoid concentrations have not been directly measured in fetal tissues, placentas of rats exposed to a low protein diet in utero have reduced 11-β hydroxysteroid dehydrogenase type 2 activity, and elevated levels of glucocorticoid-inducible enzymes in the liver and brain have also been found in these animals (Langley-Evans, Phillips et al. 1996; Langley-Evans and Nwagwu 1998). The mechanisms by which increased glucocorticoid exposure leads to hypertension may include alteration of components of the renin-angiotensin system, which is subject to glucocorticoid regulation, stimulation of hypothalamic receptors and/or increases in peripheral vascular resistance via glucocorticoid mediated changes in calcium entry to vascular smooth muscle (Ashton 2000; Langley-Evans 2001).

Maternal macronutrient imbalance may also induce other early programming mechanisms, including increased sensitivity of the hypothalamic-pituitary-adrenal axis in postnatal life and/or alterations to glomerular number in the renal cortex (Ashton 2000). For example, maternal low protein diet imposed for only the first four days of gestation in rats, corresponding to the preimplantation period, resulted in a reduction in birth weight of female offspring and early growth overcompensation in both male and female offspring for the first 12 weeks after birth. In addition, offspring had higher systolic blood pressure at 4 and 11 weeks of age, in keeping with outcomes after longer periods of low protein diet during pregnancy (Kwong, Wild et al. 2000).

Other effects of protein restriction throughout gestation and/or lactation on rat offspring include reduced bone mass and metabolism, delayed sexual maturation and accelerated aging of reproductive function in females, and adverse effects on leptin metabolism and glucose metabolism, with insulin resistance passed transgenerationally to the second generation (Guzman, Cabrera et al. 2006; Fetoui, Mahjoubi-Samet et al. 2008; Pinheiro, Salvucci et al. 2008). Several studies have demonstrated reversal of these effects by supplementation with urea, folic acid or various amino acids such as glycine and taurine, suggesting they have a key role in normal development of the fetus (Cherif, Reusens et al.
In humans, a maternal diet consisting of high carbohydrate intake in early pregnancy and low protein intake in later pregnancy is associated with reduced birth weight, ponderal index and placental weight and reduced levels of proinsulin, insulin and C-peptide in cord blood (Godfrey, Robinson et al. 1996; Godfrey, Barker et al. 1997). High protein intake in late gestation has also been associated with a reduction in birth weight (Andreasyan, Ponsonby et al. 2007). In addition, the balance of carbohydrate and protein in the maternal diet in late gestation has been shown to influence blood pressure in adulthood (Campbell, Hall et al. 1996). Although the mechanisms underlying these findings remain uncertain, it is clear that the overall balance of macronutrients is critical for the later health of the offspring (Harding 2001; Warner and Ozanne 2010).

1.2.5.1.7 Maternal micronutrient imbalance
Intrauterine deficiency of several specific micronutrients has been investigated in animal and human studies. The importance of folate in the maternal diet throughout pregnancy is well documented, with folate deficiency associated with neural tube defects, premature delivery, low birth weight and fetal growth retardation (Scholl and Johnson 2000). In addition, an association has been found between low maternal folate intake during pregnancy and childhood hyperactivity scores at 9 years of age, suggesting a role for folate in fetal brain development (Schlotz, Jones et al. 2010). Furthermore, low maternal folate status may result in increased maternal homocysteine concentration, which is associated with spontaneous abortion and hypertension of pregnancy (Scholl and Johnson 2000). However, in rats, intrauterine folate deficiency has no effect on adult blood pressure and only a minor effect on insulin secretion (Maloney, Hay et al. 2009).

The significance of maternal dietary iron has also been investigated in rats. These studies have shown reduced birth weight, increased blood pressure and reduction in the number of nephrons in offspring of iron deficient dams (Lewis, Forhead et al. 2002; Gambling, Dunford et al. 2003; Lisle, Lewis et al. 2003). Alterations in glucose and lipid metabolism were also found in pups at 3 months of age, although these findings were not consistent across all studies and did not persist into adulthood (Lewis, Petry et al. 2001; Lisle, Lewis et al. 2003). In humans, maternal iron deficiency has been associated with reduced birth weight in offspring and preterm birth (Singla, Tyagi et al. 1997; Mitchell, Robinson et al.
2004), although an association between low maternal iron status and raised blood pressure in offspring has not been found (Belfort, Rifas-Shiman et al. 2008). However, low maternal iron status in the first trimester has been associated with increased placental size and vascularity (Hindmarsh, Geary et al. 2000). This finding may have long-term health implications for offspring, given the association between increased placental size and increased blood pressure noted in epidemiological studies (Barker, Bull et al. 1990).

The effect of maternal calcium and vitamin D status on the health of offspring has also been investigated, with some evidence in humans and animals to suggest that maternal calcium supplementation during pregnancy may result in reduced blood pressure in offspring (Belizan, Villar et al. 1997; Bergel and Belizan 2002; Hatton, Harrison-Hohner et al. 2003; Bergel and Barros 2007). In addition, an association has been found between reduced maternal vitamin D stores in late pregnancy and reduced bone mineral content in offspring at 9 years of age (Javaid, Crozier et al. 2006).

1.2.5.1.8 Maternal overnutrition
Although much of the early epidemiological work focused on the effects of maternal undernutrition, an excess of maternal nutrient supply, particularly dietary fat, is an increasing problem in many developed societies (Armitage, Taylor et al. 2005; Buckley, Jaquiery et al. 2005; De Bo and Harding 2006). Therefore, a growing amount of experimental work in animals is being undertaken to investigate the effects of overnutrition on the fetus and neonate (Warner and Ozanne 2010). Rodent models of overnutrition have proved difficult to perfect due to their innate tight homeostatic regulation of nutritional intake to maintain normal body weight (Armitage, Taylor et al. 2005). Furthermore, extrapolation from rodents to humans is complicated by the fact that development of important neural circuitry involved in appetite regulation occurs before birth in humans but postnatally in rodents (Vickers, Gluckman et al. 2005; Horvath and Bruning 2006). Despite these difficulties, several animals studies of perinatal overnutrition have been developed in recent years, including high-fat feeding of rat dams (Buckley, Keser, et al. 2005), maternal obesity (Samuelsson, Matthews et al. 2008; Nivoit, Morens et al. 2009), impairment of hypothalamic satiety control in mice (Blair, Caterson et al. 1996), pharmacological induction of gestational diabetes in rats (Merzouk, Madani et al. 2000; Merzouk, Madani et al. 2002; Kiss, Lima et al. 2009), and overnutrition of adolescent sheep, who are themselves still growing (Wallace, Bourke et al. 2003). These studies have demonstrated metabolic and cardiovascular abnormalities in the offspring of overnourished mothers that resemble the
metabolic syndrome of humans, including impaired glucose tolerance and insulin resistance, abnormal lipid profile, raised blood pressure and obesity (Armitage, Taylor et al. 2005; Freeman 2010; Warner and Ozanne 2010).

In humans, the effects of maternal overnutrition on the health outcomes of offspring have been investigated in a number of ways. There is clear evidence that maternal diabetes, with exposure of the fetus to increased levels of glucose and fatty acids, leads to an increased risk of glucose intolerance and type 2 diabetes in offspring in later life (Pettitt, Aleck et al. 1988; Sobngwi, Boudou et al. 2003; Fetita, Sobngwi et al. 2006). In addition, the offspring of obese mother have increased fat mass at birth and at 9 years of age and impaired insulin sensitivity at birth (Sewell, Huston-Presley et al. 2006; Gale, Javaid et al. 2007; Catalano, Presley et al. 2009). In addition, autopsy studies of children aged 1 to 13 years found greater areas of fatty streak formation in the aortas of offspring of mothers who were hypercholesterolaemic during pregnancy than of mothers with normal cholesterol levels, suggesting that maternal hypercholesterolaemia may influence the development of atherosclerosis in offspring (Napoli, Glass et al. 1999).

Epidemiological studies have suggested transgenerational effects of overnutrition on the cardiovascular risk of subsequent generations. For example, an overabundance of food during the slow growth period of the paternal grandfather, from 8 to 12 years of age, was associated with a four-fold increase in cardiovascular and diabetes mortality in male grandchildren (Kaati, Bygren et al. 2002). A similar association between paternal overnutrition and the risk of cardiovascular disease in male offspring has also been found (Kaati, Bygren et al. 2007). The authors conclude that good nutrition in the ancestor is associated with poor survival in offspring, with effects seen across generations especially in the male line, and suggested that these findings are mediated through epigenetic modification (Kaati, Bygren et al. 2007).

Taken together, these data suggest that exposure to overnutrition prior to birth results in alterations to metabolic health which can lead to adverse health outcomes that emerge in childhood or adolescence and may contribute to a cycle of intergenerational obesity, impaired metabolic function and increased cardiovascular risk (McMillen, MacLaughlin et al. 2008).
1.2.5.1.9 Role of leptin

Leptin is important for the development of metabolic homeostasis and appetite control systems within the central nervous system. As noted previously, hypertension, hyperphagia, increased sedentary behaviour and obesity has been demonstrated in adult rats exposed to intrauterine undernutrition, particularly when fed a high fat diet postnatally. Hyperleptinaemia and hyperinsulinaemia were also found in adulthood (Vickers, Breier et al. 2000; Vickers, Breier et al. 2003). However, treatment with leptin in the neonatal period prevented these outcomes, indicating that metabolic programming induced by exposure to undernutrition in the fetal period may be reversible with appropriately timed leptin therapy (Vickers, Gluckman et al. 2005).

In the rat, leptin and insulin are thought to exert their influence through developmental effects in the early neonatal period on the neural circuitry of the hypothalamus which controls energy homeostasis (Bouret 2009). However, there is also evidence that leptin may have a peripheral role, especially in the development of fetal adiposity. For example, in humans, increased leptin levels have been found in the cord blood of offspring of obese mothers, in conjunction with increased neonatal fat mass (Catalano, Presley et al. 2009). Moreover, adult sheep undernourished in utero have leptin levels which increase markedly following catecholamine stimulation, suggesting a resetting of the adipocyte response to stress in these animals (Gopalakrishnan, Gardner et al. 2004). In addition, the offspring of ewes undernourished in early gestation have increased adipose stores and leptin levels compared to controls (Bispham, Gopalakrishnan et al. 2003). These findings may help explain the increased obesity seen in adult humans exposed to undernutrition early in gestation during the Dutch winter famine (Vickers 2007).

1.2.5.2 Postnatal nutrition and accelerated postnatal growth

As postnatal life is also a period of developmental plasticity, it is plausible that adverse environmental conditions experienced during this time may influence later health outcomes. Therefore, in recent years, increasing attention has been paid to the potential impact of early postnatal nutrition on health outcomes in later life. Particular attention has focused on the impact of rapid postnatal catch up growth in offspring born small for gestational age. Investigation of the effects of variation in postnatal nutrition may be of public health importance when considering the potential for the implementation of dietary manipulation that may reverse or ameliorate adverse health outcomes which result from exposure to an unfavourable intrauterine environment (Buckley, Jaquiery et al. 2005). The potential
underlying mechanisms have started to emerge, with evidence that hormonally induced manipulation of central nervous system pathways may play an integral role (Plagemann 2006).

1.2.5.2.1 Postnatal undernutrition
A plethora of evidence from epidemiological and cohort studies, particularly from developing countries, suggests that combined pre- and postnatal undernutrition results in deleterious outcomes in later life in humans (Victora, Adair et al. 2008; Martorell 2010; Patel, Badhoniya et al. 2010; Senarath, Dibley et al. 2010). Investigation of the effect of undernutrition in utero and during lactation has also been undertaken in animals (Armitage, Taylor et al. 2005). A limitation of these studies is that it is difficult to establish if any one of these time periods has a more critical role in determining subsequent health outcomes (Buckley, Jaquiery et al. 2005). However, undernutrition of suckling rat pups adversely affects sexual maturation, with decreased estrogen and gonadotrophin releasing hormone concentrations and accelerated aging of the reproductive axis (Chernoff, Gage et al. 2009; Ramos, Lima et al. 2010). In addition, rat pups undernourished for the first two weeks after birth displayed decreased ability on tests of spatial memory and had diminished brain and body weight compared to controls (Hemb, Cammarota et al. 2010). Rat pups exposed to undernutrition due to a low protein maternal diet during lactation show increased leptin and insulin concentrations at 10 days of age and altered feeding patterns as adults (Moura, Franco de Sa et al. 2002). Furthermore, detailed investigation of milk content and quantity in the spontaneously hypertensive rat has revealed decreased overall milk production by lactating dams, resulting in decreased ingestion of sodium and calcium by suckling pups. This difference in nutritional intake during suckling is critical to the development of hypertension, as spontaneously hypertensive rat pups cross-fostered at birth to normal rat dams do not develop hypertension in later life (Ashton 2000). Taken together, these data suggest that undernutrition during the suckling phase, either due to restricted milk supply or deficiencies of maternal diet, can have negative consequences for health in later life.

1.2.5.2.2 Postnatal overnutrition and accelerated postnatal growth
Several animal studies have investigated the consequences of postnatal overnutrition. Methods of overnutrition include provision of a high carbohydrate or fat-enriched maternal diet, culling of rat litter size to increase milk availability per pup and cross fostering of rat pups to diabetic dams. The outcomes include hyperinsulinaemia and glucose intolerance, hyperleptinaemia, hyperphagia and obesity, dyslipidaemia, hypertension and endothelial dysfunction, with detection of these abnormalities in early postnatal life and persistence into
adulthood (Fahrenkrog, Harder et al. 2004; Khan, Dekou et al. 2005; Plagemann 2006; Patel and Srinivasan 2010). Furthermore, transgenerational inheritance of this phenotype has been demonstrated (Patel and Srinivasan 2010).

Similar outcomes have been demonstrated in animals undernourished in utero, particularly if born small for gestational age. When provided with increased nutrition in the postnatal phase, either through supplementation of maternal diet during lactation and/or provision of a post-weaning “cafeteria diet” high in fat and sugar, these animals display accelerated postnatal “catch up” growth, with more severe metabolic outcomes and shorter life spans than animals who were not undernourished in utero (Vickers, Breier et al. 2000; Ozanne and Hales 2004; Armitage, Taylor et al. 2005; Vaag 2009). Furthermore, when offspring undernourished in utero have postnatal nutrition restricted, thereby preventing early catch-up growth, adverse metabolic consequences such as impaired glucose tolerance and obesity can be prevented (Jimenez-Chillaron, Hernandez-Valencia et al. 2006). These findings provide support for the Predictive Adaptive Response model discussed earlier, as adverse health outcomes in later life are exacerbated by a mismatch between the pre- and postnatal environments (Armitage, Taylor et al. 2005; Vickers 2007). The mechanisms postulated to underlie these findings include alteration of the development of hypothalamic neurohormonal circuits controlling appetite regulation, involving leptin, insulin and neuropeptide Y (Armitage, Taylor et al. 2005; Plagemann 2006; Vickers 2007; Prior and Armitage 2009; Rodrigues, de Moura et al. 2009).

These data support observations from human studies of the effects of postnatal overnutrition and catch-up growth. Although there is debate regarding the most critical time period during which accelerated growth may be disadvantageous, several epidemiological studies, together with more recent prospective studies, have provided evidence of deleterious health outcomes following provision of increased postnatal nutrient supply with or without coexisting small size at birth. In particular, insulin resistance, obesity, endothelial dysfunction and hypertension are associated with rapid weight gain in the neonatal period (Ong, Ahmed et al. 2000; Stettler, Zemel et al. 2002; Singhal, Fewtrell et al. 2003; Singhal, Cole et al. 2004; Stettler, Stallings et al. 2005; Ekelund, Ong et al. 2007; Ong 2007; Singhal, Cole et al. 2007). Furthermore, breastfeeding may confer a protective effect on the development of obesity, insulin resistance, dyslipidaemia and hypertension compared to formula feeding (Owen, Martin et al. 2006; Owen, Whincup et al. 2008; Bartok and Ventura 2009; Paul, Bartok et al. 2009; Monasta, Batty et al. 2010). While the mechanisms
underlying these observations remain unclear, lower protein and calorie content in breast milk may lead to slower growth in breast fed infants, which in turn may lead to reduced risk of adverse health outcomes, in keeping with the findings of animal and human studies discussed above (Vickers 2007).

1.2.5.3 Glucocorticoid exposure
The possibility that excess intrauterine glucocorticoid exposure may play a role in the development of long-term adverse health outcomes was first postulated by Edwards and colleagues (Edwards, Benediktsson et al. 1993). Glucocorticoids are secreted by the adrenal cortex under the control of the hypothalamic-pituitary-adrenal axis. In postnatal life, glucocorticoids play an essential role in a variety of organ systems, including modulation of cardiovascular, metabolic and immunologic function. In the developing fetus, maturation of the respiratory, cardiovascular, renal, neurological and gastrointestinal systems are all dependent upon the action of glucocorticoids.

1.2.5.3.1 Effects of increased fetal glucocorticoid exposure
Overexposure of the fetus to glucocorticoid can occur through an increase in endogenous maternal glucocorticoid production, for example, under conditions of maternal stress, or if exogenous synthetic glucocorticoids are administered to assist lung maturation in threatened preterm labour (Newnham and Jobe 2009; Parker and Douglas 2010).

Reduced birth weight and hypertension in adulthood are found in rats and sheep exposed to excess glucocorticoids in utero (Benediktsson, Lindsay et al. 1993; Levitt, Lindsay et al. 1996; Dodic, Abouantoun et al. 2002; Jensen, Gallaher et al. 2002; Woods and Weeks 2005). Physiological processes implicated include reduction in nephron number, alterations in activity of the renin-angiotensin system and changes to vascular responsiveness (Dodic, Abouantoun et al. 2002; Molnar, Howe et al. 2003; Ortiz, Quan et al. 2003; Hadoke, Lindsay et al. 2006). Impaired glucose tolerance has also been demonstrated in adult sheep and rats exposed to excess glucocorticoids in utero (Lindsay, Lindsay et al. 1996; Nyirenda, Lindsay et al. 1998; Moss, Sloboda et al. 2001). This effect is thought to be mediated through direct impairment of pancreatic β-cell development and altered expression of the hepatic enzyme phosphoenolpyruvate carboxykinase which catalyses the rate-limiting step in gluconeogenesis (Nyirenda, Lindsay et al. 1998; Blondeau, Lesage et al. 2001).

Exposure of rats to excess glucocorticoids in late gestation also results in increased red blood cell mass, haemoglobin concentration, and haematocrit in adulthood. As reticulocyte
count and erythropoietin concentration are also increased in these animals, these haematological changes are related to increased production of red blood cells, which may be due to changes in transcription of the erythropoietin gene (Tang, Seckl et al. 2011).

Overexposure to glucocorticoids in utero has also been associated with alterations in adult behaviour and neurological development, including reduced coping under stress, increased levels of anxiety and altered hippocampal development in rodents (Welberg, Seckl et al. 2000; Welberg, Seckl et al. 2001; Fujioka, Fujioka et al. 2006), and decreased brain growth and delayed myelination in sheep (Dunlop, Archer et al. 1997; Jobe, Wada et al. 1998; Huang, Beazley et al. 1999). In contrast, increased handling of rat pups in the early postnatal phase results in decreased glucocorticoid secretion in response to stress in adulthood (Meaney, Aitken et al. 1988).

In humans, a high level of self-reported maternal stress during pregnancy has been associated with reduced offspring birth weight and earlier delivery. (Wadhwa, Sandman et al. 1993) In addition, offspring of pregnant women who survived the World Trade Centre attacks of 2001 had a greater incidence of intrauterine growth restriction compared to controls, although no association was found between maternal post-traumatic stress disorder and intrauterine growth restriction. (Berkowitz, Wolff et al. 2003) Furthermore, repeat courses of antenatal corticosteroids have been associated with reduced birth weight in two large prospective randomised trials (Wapner, Sorokin et al. 2006; Murphy, Hannah et al. 2008) and reduced birth weight z-scores were found in a further large prospective study. (Crowther, Haslam et al. 2006)

Antenatal glucocorticoid exposure has also been associated with increased aortic stiffness in adults aged 25 years who were born preterm, with the vascular changes observed in these individuals similar to those seen in term-born adults 10 years older (Kelly, Lewandowski et al. 2012). As aortic stiffness is related to future development of hypertension (Redheuil, Yu et al. 2010), these findings suggest that increased glucocorticoid exposure in utero may be associated with hypertension in later life. However, at present there is minimal evidence of such an association. For example, no increase in blood pressure has been shown in children exposed to repeat course of antenatal corticosteroids when followed up at 2 to 3 years of age or in 6-year olds exposed to a single course of antenatal corticosteroids (Dalziel, Liang et al. 2004; Crowther, Doyle et al. 2007; Wapner, Sorokin et al. 2007). In contrast, in a retrospective study of 177 low birth weight individuals at 14 years of age, Doyle and
colleagues reported higher blood pressure in those exposed to glucocorticoids prior to birth compared to those not exposed to glucocorticoids (Doyle, Ford et al. 2000). However, in a much larger follow up study of 534 adults whose mothers had participated in a randomised controlled trial of antenatal betamethasone versus placebo for prevention of neonatal respiratory distress syndrome, Dalziel and colleagues reported no significant increase in blood pressure at 30 years of age in glucocorticoid exposed subjects (Dalziel, Walker et al. 2005). However, these authors did demonstrate increased insulin concentration at 30 minutes and decreased glucose concentration at 120 minutes post oral glucose load in corticosteroid exposed participants, suggesting that this exposure may confer an increased risk of insulin resistance and development of type 2 diabetes with increasing age (Dalziel, Walker et al. 2005). In support of these findings, in a cohort of young adults born preterm, impaired pancreatic β-cell function has been reported only in those exposed to antenatal glucocorticoids (Kelly, Lewandowski et al. 2012).

Although a few small uncontrolled cohort studies have reported increased behavioural problems in childhood, such as attention deficit hyperactivity disorder and aggressive and destructive behaviour, particularly after repeated courses of antenatal glucocorticoids (O'Connor, Heron et al. 2002; French, Hagan et al. 2004), follow up of participants of randomised controlled trials of antenatal glucocorticoids have generally shown favourable neurological outcomes (Roberts and Dalziel 2010). For example, a reduction in the rate of cerebroventricular haemorrhage has been found in neonates, in addition to similar or reduced rates of neurodevelopmental delay and cerebral palsy in childhood (Liggins and Howie 1972; MacArthur, Howie et al. 1982; Collaborative Group on Antenatal Steroids 1984; Schmand, Neuvel et al. 1990; Salokorpi, Sajaniemi et al. 1997). Furthermore, longer term follow up into adulthood of participants of two randomised trials of antenatal glucocorticoids revealed no evidence of cognitive or psychological effects (Dessens, Haas et al. 2000; Dalziel, Lim et al. 2005).

1.2.5.3.2 Mechanisms of glucocorticoid action in the fetus
Under normal physiological conditions, glucocorticoid concentrations are much lower in the fetal circulation than the maternal circulation, with the placenta acting as a “barrier” protecting the fetus from over-exposure to maternally derived glucocorticoids. This protective role is mediated through the action of the placental enzyme 11-β hydroxysteroid dehydrogenase type 2, which converts active glucocorticoids to inactive forms. Therefore, variation in the function of this enzyme can lead to altered exposure of the fetus to
glucocorticoid. For example, inhibition of 11-β hydroxysteroid dehydrogenase type 2 in pregnant rats leads to reduced birth weight, hypertension and glucose intolerance in later life (Edwards, Benediktsson et al. 1993; Lindsay, Lindsay et al. 1996; Langley-Evans 1997). Reduction in the activity of this enzyme is thought to form part of the mechanism by which a protein restricted maternal diet induces hypertension in offspring, suggesting that alterations in fetal nutrition and overexposure to glucocorticoids may be co-dependent programming mechanisms in some situations (Langley-Evans, Phillips et al. 1996; De Boo and Harding 2006; Harris and Seckl 2011).

Glucocorticoid actions are exerted through glucocorticoid receptors that are expressed in almost all fetal tissues (Drake, Tang et al. 2007). This expression can be altered by increased exposure of the fetus to glucocorticoids. For example, fetal rats exposed to excess glucocorticoids in the last trimester have reduced hippocampal glucocorticoid receptor expression. This results in impairment of negative feedback on the hypothalamic-pituitary-adrenal axis with consequent up-regulation of hypothalamic-pituitary-adrenal axis function in postnatal life (Levitt, Lindsay et al. 1996). Similar changes in hypothalamic-pituitary-adrenal axis responsiveness have been found in sheep and guinea pigs following excess glucocorticoid exposure in utero, with effects dependent upon timing of in utero exposure and sex of the offspring (Dean, Yu et al. 2001; Sloboda, Moss et al. 2002). These hypothalamic-pituitary-adrenal axis changes and the resultant increase in circulating glucocorticoid concentrations are thought to be integral to the adverse cardiovascular and metabolic outcomes seen in adult offspring exposed to excess glucocorticoid prior to birth (Cottrell and Seckl 2009).

Intergenerational transmission of the effects of increased fetal glucocorticoid exposure has also been demonstrated. For example, second generation offspring of male and female rats exposed to excess glucocorticoid concentrations in utero have reduced birth weight, glucose intolerance and elevation of hepatic phosphoenolpyruvate carboxykinase activity similar to that seen in the F1 generation, with these effects disappearing in the third generation (Drake, Walker et al. 2005). These findings indicate that epigenetic changes may, at least in part, explain the observed effects of excess fetal glucocorticoid exposure (Drake, Tang et al. 2007; Cottrell and Seckl 2009).
1.2.5.4 Preterm delivery

The Developmental Origins hypothesis had its genesis in the observation of associations between low birth weight and subsequent cardiovascular disease. However, these retrospective cohort studies were not able to investigate the effect of gestational length on these associations, as this data was lacking from the historical records (Dalziel, Parag et al. 2007). Until recently, animal and human studies have continued to focus on the relationship between low birth weight and risk factors for cardiovascular disease without independently investigating the effect of gestation length (Regan, Cutfield et al. 2006). However, preterm birth, defined as delivery at less than 37 weeks’ gestation, is a frequent cause of low birth weight. Although the rate of preterm birth may be falling from a peak of 12.8% of all deliveries in 2006, it continues to account for approximately 12% of all births in the United States (Hamilton, Martin et al. 2010; Martin, Osterman et al. 2010). Therefore, over the last decade, research has begun to focus on whether an association exists between preterm birth and health outcomes in later life, independent of the effect of low birth weight.

1.2.5.4.1 Effects on blood pressure

Siewart-Delle and Ljungman studied a cohort of 430 subjects at age 49 years and found an inverse correlation between gestational age and systolic blood pressure for subjects born preterm (Siewert-Delle and Ljungman 1998). Several other cohort studies have subsequently demonstrated a similar association, with the effect of gestational age on adult blood pressure independent of any effect of birth weight (Bonamy, Bendito et al. 2005; Johansson, Iliadou et al. 2005; Keijzer-Veen, Finken et al. 2005; Kistner, Celsi et al. 2005; Dalziel, Parag et al. 2007; Rotteveel, van Weissenbruch et al. 2008). For example, Dalziel and colleagues found that preterm delivery at a median gestational age of 34 weeks resulted in a 3.5 mmHg increase in systolic blood pressure at 30 years of age, with a 0.5 mmHg increase in systolic blood pressure for each one week decrease in gestational age. This finding was consistent throughout all gestational ages, including those born at term. Low birth weight was also associated with an increase in systolic blood pressure, but adjustment for preterm birth using birth weight z-score abolished this relationship, indicating that preterm birth, rather than fetal growth, was the major determinant of increased systolic blood pressure (Dalziel, Parag et al. 2007). In an earlier study of over 165,136 Swedish male conscripts at 18 years of age, Leon and colleagues demonstrated a similar inverse relationship between systolic blood pressure and gestation length from 35 to 44 weeks, but also showed an independent effect of birth weight. It is possible that this effect was apparent due to the very large sample size (Leon, Johansson et al. 2000). However, taken together,
these studies show that the effect of gestational length on blood pressure is continuous across all gestational ages and not just a consequence of preterm birth. More recently, several other studies have also demonstrated an association between gestational age and systolic blood pressure in children (Bonamy, Bendito et al. 2005; Bayrakci, Schaefer et al. 2007; Mikkola, Leipala et al. 2007; Relton, Pearce et al. 2008). In addition, a relationship between gestation length and increased pulse pressure has also been reported, with early vascular changes, in particular increased arterial stiffness, postulated to account for this finding (Relton, Pearce et al. 2008).

In keeping with these studies, higher systolic blood pressure has been reported in adults born preterm to hypertensive or normotensive mothers, in comparison to term offspring of normal pregnancies. However, those born preterm to normotensive mothers had greater arterial stiffness than preterm offspring of hypertensive mothers, who in turn, were found to have greater carotid intima-media thickness than preterm offspring of normotensive mothers. These findings suggest that vascular changes are responsible for the increase in systolic blood pressure observed in these two groups, and that the vascular phenotype of preterm offspring may be dependent upon the presence or absence of maternal hypertension (Lazdam, de la Horra et al. 2010).

1.2.5.4.2 Effects on insulin resistance and other endocrine outcomes
An association between preterm delivery and altered insulin concentration in later life has been reported in several studies (Irving, Belton et al. 2000; Hofman, Regan et al. 2004; Dalziel, Parag et al. 2007). Initially, preterm birth was found to be associated with increased fasting insulin levels in young adulthood, but the relative importance of preterm birth and low birth weight could not be determined in this small cohort (Irving, Belton et al. 2000). Hofman and colleagues subsequently demonstrated insulin resistance in children born prior to 32 weeks’ gestation and tested at 4 to 10 years of age. Their results indicated no effect of gestation length or severity of neonatal course on insulin resistance, nor any effect of being born small for gestational age (Hofman, Regan et al. 2004). However, children who experienced greater weight gain in postnatal life had greater insulin resistance (Regan, Cutfield et al. 2006). These findings are in keeping with the observed effects of alterations to maternal diet in animal studies, for example of protein restriction of rat dams (Hofman, Regan et al. 2006).
The relationship between preterm delivery and insulin resistance, assessed by oral glucose tolerance test, has also been investigated in 30 year olds born moderately preterm. The association between preterm birth and insulin resistance was consistent across the range of gestational ages studied and indicated that even mild degrees of prematurity are associated with alterations to the glucose/insulin axis in adulthood. As with blood pressure, birth weight was not associated with insulin resistance when adjusted for gestational age (Dalziel, Parag et al. 2007).

Other endocrine and metabolic changes have been described in children following preterm birth, including reduced insulin-like growth factor-1 and insulin-like growth factor binding protein-3 levels, suggesting partial growth hormone resistance (Cutfield, Regan et al. 2004), and elevated insulin-like growth factor-2 levels, proposed to play a role in the development of obesity (Cutfield, Hofman et al. 2007). Premature pubarche has also been associated with preterm birth (Neville and Walker 2005).

1.2.5.4.3 Effects on adiposity
A small number of studies have examined the relationship between preterm birth and body composition in infancy or young adulthood. Infants born before 32 weeks have reduced subcutaneous but increased intra-abdominal adipose stores at term compared to term-born infants, and accelerated postnatal weight gain is associated with increased total body adipose stores (Uthaya, Thomas et al. 2005). Moreover, in a study of 403 individuals born at less than 32 weeks’ gestation, rapid weight gain in the first 12 months was associated with increased body mass index, increased percentage body fat and greater abdominal fat stores at 19 years of age (Euser, Finken et al. 2005). Similar body mass index outcomes have been reported from a cohort of extremely low birth weight individuals, examined at 20 years of age (Doyle, Faber et al. 2004). This aberrant adipose deposition and increased adiposity may predispose those born preterm to a higher risk of metabolic disease in later life.

1.2.5.4.4 Effects on myocardium
Preterm birth exposes an individual to life ex utero before most organ systems have completed maturation. This may be particularly relevant to the cardiovascular system, which is not only morphologically immature in infants born preterm (Rudolph 2000), but must also undergo a number of physiological adaptations in response to the extrauterine environment (Thornburg and Shaut 2009). However, only one study has investigated the effect of preterm birth on the myocardium, reporting histological findings from sheep born 14 days preterm, equivalent to a gestational age of 32 to 36 weeks in humans (Bensley,
Stacy et al. 2010). These animals were found to have increased left and right ventricular collagen deposition and cardiomyocyte volume at 9 weeks after term equivalent age, when cardiomyocyte proliferation and maturation have ceased. Alterations in cardiomyocyte maturation were also evident in lambs born preterm, with increased number of mononucleated cells and a marked increase in ploidy. Although there was no evidence of cardiac dysfunction at 8 weeks of age, these cellular changes could lead to adverse cardiovascular function in later life, with the potential for even greater adverse cardiovascular outcomes in those born at an earlier gestation (Bensley, Stacy et al. 2010).

1.2.5.4.5 Potential mechanisms of the programming action of preterm delivery

It is not possible to conclude from the findings discussed above that preterm delivery is itself a causal factor in the development of adverse health outcomes in later life. It is possible that preterm delivery simply acts as a marker of an unfavourable fetal environment (Dalziel, Parag et al. 2007). For example, adverse intrauterine factors, such as maternal or fetal disease or poor placental function may result in preterm delivery. (Bloomfield, Oliver et al. 2003b) Thus, the same adverse intrauterine factors that precipitate preterm delivery may also act as programming stimuli in the developing fetus (Gluckman, Cutfield et al. 2005). Furthermore, despite provision of the very best neonatal care, preterm birth exposes an infant to nutritional, metabolic, hormonal, sensory and respiratory challenges at a developmental stage equivalent to the third trimester; a period of gestation thought to be critical for the developmental programming of the fetus (Gluckman, Cutfield et al. 2005; Hofman, Regan et al. 2006). These adverse extraterine environmental factors may themselves act as programming stimuli and predispose the infant to adverse health outcomes in later life (Gluckman, Cutfield et al. 2005; Dalziel, Parag et al. 2007). For example, the differences between in utero fetal and ex utero neonatal nutrition, with resultant changes to weight gain over critical developmental periods, may play a role in determining long-term insulin sensitivity (Regan, Cutfield et al. 2006). It is also possible that accelerated maturation of organ systems following preterm delivery may have implications for later health outcomes, such as the maturational changes seen in the cardiomyocytes of lambs born preterm (Bensley, Stacy et al. 2010).

Ultimately, it is likely that all these mechanisms play a role in determining long-term health outcomes following preterm birth, with preterm birth simply acting as a surrogate for various adverse in and ex utero factors which may have a causal role in determining health
in later life, similar to the observed associations in individuals of low birth weight (Gluckman, Cutfield et al. 2005; Dalziel, Parag et al. 2007).

1.2.6 Developmental Origins Hypothesis and Other Diseases

Although the majority of work on the Developmental Origins hypothesis has focused on cardiovascular disease and its associated risk factors, there has been a growing realisation in recent years that adverse conditions in utero or early life may play a role in the causation of a number of other diseases (Phillips 2006), particularly respiratory conditions such as chronic bronchitis and asthma, autoimmune disorders and osteoporosis.

1.2.6.1 Respiratory disease

Barker and colleagues initiated the investigation of the role of adverse intrauterine conditions on the development of respiratory disease in the mid 1980s, demonstrating a strong geographical relationship between death rates from chronic bronchitis and emphysema in 1959-1978 and infant mortality from bronchitis and pneumonia during 1921-1925, suggesting a potential causal link between acute lower respiratory tract infection in childhood and chronic bronchitis in adult life (Barker and Osmond 1986b). A subsequent long-term follow up study of nearly 6,000 men revealed a strong inverse relationship between birth weight and chronic obstructive pulmonary disease morbidity and mortality at age 59 to 70 years. The authors hypothesised that irreversible constraint on the growth of airways may occur as a result of adverse intrauterine conditions that retard fetal weight gain (Barker, Godfrey et al. 1991).

The role of adverse intrauterine conditions in the development of asthma has also been investigated. The Dunedin Multidisciplinary Child Development Study showed that low birth weight was associated with a lower prevalence of asthma at 13 years of age but conversely, increased birth length was associated with increased asthma prevalence (Leadbetter, Pearce et al. 1999). An association has also been found between reduced head circumference at 10 to 15 days of age and wheeze at 7 years (Carrington and Langley-Evans 2006). Both these studies led the authors to suggest that factors determining fetal growth may also influence the developing respiratory and immune systems (Leadbetter, Pearce et al. 1999; Carrington and Langley-Evans 2006). Investigations into the relationship between adverse early life events other than those represented by birth weight and the development of atopy and wheeze have also been conducted in animals and humans. For example, Hollingsworth and colleagues demonstrated that intrauterine supplementation of mice with
methyl donors increased the severity of allergic airways disease in offspring, an outcome that was inherited transgenerationally. The authors concluded that exposure to adverse dietary factors during a critical period of fetal development can increase the risk of allergic airways disease through epigenetic modification (Hollingsworth, Maruoka et al. 2008). Epidemiological studies in humans have also suggested that prenatal exposure to tobacco smoke and maternal stress may increase the risk of childhood asthma, through altered DNA methylation (Shaheen and Adcock 2009).

1.2.6.2 Immune function
Given that much of the development of the immune system occurs antenatally or in early neonatal life, there is considerable potential for adverse fetal and neonatal conditions to influence the developing immune system (Phillips 2006). Exposure of pregnant mice to specific chemical agents such as Dioxin, mercury and diethyl stilboestrol has been shown to result in sustained immunosuppression in offspring and earlier development of autoimmune disease in susceptible animals (Holladay, Blaylock et al. 1993; Holladay 1999). A similar effect may be seen in humans exposed to diethyl stilboestrol (Phillips 2006). In addition, the occurrence of autoimmune thyroiditis has been linked to birth size, with the development of thyroid autoantibodies inversely related to birth weight in women, (Phillips, Cooper et al. 1993) and spontaneous hypothyroidism in women has also been shown to be associated with lower birth weight and length (Kajantie, Phillips et al. 2006). Furthermore, high birth weight has been linked to the later development of rheumatoid arthritis (Jacobsson, Jacobsson et al. 2003), and type 1 diabetes (Dahlquist, Bennich et al. 1996), although for the latter a number of other environmental factors may be relevant, such as delivery complications and maternal age (Stene, Barriga et al. 2004). There is also growing evidence from both humans and animals to suggest that exposure to various infections in early life may alter an individual’s susceptibility to a number of autoimmune disorders including systemic lupus erythematosus and multiple sclerosis (Edwards and Cooper 2006).

1.2.6.3 Osteoporosis
Studies of osteoporosis and bone mineral density in elderly subjects have demonstrated a relationship between birth weight, weight at one year of age and adult bone mass (Cooper, Harvey et al. 2009). In one study of a cohort of elderly patients in Britain, the relationship between lumbar spine bone mineral density and vitamin D receptor genotype varied according to birth weight, suggesting that intrauterine nutrition may modify genetic influences on vitamin D response, thus influencing adult bone mass (Dennison, Arden et al. 2001). Furthermore, maternal diet, smoking and physical activity modify bone mineral
acquisition *in utero*. These effects appear to be mediated through influence on the hypothalamic-pituitary-adrenal and growth hormone/insulin-like growth factor-1 axes (Cooper, Harvey et al. 2009).

1.3 Fetal Anaemia and the Developing Heart

1.3.1 Fetal Circulation

The cardiovascular system required to support the fetus *in utero* is significantly different from that of an infant or adult. These differences are based primarily on the fact that the site of gas exchange in the fetus is the placenta, whereas in the adult gas exchange occurs in the lungs (Park 1996). In addition to the essential anatomical changes which occur in the cardiovascular system following birth, the fetal myocardium undergoes a process of maturation *in utero* over the course of normal gestation. Therefore, exposure of the immature fetal heart to atypical physiological stress, such as anaemia or hypoxia, may influence the functioning of the cardiovascular system *in utero* and the ability of the fetal circulation to adapt to life *ex utero*, as well as cardiovascular outcome in later life (McMillen, MacLaughlin et al. 2008).

The fetal circulation is dominated by the right ventricle, which receives 66% of the cardiac output and has a larger chamber volume and thicker wall than the left ventricle (Thornburg and Reller 1999; Bensley, Stacy et al. 2010). The majority of blood flow to the right ventricle is from the inferior vena cava, which drains the low resistance, high flow placental circulation. In comparison, the pulmonary circulation of the fetus has high resistance and low flow (Bensley, Stacy et al. 2010). This facilitates the shunting of blood from the right ventricle to the left atrium via the foramen ovale, ensuring delivery of well-oxygenated blood to the brain and coronary circulation (Park 1996).

After birth, removal of the low resistance placental vascular bed causes systemic arterial pressure to rise significantly, which increases the work of the left ventricle. Concomitantly, lung expansion results in a marked increase in oxygenation, decreased pulmonary vascular resistance and increased pulmonary blood flow with a consequent reduction in the work of the right ventricle. Hence, in the adult circulation, the left ventricle is dominant (Park 1996; Thornburg and Reller 1999; Bensley, Stacy et al. 2010). These changes in flow, pressure and oxygen saturation result in functional closure of the ductus arteriosus and foramen ovale, which permit right to left blood flow during intrauterine life (Park 1996).
1.3.2 Concepts of Coronary Flow Reserve and Conductance

Flow of blood through a vessel in any organ is governed by the relationship between resistance to flow and driving pressure. Resistance to flow in the heart is determined by a number of factors including tissue pressure, neural and endocrine stimulation, myocardial metabolism and the response of vascular smooth muscle. However, coronary perfusion pressure is hard to define, as the heart is a dynamic organ with inflow and outflow pressures dependent upon the phase of the cardiac cycle (Thornburg and Reller 1999). Despite this, under resting conditions, coronary blood flow is closely regulated, such that changes in coronary perfusion pressure do not cause a significant alteration to coronary flow. This consistency of coronary flow across a wide range of perfusion pressures is termed autoregulation (Hoffman 1984; Thornburg and Reller 1999). However, under conditions of maximal vasodilation, coronary blood flow increases in direct proportion with increases in perfusion pressure (Figure 1.2) (Hoffman 1984).

Figure 1.2: Relationship between left ventricular pressure and coronary blood flow at rest and with maximal vasodilation (redrawn from Hoffman et al, 1984)

Coronary perfusion pressures of 70 mmHg and 100 mmHg result in coronary flow reserves (CFR) 1 and 2 respectively, when heart rate and aortic pressures are constant.
Coronary vasodilation can be induced by a number of stimuli, including exercise, pacing, transient coronary occlusion or a pharmacological agent such as adenosine (Bookstein and Higgins 1977; Warltier, Gross et al. 1981; White, Sanders et al. 1981; Davis, Hohimer et al. 1999). If maximal coronary vasodilation is achieved, coronary flow will be maximal for a given perfusion pressure (Hoffman 1984). Thus, the difference between resting and maximal coronary flow is the coronary flow reserve (Hoffman 1984; Davis, Hohimer et al. 1999). There is no one single value for coronary flow reserve as this variable is dependent upon perfusion pressure (Figure 1.2) (Hoffman 1984). The functional significance of coronary flow reserve lies in the ability of the coronary vasculature to increase flow in response to an increased workload or physiological stressor (Vassalli and Hess 1998).

The relationship between coronary blood flow at maximal vasodilation and perfusion pressure has been termed coronary conductance (Hoffman 1984). As the maximal flow in coronary vessels at a given perfusion pressure is a function of the total cross sectional resistance vessels, coronary conductance can be thought of as an index of the vascular growth of resistance vessels (Davis, Thornburg et al. 2005). In adults, coronary conductance does not increase in response to physiological stress (Hoffman and Spaan 1990). In fact, some authors have demonstrated a fall in coronary conductance in adults under conditions of stress. For example, reduced coronary perfusion has been demonstrated in adult rats following coronary artery ligation or aortic banding due to inadequate vascular growth in relation to the degree of ventricular hypertrophy that resulted from the vascular insult (Kalkman, Bilgin et al. 1996). Similarly, aortic banding of adult sheep results in left ventricular hypertrophy with reduced subendomyocardial capillary density and consequent reduction in coronary conductance and reserve (Flanagan, Aoyagi et al. 1994). In contrast, capillary density and coronary conductance is preserved in young lambs following aortic banding, suggesting that age is a factor in the ability of the animal to compensate for ventricular hypertrophy by angiogenesis (Flanagan, Aoyagi et al. 1994). This suggests that exposure of the developing fetus to cardiovascular stress may also lead to an increase in coronary conductance (Davis, Hohimer et al. 1999).

### 1.3.3 Physiological Effects of Anaemia in Adults

Anaemia is defined as a reduction in the number of red blood cells or quantity of haemoglobin in blood (Schwartz 2000), resulting in a decrease in oxygen carrying capacity. Therefore, under anaemic conditions, the need to preserve tissue oxygenation results in a number of physiological adaptations (Varat, Adolph et al. 1972; Holzman, Tabata et al. 2000).
1986), which depend on the rapidity with which anaemia develops, the severity of haematocrit reduction and the age of the affected individual.

In both acute and chronic anaemia in adults, the most important mechanism for maintaining an adequate oxygen supply to the tissues is an increase in cardiac output. This occurs through increases in heart rate and stroke volume, with the former of greater importance in acute anaemia and the latter more relevant in chronic anaemia (Varat, Adolph et al. 1972; Fan, Chen et al. 1980). The increase in stroke volume in chronic anaemia is largely the result of a reduction in afterload due to decreased blood viscosity and reduced peripheral vascular resistance (Vatner, Higgins et al. 1972). Thus, in adults, chronic anaemia leads to peripheral vasodilation, whereas acute anaemia is associated with vasoconstriction of nonessential vascular beds, such as those supplying the intestines, skin and muscle (Vatner, Higgins et al. 1972; Fan, Chen et al. 1980; Davis and Hohimer 1991). However, in both acute and chronic anaemia, regional blood flow to the brain and heart increases to maintain adequate oxygen supply to these essential organs (Vatner, Higgins et al. 1972; Fan, Chen et al. 1980).

Chronic anaemia in adult rats results in increased heart mass and eccentric myocardial hypertrophy, with increased capillary length and diameter (Rakusan and Rajhathy 1972; Olivetti, Lagrasta et al. 1989). Chronic anaemia also results in ventricular remodelling in humans. For example, patients with chronic anaemia due to renal failure have a reduction in end diastolic volume when anaemia is corrected with erythropoietin (Goldberg, Lundin et al. 1992).

1.3.4 Physiological Effects of Anaemia in the Fetus
As in adults, fetal anaemia leads to lowered blood viscosity and decreased delivery of oxygen to tissues, inducing a variety of physiological adaptations in the fetus in order to preserve myocardial and systemic oxygen supply.

1.3.4.1 Changes to systemic haemodynamics and regional blood flow
In response to an acute reduction in fetal haematocrit, blood flow to the brain, heart and adrenal glands increases in fetal sheep, in order to maintain delivery of oxygen to these organs. However, blood flow to the gastrointestinal tract, spleen, kidneys, placenta and carcass varies very little, resulting in decreased oxygen supply to these tissues (Fumia, Edelstone et al. 1984).
By comparison, chronic anaemia, induced by daily isovolaemic haemorrhage over 5 to 7 days, results in a 50% increase in cardiac output in fetal sheep. As a result, blood flow to all regional circulations except the placenta also increases (Davis and Hohimer 1991). However, as oxygen extraction in the ovine fetal heart is 65% under normal conditions, compared to 34% in the brain (Davis, Hohimer et al. 1999), under conditions of hypoxic stress, blood flow to the coronary circulation must increase to a much greater extent than in other organs in order to maintain adequate myocardial oxygen supply (Davis, Thornburg et al. 2005). Thus, blood flow to the coronary circulation of the anaemic fetus increases six-fold in comparison to, at most, a two-fold increase in flow to other regional circulations (Davis and Hohimer 1991).

The increased cardiac output observed in chronically anaemic fetal sheep is primarily due to an increase in stroke volume (Davis and Hohimer 1991; Davis, Hohimer et al. 1996). This is in keeping with the findings from studies of chronic anaemia in adult animals (Vatner, Higgins et al. 1972). The factors postulated to account for the increase in stroke volume include reduced afterload secondary to decreased peripheral vascular resistance in all tissues except the placenta, and remodelling of the fetal heart, resulting in a larger end-diastolic volume (Davis and Hohimer 1991; Davis, Hohimer et al. 1996). The ratio of heart to body weight in anaemic fetuses is reported to increase by 30%, suggesting cardiac hypertrophy as seen in the chronically anaemic adult (Davis and Hohimer 1991).

There are few studies of cardiac output in the anaemic human fetus. However, pulsed Doppler flow analysis in 13 fetuses severely affected by Rhesus Haemolytic Disease (RhHD) showed a 35% increase in biventricular cardiac output in affected fetuses compared to controls matched for fetal weight and gestational age, which was not accounted for by an elevation in heart rate (Copel, Grannum et al. 1989). Several other authors subsequently demonstrated a fall in cardiac output following intrauterine transfusion (IUT) in fetuses affected by RhHD (Moise, Mari et al. 1990; Rizzo, Nicolaides et al. 1990).

### 1.3.4.2 Coronary vascular changes

The effect of anaemia on coronary conductance has been assessed using chronically instrumented near-term fetal sheep with occluders placed in the inferior vena cava and aorta to achieve a range of perfusion pressures, and adenosine infusion to achieve maximal vasodilatation (Davis, Hohimer et al. 1999). Measurements before and after induction of anaemia to a fetal haematocrit of 16% over 7 days demonstrated that coronary blood flow
was significantly elevated in the anaemic state, both at rest and at maximal vasodilation (Figure 1.3). The authors postulated that the increased coronary blood flow permitted conservation of myocardial oxygen consumption and hence cardiac function (Davis, Hohimer et al. 1999). However, coronary flow reserve was unchanged. These findings suggest increased growth of coronary resistance vessels in the fetus in response to chronic anaemia and thus plasticity of the coronary circulation during fetal life (Davis, Hohimer et al. 1999; Davis, Thornburg et al. 2005).

Figure 1.3: Pressure-flow measurements in a single fetus at rest and at maximal vasodilation before and after anaemia (redrawn from Davis et al, 1999)

Solid and dashed lines show coronary blood flow at varying perfusion pressures in nonanaemic and anaemic states respectively. At maximal vasodilation in the nonanaemic state, the fetus demonstrated loss of autoregulation. In the anaemic state, resting coronary blood flow was almost identical to that of the nonanaemic state at maximal vasodilation. Coronary conductance is represented by the slope of the relationship between perfusion pressure and flow at maximal vasodilation. In the anaemic state, coronary conductance was nearly twice that of the fetus in the nonanaemic state (Davis, Hohimer et al. 1999).

To investigate the contribution of decreased viscosity to these results, 5 of the anaemic fetuses were transfused in utero. Following transfusion, coronary blood flow at maximal vasodilation was less than in the anaemic state but did not return to the values documented in the nonanaemic state. The authors calculated that the reduction in blood viscosity contributed 57.3% +/- 18.9% of the total increase in conductance found in the anaemic state, indicating that approximately 40% was due to vascular remodelling through angiogenesis (Davis, Hohimer et al. 1999). These findings are in keeping with investigations in adult
dogs of the effect of changes in blood viscosity on coronary blood flow (Baer, Vlahakes et al. 1987; Drossner and Aversano 1990).

While these experiments clearly demonstrate an increase in fetal coronary blood flow and conductance in response to anaemia, it is not possible to determine whether the primary stimulus for angiogenesis is hypoxia, resulting from anaemia, or increased coronary flow. To investigate this question, the same research group assessed maximal coronary blood flow at varying perfusion pressures in near-term fetal sheep before and after periodic chronic infusions of adenosine, thereby inducing increased coronary flow without hypoxia (Wothe, Hohimer et al. 2002). Following chronic adenosine infusion, coronary blood flow and conductance at maximal vasodilation was significantly greater in fetuses exposed to chronic adenosine infusion. Hypoxia did not account for this result, as oxygen content was similar in experimental and control fetuses. Furthermore, the induction of hypoxia in 4 of the 6 fetuses exposed to chronic adenosine infusion did not significantly alter coronary conductance. Therefore, the authors concluded that coronary flow, resulting in shear wall stress, is likely to be the key factor regulating vascular development in the coronary circulation. They speculated that under normal environmental conditions, fetal growth leads to increased cardiac work and consequently increased coronary flow, thereby acting as a stimulus for angiogenesis in the developing fetus. As fetal anaemia leads to further increases in coronary flow, this is likely to be the stimulus for increased growth of vascular resistance vessels in the coronary circulation of anaemic fetuses (Wothe, Hohimer et al. 2002).

In keeping with these findings, increased myocardial vascularisation has been demonstrated in the hearts of anaemic fetal sheep, with increased minimal capillary diameter, reduced intercapillary distance and greater capillary volume density in comparison to controls (Martin, Yu et al. 1998). Although the hearts of anaemic fetuses were hypertrophied, the overall vascularity of the hypertrophied hearts was equal to or greater than the vascularity of control hearts. Similar findings have been documented in adult rats in response to chronic anaemia (Olivetti, Lagrasta et al. 1989). In addition, cardiac expression of vascular endothelial growth factor, a potent endothelial cell mitogen, and hypoxia-inducible factor-1, a transcription factor for genes producing proteins involved in oxygen homeostasis, were also significantly increased in anaemic fetal sheep, suggesting that these factors may, at least in part, modulate increased myocardial vascularisation in the anaemic fetal heart (Martin, Yu et al. 1998). Increased expression of several myocardial angiogenic and metabolic genes regulated by hypoxia-inducible factor-1 has since been demonstrated in
chronically anaemic fetal sheep, causing these authors to conclude that hypoxia-inducible factor-1 plays an important role in coordinating the metabolic and myocardial response to chronic anaemia in the fetus (Mascio, Olison et al. 2005).

There are few studies investigating the vascular effects of anaemia on the fetal coronary circulation in humans. However, in keeping with findings from sheep studies, significantly increased diastolic coronary flow has been demonstrated using pulsed-wave Doppler in severely anaemic human fetuses (Baschat, Muench et al. 2003). Furthermore, in vitro studies have demonstrated that expression of vascular endothelial growth factor by human cardiomyocytes can be induced by hypoxia (Shifren, Doldi et al. 1994; Liu, Cox et al. 1995).

1.3.4.3 Myocardial changes
Throughout most of gestation, heart growth occurs by cardiomyocyte proliferation. However, in the last third of gestation, a transition occurs from proliferation to enlargement of cardiomyocytes. This is accompanied by an increase in terminal differentiation, evidenced by an increasing number of binucleated cells (Jonker, Faber et al. 2007). Thus, maximal cardiomyocyte number and cardiomyocyte maturity is established in the perinatal period, which likely results in a limited capacity for cellular regeneration after cardiac injury in adulthood (Jonker, Giraud et al. 2010). Therefore, any intrauterine environmental stimulus that alters the timing or nature of cardiomyocyte maturation may have long-term consequences for cardiac function in later life (McMillen, MacLaughlin et al. 2008).

Myocardial changes in response to fetal anaemia have been investigated most recently in sheep. A 50% reduction in fetal haematocrit from gestational day 129 to 138 (term gestation 145 days) results in a 39% increase in heart weight, due to increased cardiomyocyte proliferation, enlargement and terminal differentiation, rather than cardiac oedema or expansion of non-myocyte connective tissue (Jonker, Giraud et al. 2010). However, the cardiomyocytes of fetal sheep exposed to a similar degree of anaemia earlier in gestation (from gestational day 109 to 119) showed no evidence of increased terminal differentiation or enlargement. Instead, the 45% increase in heart weight in these fetuses was due to increased cardiomyocyte proliferation, which decreased considerably following intrauterine transfusion. Thus, IUT results in a slowing but not cessation of increased cardiac growth after anaemia (Jonker, Scholz et al. 2011). These findings suggest that the timing of exposure to anaemia and intrauterine transfusion determine the myocardial changes seen in the anaemic fetus.
Myocardial hypertrophy has also been demonstrated in anaemic human fetuses suffering RhHD, with symmetrical hypertrophy involving the right ventricular free wall, interventricular septum and left ventricular posterior wall (Oberhoffer, Grab et al. 1999). Following IUT, decreased left and right ventricular output and myocardial contractility have also been documented in human fetuses (Moise, Mari et al. 1990; Sikkel, Klumper et al. 2005).

1.3.4.4 The role of hypoxia versus anaemia in fetal heart development

One of the main physiological consequences of anaemia is a decrease in the oxygen carrying capacity of blood, resulting in impairment of tissue oxygenation and hypoxia. Although anaemia also leads to a number of other physiological effects including a reduction in blood viscosity and alterations to shear wall stresses in the vascular system due to compensatory increases in cardiac output, many of the physiological adaptations to anaemia occur in order to mitigate the effects of hypoxia.

Fetal arterial oxygen content is much lower than that found in adults. Therefore, development of the fetus occurs in a state of relative hypoxia, and this physiologically “normal” hypoxia may be necessary to ensure normal cardiac development (Patterson and Zhang 2010). For example, an environment of relative hypoxia is required for the expression of vascular endothelial growth factor and hypoxia-inducible factor-1, necessary for normal vasculogenesis, angiogenesis and remodelling of the fetal heart (Sugishita, Leifer et al. 2004). However, if the fetus is exposed to a pathologically hypoxic intrauterine environment, adverse cardiovascular development occurs, including changes in the structure, function and gene expression of the fetal heart that may persist in later life (Patterson and Zhang 2010).

A number of factors can contribute to the development of a sustained pathological hypoxic fetal environment. For example, maternal pre-eclampsia, smoking, aberrant placental development and residence at high altitude may induce intrauterine hypoxia. In addition, conditions that induce fetal anaemia, such as haemoglobinopathies, RhHD or other nonimmune conditions which affect red blood cell formation or longevity, induce fetal hypoxia through impairment in oxygen carrying capacity. In animal studies, a number of different techniques have been employed to simulate conditions of fetal hypoxia, including high altitude, induction of placental insufficiency through umbilico-placental embolisation, restriction of uterine blood flow and reduction in maternal inspired oxygen content.
Bocking, Gagnon et al. 1988; Kamitomo, Longo et al. 1994; Steyn and Hanson 1998; Louey, Jonker et al. 2007). These studies have conclusively demonstrated that sustained intrauterine hypoxia results in abnormal fetal heart development, with cardiomyocyte enlargement, increased apoptosis and reduced proliferation in fetal rodents and sheep exposed to intrauterine hypoxia (Bae, Xiao et al. 2003; Louey, Jonker et al. 2007; Morrison, Botting et al. 2007; Ream, Ray et al. 2008). Decreased terminal differentiation has also been reported in response to hypoxia in fetal sheep, suggesting that the hypoxic fetus may have a less mature myocardium (Louey, Jonker et al. 2007).

Impairment of cardiac function has also been demonstrated in animals exposed to intrauterine hypoxia. For example, impaired cardiac contractility, left ventricular dilation, reduced left ventricular ejection fraction and failure to maintain blood pressure under conditions of acute stress have been found in chick embryos following exposure to chronic hypoxia (Rouwet, Tintu et al. 2002; Tintu, Noble et al. 2007; Tintu, Rouwet et al. 2009). Impaired myocardial contractility has also been demonstrated in fetuses of high altitude sheep (Browne, Stiffel et al. 1997), as has decreased right and left ventricular outputs in fetal sheep exposed to sustained hypoxic conditions for two weeks late in gestation (Kamitomo, Longo et al. 1994). However, short episodes of acute hypoxia on a daily basis over this same time period did not result in any adverse cardiovascular outcomes, indicating that the fetus may be relatively tolerant of brief bouts of hypoxia, as may occur normally in late gestation, for example, due to uterine artery compression (Steyn and Hanson 1998; Patterson and Zhang 2010).

Circulatory responses to fetal hypoxia have also been demonstrated. The distribution of cardiac output is altered in response to intrauterine hypoxia in order to preserve blood flow to the brain, heart and adrenal gland (Bocking, Gagnon et al. 1988). In addition, hypertrophy of the ascending aorta and increased baseline tone due to sympathetic hyperinnervation in resistance vessels has been demonstrated in chronically hypoxic chick embryos (Rouwet, Tintu et al. 2002). Furthermore, chronic hypoxia has been shown to induce remodelling of the coronary vasculature in fetal sheep similar to that seen in response to fetal anaemia. For example, the resting coronary flow of fetal sheep exposed to chronic hypoxia is equal to the coronary flow at maximal vasodilation in normoxic fetuses, and coronary blood flow at maximal vasodilation also increases considerably in these animals (Reller, Morton et al. 1992). However, as indicated above, these changes to
coronary vasculature are due primarily to alterations to coronary flow, rather than hypoxia (Wothe, Hohimer et al. 2002).

Taken together, these data show that exposure of the fetus to an hypoxic environment can significantly alter fetal cardiac and vascular development, with many of the cardiovascular consequences similar to those seen as a result of fetal anaemia.

1.3.5 Cardiovascular Outcome Following Intrauterine Anaemia

Davis and colleagues, who performed the original work on coronary blood flow and conductance in anaemic fetal sheep, investigated whether the changes they had observed in the fetal period persisted into adulthood. Using twin sheep pregnancies, with one twin made anaemic in utero with subsequent transfusion prior to delivery and the other twin acting as a control, they demonstrated that maximal coronary conductance in sheep who were anaemic in utero was nearly twice that of non-anaemic controls, even if anaemia was corrected prior to delivery (Davis, Roullet et al. 2003). Furthermore, coronary reserve in adult animals was also nearly twice that of non-anaemic controls. These data indicate that exposure to anaemia in fetal life induced increased growth of resistance vessels and that these changes persisted into adulthood. The authors postulated that this “coronary supertree” might be better able to compensate for hypoxic stress in adulthood and thus confer a functional advantage. However, as resting blood flow was unchanged in the previously anaemic adult animal, they also suggested that the coronary circulation of these animals was relatively vasoconstricted. Therefore, they speculated that the increased coronary flow to which previously anaemic adults are exposed to under conditions of stress may have long-term negative effects on coronary endothelial function or vascular reactivity (Davis, Roullet et al. 2003).

Using a similar experimental approach, the same group showed that sheep exposed to fetal anaemia had improved indices of left ventricular function in response to acute hypoxia (Broberg, Giraud et al. 2003). Specifically, end-systolic elastance and the maximum derivative of left ventricular pressure with respect to time were increased, suggesting that previously anaemic animals had a functional cardiac advantage as adults under conditions of hypoxic stress. However, as this experiment was conducted in early adulthood, it is possible that this functional advantage may not be seen in older animals (Broberg, Giraud et al. 2003).
The authors also expected, but did not find, an increase in arteriolar density in experimental animals to account for the differences in cardiac function. They speculated that the vascular changes accounting for the change in coronary conductance may have occurred at the level of the capillaries rather than the arterioles. Alternatively, the arteriolar endothelium of previously anaemic adults may have been programmed to respond differently under conditions of hypoxic stress, with increased vasodilation secondary to increased sympathetic innervation or other subcellular mechanisms (Broberg, Giraud et al. 2003).

Endothelial dysfunction in adulthood has also been suggested to occur following intrauterine exposure to hypoxia, with impaired endothelium dependent relaxation and nitric oxide mediated endothelial function found in adult rats (Williams, Hemmings et al. 2005). Furthermore, adult rats demonstrated an increased area of myocardial infarction and decreased post-ischaemic recovery following exposure to intrauterine hypoxia (Li, Xiao et al. 2003). Previously hypoxic animals had decreased ventricular levels of heat shock protein 70 and endothelial nitric oxide synthase, which both play a protective role against ischaemia-reperfusion injury in the myocardium. They also had elevated caspase 3 activity, a protease that facilitates programmed cell death (Li, Xiao et al. 2003). The authors concluded that chronic exposure to intrauterine hypoxia increased the susceptibility of the adult heart to ischaemia-reperfusion injury through increased apoptosis and programmed alteration of expression of critical endothelial proteins (Li, Xiao et al. 2003). A subsequent study replicated these findings and also demonstrated left ventricular hypertrophy and impaired cardiac remodelling in adult rats that suffered intrauterine growth restriction through exposure to hypoxia and/or nutrient restriction (Xu, Williams et al. 2006).

Ischaemia-reperfusion injury has also been studied in sheep exposed to intrauterine anaemia, with previously anaemic animals shown to have an increase in infarct size when expressed as a percentage of the area of left ventricle at risk of infarction (Yang, Hohimer et al. 2008). These findings suggest that exposure to intrauterine anaemia may decrease tolerance to myocardial ischaemia in adulthood and are in keeping with findings in adult rats exposed to prenatal hypoxia. Furthermore, in previously anaemic sheep there were no alterations in the responsiveness of coronary vessels to mediators of vasoconstriction or relaxation, suggesting that increased coronary conductance and coronary reserve cannot be explained by changes in vasomotor responsiveness to these agents (Yang, Hohimer et al. 2008).
No studies of cardiovascular outcome in adult humans following intrauterine anaemia have been reported to date. However, a small case-control study reported cardiovascular outcome in 25 children aged 3 to 16 years who received IUT for the treatment of fetal anaemia due to red cell isoimmunisation, and 25 controls (Dickinson, Sharpe et al. 2010). Cases tended to have lower systolic blood pressure than controls (105 versus 108 mmHg, p=0.06). Cases also had 10% less ventricular mass and 9% less atrial area than controls on echocardiography, and mitral valve atrial duration was on average 11 ms less in cases. Adjustment for severity of haemolytic disease did not alter these findings. However, overall ventricular function was not different between cases and controls at rest and assessment was not performed under conditions of stress. The authors speculated that the reduction in left ventricular mass may reflect accelerated cardiomyocyte proliferation and terminal differentiation in children exposed to intrauterine anaemia. They also suggested that the reduction in left atrial size was correlated with the reduced overall size of the left heart. The significance of the finding of shortened mitral valve atrial duration was not known as this measurement is not usually interpreted as an isolated finding, but rather used as part of the assessment of left ventricular diastolic function. However, the authors postulated that the finding of reduced myocardial mass may imply fewer coronary capillaries and thus predispose children exposed to anaemia prior to birth to an increased risk of cardiovascular disease in later life (Dickinson, Sharpe et al. 2010).

### 1.4 Rhesus Haemolytic Disease of the Fetus and Newborn

#### 1.4.1 Historical Background

##### 1.4.1.1 ABO and rhesus incompatibility

The foundation upon which our knowledge of Rhesus Haemolytic Disease of the Fetus and Newborn (RhHD) is based was laid at the turn of last century by Karl Landsteiner, with his discovery of the ABO blood group system (Landsteiner 1901). Landsteiner hypothesised that the occurrence of shock and haemolysis in some patients following blood transfusion was due to the presence of polymorphisms on red cells. To test his hypothesis, he mixed red cells from one patient with sera from another, noting that in some instances there was no adverse reaction, while in others agglutination of red cells occurred (Zetterstrom 2007). Landsteiner’s realisation that this red cell agglutination was due to the presence of an individual serological factor led to the discovery of three different human blood groups, which he named A, B and O. A fourth blood group, AB, was identified a year later (Decastello and Sturli 1902). Landsteiner established that these serological factors, which he termed isoagglutinins but which we now know as antibodies, were present in groups A, B.
and O. He surmised that isoagglutinins interacted with carbohydrate structures on the surface of red cells, which he termed isoagglutinogens, now referred to as antigens. He also determined that such serological factors and carbohydrate surface structures were absent in blood group O (Zetterstrom 2007).

The significance of Landsteiner’s discoveries with regard to blood transfusion was slow to dawn on scientific and medical communities. Once the inheritance of blood groups had been shown to follow a simple Mendelian pattern, with A, B and AB inherited dominantly and O recessively (von Dungern and Hirschfeld 1910), the initial application of this knowledge was to paternity testing and forensic identification of human blood. In fact, when Landsteiner was awarded the Nobel prize in 1930 for his work on blood typing, the chairman of the Nobel committee commended him for his contribution to forensic medicine, with no mention of its application to blood transfusion. However in his Nobel Prize lecture, Landsteiner’s focus was on the importance of blood group testing prior to blood transfusion, a practice which had become widely adopted during the First World War (Zetterstrom 2007).

Landsteiner and a number of other researchers went on to identify several more human blood groups, although the reactions precipitated by incompatibility of these groups were less significant than those induced by ABO group incompatibility (Zetterstrom 2007).

The next major advance in the understanding of blood groups came in 1939, with the publication of a case report by Philip Levine, describing “an unusual case of intra-group agglutination” (Levine and Stetson 1939.) Levine described a young woman in her second pregnancy who delivered a stillborn macerated fetus at 33 weeks’ gestation and subsequently suffered a significant post partum haemorrhage. She was transfused with blood from her husband who, like the patient, was blood group O. However, soon after transfusion, she developed chills, leg pains and headache consistent with a transfusion reaction. Subsequent investigation revealed that the patient’s serum agglutinated not only her husband’s blood, but also nearly 80% of the group O specimens held by Levine in his laboratory. Levine hypothesised that “products from the disintegrating fetus were responsible… for this iso-immunisation” and that “the immunising property from the blood and/or tissues of the fetus must have been inherited from the father” (Levine and Stetson 1939).
The following year, Landsteiner and his co-worker Wiener identified an agglutinable factor in human blood which was recognised by the sera of rhesus monkeys and independent of other known blood types (Landsteiner and Wiener 1940). They termed this new blood group system “rhesus”. Further work led them to realise that when patients who were rhesus negative were exposed to rhesus positive blood, they could develop antibodies to the rhesus antigen. By studying previous case reports in the literature and from their own clinical experience, they established that this exposure could occur not only by direct transfusion of rhesus positive blood but also perinatally, when a rhesus negative woman was pregnant with a rhesus positive fetus (Landsteiner and Wiener 1941; Zetterstrom 2007). In this way, Levine was quick to recognise the rhesus blood group system as the “immunising property” in his previously described patient (Levine, Katzin et al. 1941). Landsteiner and Wiener correctly estimated that 85% of the white American population would be rhesus positive (Landsteiner and Wiener 1940), and they confirmed that inheritance of the Rh blood group system followed a simple Mendelian pattern (Landsteiner and Wiener 1941).

In 1961, Levine discovered that animal and human rhesus antibodies were not identical. Rather, animal serum detected a “D-like” antigen in human rhesus positive blood (Levine, Celano et al. 1961). However as the name “rhesus” was well established in the literature by this time, the original terminology remained in place, with the addition of the term “anti-D” to refer to the rhesus antibody in humans.

With the advent of molecular DNA sequencing technology, much has been discovered about the molecular structure, complexity and diversity of the rhesus system in humans (Avent and Reid 2000), and genetic testing for rhesus status has become possible. Yet despite the vast amount of knowledge about the molecular structure of the rhesus complex, its function remains poorly elucidated. It is thought to be critical to the structure of the red cell membrane and may play a part in the sequestration of ammonia from blood (Westhoff 2007).

1.4.1.2 Rhesus haemolytic disease of the fetus and newborn
There is historical evidence to suggest that the clinical entity of RhHD has been known for many centuries. It has been suggested that the offspring of Henry VIII and Katherine of Aragon were affected by RhHD, with two boys and three girls dying in utero or infancy and only one child, Mary Tudor, surviving (Bowman 1998). If this was indeed the underlying pathology, it could be inferred that RhHD was responsible for significant political and
religious upheaval in the 16th century, as Henry sought annulment of his marriage to Katherine due to her inability to produce a son. When the Pope was reluctant to agree to an annulment, Henry requested permission from the Archbishop of Canterbury and so began the split from Rome and the English Reformation.

A more definitive description of RhHD was made in 1609 following the delivery of twins by a French midwife. The first twin was hydropic and stillborn and the second deeply jaundiced at birth, subsequently dying from what we now refer to as kernicterus (Bowman 1998). However it was not until 1932 that the previously unrelated syndromes of severe neonatal jaundice (“icterus gravis neonatorum”), generalised oedema (“universal oedema of the fetus”) and severe anaemia (“congenital anaemia of the newborn”) were linked by Louis Diamond (Diamond, Blackfan et al. 1932). Diamond and his colleagues drew on their own clinical experience and the literature of the day to conclude that these syndromes were likely to be manifestations of the same disease process (Naiman 2001). Diamond collectively named these syndromes “erythroblastosis fetalis”, a term originally used in 1912 by Rautmann to describe the blood appearance in cases of universal oedema (Rautmann 1912). “Haemolytic disease of the newborn” came into use in subsequent years, with the nomenclature extended to include the fetus once intrauterine transfusion became accepted practice (Naiman 2001).

Diamond’s work was ground breaking, especially considering that it would be another nine years before the rhesus blood group system was described by Levine and Landsteiner. However, with the discovery of the rhesus system came the identification of the cause of haemolysis in erythroblastosis fetalis and the understanding that the disease occurred in rhesus positive offspring of rhesus negative mothers (Levine, Katzin et al. 1941; Bowman 1998). From this, the development of therapeutic strategies to ameliorate effects of RhHD began. In 1944, Wiener was the first to describe the technique of exchange transfusion for neonates suffering from severe RhHD, the aim being to replace the infant’s circulating rhesus positive blood with blood from a rhesus negative donor, thereby preventing haemolysis and the resulting hyperbilirubinaemia and kernicterus (Wiener 1944). However, his technique involving cannulation of the radial artery and saphenous vein was cumbersome and bloody, required heparinisation of the infant and most importantly, did not allow for multiple transfusions (Stockman 2001). Wallerstein refined the technique in 1946 with a method involving removal of blood via the longitudinal sinus of the anterior fontanelle and replacement of blood into a wrist vein (Wallerstein 1946). However, when
Diamond pioneered the much easier technique of umbilical venous catheterisation in the same year, exchange transfusion rapidly became standard treatment for neonates suffering from RhHD (Diamond 1948), leading to a reduction in perinatal mortality of more than fifty percent within only a few years (Lowenstein and Sabin 1957).

While exchange transfusion was useful for neonates who could be delivered at or close to term, the problem of the hydropic fetus who was too immature to deliver remained. Early induction of labour or delivery by Caesarean section after 34 weeks was possible, thereby offsetting the risks of worsening hydrops fetalis if the fetus remained in utero, with the risks of premature delivery. However, at least half the cases of RhHD occurred before 34 weeks’ gestation. In addition, the diagnostic tools available for assessment of the fetus and quantification of the severity of RhHD were rudimentary. Attempts to predict the severity of fetal disease based on the titre of anti-D in maternal blood proved to be unreliable (Mackay 1961). Therefore, in the 1950s, further work focused on the development of methods to allow not only antenatal diagnosis of RhHD but also an estimation of disease severity. The first steps in this regard came from Bevis and colleagues, who developed a technique for measuring bilirubin and oxyhaemaglobin concentration in amniotic fluid using spectrophotometry (Bevis 1956). Having noted that many infants of rhesus negative mothers were either not affected at all, or only mildly affected by RhHD, their main objective was to find a technique to predict which infants were at risk of developing kernicterus and therefore would require exchange transfusion following delivery. Several others adopted this method and the technique of amniocentesis was developed over this time (Walker 1957; Mackay 1961).

Working at National Women’s Hospital in Auckland in the early sixties, William Liley developed this principle further. Liley obtained amniotic fluid by amniocentesis from all rhesus negative mothers for whom the indirect Coombs titre exceeded 1:8. By collecting this data, he was able to develop a chart of changing amniotic fluid bilirubin levels, measured by assessment of the optical density of amniotic fluid, from 27 to 40 weeks (Figure 1.4) (Liley 1961). This chart could then be used to predict with some precision those fetuses requiring delivery compared with those who could be safely left in utero (Stockman 2001). Using this method, with a policy of selective induction based on amniocentesis findings, Liley was able to reduce the perinatal mortality due to RhHD at National Women’s Hospital from 22% in 1957 to 1958 to 9% in 1962 (Liley 1963).
Despite these advances, there was still no solution for babies who were too immature to deliver and transfuse (Green 1985). Liley realised that the answer lay in the ability to transfuse these infants \textit{in utero} and in 1963 he pioneered the technique of intrauterine transfusion (IUT) at National Women’s Hospital. By this time he had significant experience with amniocentesis. However, whilst performing an amniocentesis with his colleague Mont Liggins, Liley inadvertently entered the fetal abdomen, withdrawing ascitic fluid. He subsequently wondered whether deliberate placement of a needle into the fetal abdomen could be used as a route for red cell transfusion. Liggins and Liley were about to commence animal experiments to test this hypothesis, when a chance visit from a British geneticist, who had been working with patients with sickle cell disease in Nigeria, provided the information that intraperitoneal transfusion was used routinely and with success in sickle cell patients. Liley subsequently reported that “this was good enough evidence for us” (Liley 1971) and in mid-1963, the first intraperitoneal IUT was performed, using type O rhesus negative blood. Unfortunately, the first 3 fetuses were severely hydropic at the time of IUT and died soon afterwards. However, the fourth patient survived IUT at 32+1 and 33+4 weeks’ gestation, and was delivered at a gestational age of 34+3 weeks. The infant required exchange transfusion following delivery and had mild respiratory distress but his neonatal course was otherwise unremarkable (Liley 1963).
Liley’s technique of intraperitoneal IUT remained the standard of care worldwide for nearly two decades. The replacement of X-ray imaging with ultrasound guidance for placement of the transfusion needle in the intraperitoneal cavity was the only significant modification of the technique over this time (Schumacher and Moise 1996). In 1981, Rodeck and colleagues performed the first intravascular IUT, using the chorionic plate for vascular access (Rodeck, Kemp et al. 1981) and a year later Bang and colleagues performed intravascular IUT via the umbilical vein under ultrasound guidance (Bang, Bock et al. 1982). Since then, several other techniques for intravascular IUT have been described, involving both simple direct intravascular transfusion and intravascular exchange transfusion, with or without intraperitoneal transfusion (Schumacher and Moise 1996).

1.4.1.3 Development of rhesus prophylaxis
Concomitant with an increased understanding of the immunological basis of RhHD, came research into the possibility of disease prevention. By 1956, Levine had deduced that rhesus antibodies were more likely to develop in mothers who lacked A and B antibodies than those in whom these were present (Wegmann and Gluck 1996). This was the first recognition of the phenomenon of antibody mediated immune suppression in which passive acquisition of antibody prevents active sensitisation by the corresponding antigen (Urbañiak and Greiss 2000). Stern and colleagues investigated this concept further by repeatedly injecting rhesus negative male volunteers with rhesus positive blood. They showed that rhesus sensitisation occurred in 58% of those injected with ABO compatible rhesus positive blood, compared to only 9% injected with ABO incompatible rhesus positive blood (Stern, Davidson et al. 1956). In 1961 Stern went on to investigate whether anti-D serum could exert a protective effect against rhesus sensitisation by injecting rhesus negative males with rhesus positive red cells coated with anti-Rh serum. None of the 16 subjects involved became rhesus sensitised (Stern, Goodman et al. 1961). In 1960, Finn postulated that it might be possible to destroy fetal red blood cells in the maternal circulation by administration of an antibody. He noted that “if successful, this would prevent the development of erythroblastosis, so mimicking the natural protection afforded by A,B,O incompatibility” (Wegmann and Gluck 1996).

Finn’s theory was initially tested and proved correct in experiments conducted on male inmates in Sing Sing prison (Freda, Gorman et al. 1964). Interestingly, the recommendation that anti-D immunoglobulin prophylaxis be given no more than 72 hours after delivery is a direct result of the security measures at Sing Sing, which mandated that the research team
could only be readmitted to the prison at 72-hour intervals. No subsequent studies have tested the utility of anti-D administration at a time interval greater than this (Stockman 2001). The use of anti-D for rhesus prophylaxis in pregnancy was first reported by Clarke and Sheppard in 1965. They reported that of the 27 rhesus negative mothers treated with anti-D immunoglobulin following delivery, none showed signs of rhesus sensitisation at 3 or 6 months post partum, compared with 29 rhesus negative mothers who were not treated with anti-D following delivery, 9 of whom were subsequently found to be rhesus sensitised (Clarke and Sheppard 1965).

A torrent of research into the use of anti-D immunoprophylaxis followed. This culminated in the introduction of routine post partum anti-D administration in the United States in 1968, New Zealand in 1969 and the United Kingdom in the early 1970s and resulted in a significant reduction in the incidence of RhHD (Chavez, Mulinare et al. 1991; Hussey and Clarke 1991; Pattison, Roberts et al. 1992). However immunoprophylaxis failures were soon noted. Thus it was recognised that rhesus sensitisation can occur prior to delivery and trials of antepartum administration of anti-D immunoprophylaxis demonstrated the utility of this approach (Urbaniak and Greiss 2000; Stockman 2001). Use of antepartum anti-D has now become standard practice in most countries (Royal Australian and New Zealand College of Obstetricians and Gynaecologists 2011; Faed 2013). However, it is not yet universally applied, with factors such as the incidence of rhesus negativity in a population and the availability and cost of anti-D critical to uptake rates (Urbaniak and Greiss 2000; Payam Khaja Pasha and Shokri 2008).

Given the declining incidence of pregnancy related rhesus sensitisation of rhesus negative women, the pool of potential anti-D donors is also declining. Thus, in recent years, work has focused on the production of monoclonal and recombinant anti-D (Kumpel 2007). Although in vitro testing of monoclonal and recombinant anti-D produced promising results, the results of in vivo tests have been disappointing, with none demonstrating clinical efficacy equivalent to plasma-derived polyclonal anti-D, and some even inducing an augmented antibody response to the rhesus antigen (Brinc, Denomme et al. 2009). Abnormal glycosylation, or the presence of extra oligosaccharide chains preventing binding to the Fc receptor may be responsible for the failure of monoclonal and recombinant anti-D to adequately prevent rhesus isoimmunisation (Kumpel 2007).
1.4.2 Epidemiology

The incidence of RhHD reflects the frequency of rhesus negativity in the community. Rhesus negativity is uncommon in Asian and African populations (Urbaniak and Greiss 2000; Urbaniak 2006). However in populations of European origin, rhesus negativity has a frequency of approximately 17%, with the combination of female rhesus negativity and male rhesus positivity occurring for an estimated 1 in 7 couples (McConnell 1966).

Prior to the introduction of anti-D immunoprophylaxis, clinically significant RhHD was estimated to occur at a frequency of 5 per 1,000 births (McConnell 1966). In the 1940s and 50s, before the development of exchange transfusions and IUT, 50% of infants affected by RhHD died in the perinatal period and 10% of all neonatal deaths were due to RhHD (Bowman 1998). It was estimated that in England and Wales in the 1950s, two to three mothers lost an infant every day due to RhHD (Tovey 1992). By the 1960s, with the development of antenatal diagnostic techniques and IUT, together with improvements in ante- and postnatal care, the perinatal death rate had reduced to approximately 1.2 per 1,000 births (Stockman 2001). However the introduction of ante- and postpartum anti-D immunoprophylaxis led to a dramatic decrease in the burden of rhesus related haemolytic disease. In the 1990s, large population based studies revealed a significant reduction in the frequency of rhesus sensitisation in pregnant woman, from 43.3 per 1,000 pregnancies in 1967 to 2.6 per 1,000 pregnancies in 1996 in the United States (Geifman-Holtzman, Wojtowycz et al. 1997).

Concomitant with this came a reduction in clinical disease, with an estimated 10.6 births per 10,000 affected by RhHD in the USA by 1986 (Chavez, Mulinare et al. 1991; Schumacher and Moise 1996), and a perinatal death rate of between 0.7 and 1.5 per 100,000 births in the late 1980s (Hussey and Clarke 1991). Modifications to the technique of IUT have also improved survival rates. In New Zealand, the reported survival rate between 1986 and 1990 following the introduction of intravascular IUT was 90%, compared with 60% survival for fetuses receiving intraperitoneal IUT prior to 1986 (Pattison, Roberts et al. 1992). This difference in survival was found to be due to the reduction in procedure-related stillbirths following intravascular transfusion. A survival rate of 90% or more following IUT is in keeping with survival data reported from other centres (Harman, Manning et al. 1983; Peterec 1995; Stockman 2001; Moise 2008a).
Despite ongoing routine and widespread use of anti-D immunoprophylaxis, the incidence of RhHD has remained relatively constant over the last two decades, albeit with associated morbidity rather than mortality (Hughes, Craig et al. 1994). Rhesus sensitisation in pregnancy is now estimated to occur at a frequency of 6 cases per 1,000 live births (Moise 2008a).

1.4.3 Genetics

The rhesus group is one of 26 known blood group systems in humans and has the most complex molecular structure of all blood groups (Urbaniak 2006). The rhesus antigen complex is embedded in the red cell membrane and is only expressed on red blood cells. Three major rhesus antigens, C/c, D and E/e determine rhesus status, and are located on the short arm of chromosome 1 (Cherif-Zahar, Mattei et al. 1991). The rhesus complex also contains the rhesus-associated glucoprotein and rhesus accessory proteins (Avent and Reid 2000). In most rhesus negative Caucasians the gene encoding the D antigen is deleted.

Rhesus status is inherited according to the rules of simple Mendelian genetics, with rhesus positivity the dominant state. Therefore, a rhesus positive individual may be homozygous or heterozygous, while a rhesus negative individual must be homozygous for rhesus negativity, having inherited a rhesus complex which does not express the D antigen from each parent (Ramasethu and Luban 2006).

Although inheritance of rhesus genotype is straightforward, expression of the rhesus phenotype is more complex in some individuals. Rhesus variant phenotypes, in which rhesus status may be partial or weakly expressed, are thought to arise through a variety of genetic mechanisms including point mutations, nonsense mutations, gene rearrangements and gene deletions (Avent and Reid 2000). These individuals have a quantitative or qualitative variation in their expression of the D antigen and consequently, variation in their propensity to make anti-D if exposed to rhesus positive red cells. For practical purposes in perinatal medicine, pregnant women with a partial rhesus status are classed as rhesus negative and thus receive the usual treatment and prophylactic management of this state (Hughes, Craig et al. 1994).
1.4.4 Molecular Basis of Rhesus Haemolytic Disease

1.4.4.1 Sensitisation and antibody development

In the clinical setting, alloimmunisation, that is, development of antibodies against a foreign blood group antigen, occurs when an individual who is negative for a particular red cell antigen is exposed to red cells expressing that antigen. This can occur in the context of red blood cell transfusion or when fetal blood enters the circulation of a pregnant woman, for example at the time of delivery, miscarriage or abortion or following an invasive fetal procedure such as amniocentesis or chorionic villous sampling. However, spontaneous fetomaternal haemorrhage can occur without an associated clinical event and at any stage of pregnancy, although it is most common in the third trimester (Sebring and Polensky 1990). Therefore, the previously held adage that RhHD is “very rare among first born” due to the necessary presence of an “immunising fetus” (McConnell 1966) is now known to be untrue. In fact, although the incidence of rhesus sensitisation has decreased considerably thanks to the widespread use of anti-D immunoprophylaxis, an increasing proportion of those who are sensitised develop antibodies in their first pregnancy (Pattison, Roberts et al. 1992).

After A and B antigens, rhesus is the most immunogenic blood group antigen in humans. However, exposure of a rhesus negative individual to rhesus positive blood does not always lead to sensitisation and production of anti-D. Several factors may influence this immune response. Firstly, the volume of rhesus positive blood to which a rhesus negative individual is exposed to is critical; development of a primary immune response requires exposure to a considerably greater volume of blood than that required for initiation of a secondary immune response. Fetal blood is detectable in the circulation of most women at the end of pregnancy and following delivery. However, the volume detected is less than 2 ml in 98% of patients, as determined by Kleihauer’s acid elution test (Sebring and Polensky 1990). A fetomaternal haemorrhage of more than 30 ml places a rhesus negative mother at significant risk of sensitisation, with this degree of fetomaternal haemorrhage occurring in an estimated 3 in 1,000 pregnancies (Sebring and Polensky 1990). By contrast, development of a secondary (anamnestic) immune response requires exposure to a very small volume, at times as little as 0.03 ml of rhesus positive red cells (Urbanik and Greiss 2000).

The second critical factor determining the likelihood of rhesus sensitisation relates to the ABO status of the mother and fetus. ABO incompatibility between mother and fetus provides a degree of protection from rhesus sensitisation due to the ability of anti-A and anti-B antibodies to rapidly remove fetal blood cells from the maternal circulation before an
immune response to the rhesus antigen can be mounted. (Urbaniak and Greiss 2000) The influence of this effect is considerable, with a risk of rhesus sensitisation of 16% for an ABO compatible mother and fetus compared to 1.5 to 2% for and ABO incompatible pair (Bowman 1997).

The other two main factors determining the likelihood of rhesus sensitisation relate to the specific expression of the rhesus antigen on the fetal red cell membrane and the individual potency of the mother’s immune response (Urbaniak and Greiss 2000). It is likely that as yet undefined genetic factors determine whether an individual is capable of production of a strong immune response to a small number of red cells (Urbaniak 2006).

A primary immune response occurs in a rhesus negative mother who has not been previously exposed to the rhesus antigen. This primary response is slow and antibodies may not be detectable for up to 4 weeks after exposure. The initial response involves production of IgM antibodies, which do not cross the placenta (Urbaniak 2006). Subsequent exposure to the rhesus antigen results in a rapid secondary immune response involving production of IgG anti-D, which may be detected within 48 hours of exposure. The IgG anti-D produced may be of subclass 1 and/or 3, both of which can cross the placenta. IgG1 crosses the placenta early in gestation compared to IgG3, which is not detectable in fetal blood until the third trimester. Therefore, IgG1 anti-D is associated with more severe intrauterine anaemia (Urbaniak and Greiss 2000). Following a large fetomaternal haemorrhage, primary and secondary immune responses may merge, presumably due to the persistence of antigenic stimulation (Urbaniak 2006).

1.4.4.2 Immunological mechanisms of red cell destruction
Following transplacental passage, maternal anti-D IgG binds to fetal red cells expressing the rhesus antigen, initiating a cascade of events which result in red cell destruction (Avent and Reid 2000). Binding of anti-D to the rhesus antigen allows presentation of the coated red cells to macrophages, primarily in the spleen. This leads to either complete cell destruction by phagocytosis and lysis or partial cell membrane disruption and formation of spherocytes, which are susceptible to entrapment within the spleen and early destruction (Urbaniak and Greiss 2000). The extent of haemolysis is influenced by the amount of anti-D produced by the mother and the efficiency of its transplacental passage. In addition, the avidity of the antibody for the fetal rhesus antigen, the functional maturity of the fetal spleen and the
presence of blocking antibodies may influence the degree of red cell destruction (Urbaniak and Greiss 2000).

1.4.4.3 Immunological mechanisms of anti-D immunoprophylaxis
The exact mechanism of action of anti-D immunoprophylaxis is not completely understood (Copel, Gollin et al. 1991). In mice, binding of passively administered anti-D to the D antigen on red cells, so-called epitope masking, has been shown to be the main mechanism for the reduction of the primary antibody response to the D antigen (Kumpel 2002a). However, in humans the effective dose of anti-D is insufficient to bind the majority of vacant D antigen sites. Therefore, epitope masking is not thought to be relevant in humans (Kumpel 2002b).

Instead, two immunological processes are thought to be responsible for the prophylactic action of passively administered anti-D. The first involves rapid clearance from the circulation of rhesus positive red cells before they can become immunogenic. Red cells expressing the D antigen and bound with anti-D are removed to the spleen, where they are phagocytosed or lysed by macrophages (Kumpel 2002b). This is similar to the mode of destruction of red cells in the circulation of a fetus affected by RhHD. However, macrophages are poor antigen presenting cells and this mechanism can only clear a few millilitres of rhesus positive fetal cells from the maternal circulation. The fact that maternal sensitisation can be prevented with anti-D immunoprophylaxis following transfer of much larger volumes of rhesus positive fetal blood suggests that there is another mechanism at play (Kumpel 2002b).

The second postulated mechanism involves the down regulation of B cell responses by cross-linking of heterologous receptors on B cells with anti-D bound to the D antigen on red cells. This results in inhibition of B cell activation and apoptosis (Kumpel 2002c). It is likely that cytokines such as transforming growth factor-β also play a role in this process by recruiting anergic T cells, thereby accounting for the relatively long term suppression of the anti-D response observed following administration of anti-D immunoprophylaxis (Kumpel 2002a).

1.4.4.4 Other causes of haemolytic disease of the fetus and newborn
The use of anti-D immunoprophylaxis has led to a significant reduction in the prevalence of anti-D antibodies and other rhesus associated antigens (C, c and E) are now detected with increased frequency in pregnancy and implicated as causative agents in RhHD. However,
the D antigen continues to be the most common cause of morbidity and mortality from RhHD (Howard, Martlew et al. 1998; Avent and Reid 2000; Roberts 2008). Reporting on a cohort including almost all women who received IUT in New Zealand between 1963 and 1990, Pattison and colleagues found that the anti-D antibody was the cause of haemolytic disease in 97% of cases (Pattison, Roberts et al. 1992). However, the single largest cause is now ABO incompatibility, although it is rare for this to result in neonatal anaemia (Murray and Roberts 2007; Roberts 2008). Antibodies directed against other red cell antigens, such as Kell, Duffy (Fya), Kidd (Jka) and MNS have also been reported to cause haemolytic disease in the fetus and neonate (Moise 2008b).

1.4.5 Antenatal Diagnosis and Monitoring of Rhesus Haemolytic Disease

1.4.5.1 Blood grouping and antibody detection

Successful management of RhHD requires early diagnosis and the ability to reliably monitor fetal condition as pregnancy progresses. As identification of a fetus at risk of RhHD first requires knowledge of maternal blood group, it is routine practice for women to have blood group determined early in pregnancy. If a woman is found to be rhesus negative, the paternal blood group is also determined. Given the Mendelian inheritance of rhesus status, if both parents are rhesus negative the fetus will also be negative. However the situation is more complex if the father is rhesus positive, as paternal heterozygosity will result in rhesus negativity in 50% of offspring (Lo, Hjelm et al. 1998). In Liley’s day, determination of paternal genotype was not possible. Instead, it was inferred from knowledge of the rhesus phenotype of any other offspring and the parents of the father (Liley AW, unpublished teaching notes, 1973). With the advent of molecular DNA techniques, this process has become more straightforward and the results more reliable.

An indirect antiglobulin (Coombs) test is also performed early in pregnancy to determine if sensitisation to the rhesus antigen has previously occurred. As antibodies can develop during pregnancy, a rhesus negative mother with a negative initial antibody screen has repeat testing later in pregnancy, usually between 28 to 32 weeks and at the time of delivery (Urbaniak and Greiss 2000). However if anti-D antibodies are detected in a rhesus negative mother, the antibody level is quantified and measured at regular intervals, the frequency of monitoring being determined by gestation, the trend in antibody concentration and the mother’s previous history (Whittle 1992). Although the trend in antibody concentration is more predictive of outcome for the fetus than the absolute value, an antibody concentration of greater than 4 IU.ml⁻¹ is used to indicate the need for further evaluation in a specialised
centre, with invasive testing recommended if the concentration exceeds 15 IU.ml⁻¹ (Contreras 1994; Urbaniak and Greiss 2000; Kumar and Regan 2005). However, the antibody concentration is of limited value in predicting the degree of fetal anaemia for mothers with a history of a previous affected fetus (Moise 2008a).

1.4.5.2 Amniocentesis
Once a fetus at risk of RhHD is identified, the requirement for further management depends on whether the fetus becomes anaemic. Only 10% of fetuses at risk of haemolytic disease develop anaemia severe enough to require IUT before 34 weeks’ gestation. The remaining 90% are either unaffected or have only mild disease (Mari, Deter et al. 2000). Therefore ongoing investigations are directed at detecting fetal anaemia so that appropriate obstetric management, in particular the need for IUT, can be determined.

The use of serial amniocentesis to evaluate the bilirubin content of amniotic fluid using spectrophotometry became the standard tool for assessment of fetal anaemia from the mid 1960s (Whittle 1992). The chart originally designed by Liley to guide treatment decisions based on amniotic fluid assessment remains in use (Liley 1961), although various modifications have been made by others in an attempt to allow extrapolation of data to the second and first trimesters (Whitfield 1970; Queenan, De Catte et al.). However, the reliability of this technique prior to 27 weeks’ gestation remains questionable (Nicolaides, Rodeck et al. 1986; Mari, Deter et al. 2000; Urbaniak and Greiss 2000). In addition, there are several serious potential complications with the use of repeated amniocentesis. Intrauterine death following amniocentesis is estimated to occur at a rate of approximately 1 in 400 procedures (Stockman 2001). Worsening of anti-D sensitisation can also result if the procedure causes spill of fetal red blood cells to the maternal circulation (Urbaniak and Greiss 2000; Roberts, Mitchell et al. 2001; Oepkes, Seaward et al. 2006). Other complications including premature rupture of membranes and/or preterm labour, chorioamnionitis and fetal bradycardia can also occur (Roberts, Mitchell et al. 2001; Nishie, Brizot et al. 2003; Oepkes, Seaward et al. 2006). The requirement for repeated monitoring of amniotic fluid samples also exposes the mother to multiple invasive procedures (Bennett, Kim et al. 1993).

1.4.5.3 Fetal blood sampling
The technique of ultrasound guided cordocentesis for fetal blood sampling was developed in the early 1980s. This offers the benefit of direct assessment of fetal haematocrit (Daffos, Capella-Pavlovsky et al. 1985; Urbaniak and Greiss 2000), and enables determination of
fetal blood group, which is particularly useful if the father is heterozygous (Whittle 1992). A further advantage of fetal blood sampling is the ability to perform direct intravascular IUT following blood sampling (Stockman 2001). However, fetal loss following fetal blood sampling occurs at a rate of 1 to 2% for non-hydropic fetuses, 15% for hydropic fetuses sampled prior to 20 weeks’ gestation and 5% for hydropic fetuses sampled after 20 weeks (Urbaniak and Greiss 2000). Fetal blood sampling is also associated with the other complications encountered following amniocentesis, including the potential for elevation of anti-D levels secondary to fetomaternal haemorrhage.

1.4.5.4 Ultrasonography
Due to the risks associated with the invasive procedures of amniocentesis and fetal blood sampling, experimentation with noninvasive techniques for the diagnosis and monitoring of fetuses affected by RhHD has burgeoned over the last decade (Nishie, Brizot et al. 2003; Moise 2008c). Ultrasound has been used for many years to monitor fetal status during pregnancy. In severe RhHD, ultrasound can detect polyhydrannios, placental thickening, enlargement of the fetal liver and overt fetal hydrops (Urbaniak and Greiss 2000). An audit of imaging performed on all patients examined at the Rhesus Clinic at National Women’s Hospital from 1986 to 1999 demonstrated the utility of ultrasonographic assessment of fetal liver size. All fetuses with anaemia, determined by fetal blood sampling, were found to have an enlarged liver on ultrasound, with 93% of liver measurements greater than or equal to the 95th percentile (Roberts, Mitchell et al. 2001). However the reliability of ultrasound for the detection of early changes of hydrops has been questioned (Whittle 1992).

The widespread availability of pulsed wave Doppler ultrasound broadened the applications of ultrasound for non-invasive fetal assessment in RhHD. Observations of increased fetal blood flow velocities in anaemic animals led to studies of blood velocity at various sites in the human fetus, including the descending aorta, umbilical vein, splenic artery, common carotid artery and middle cerebral artery (Moise 2008c). In 2000, the Collaborative Group for Doppler Assessment of the Blood Velocity in Anaemic Fetuses confirmed the ability of Doppler ultrasonography to detect moderate to severe anaemia in the non-hydropic fetus by increased middle cerebral artery peak systolic velocity (MCA-PSV) (Mari, Deter et al. 2000). The middle cerebral artery was chosen as this vessel is easily visualised and the cerebral arteries respond quickly to hypoxaemia. Doppler ultrasonography of this vessel was found to have a sensitivity of 100% with a false positive rate of 12%, using a threshold of 1.5 multiples of the median of peak systolic velocity for prediction of moderate to severe
anaemia (Mari, Deter et al. 2000; Moise 2008c). Subsequent work has confirmed these findings and determined that Doppler studies are as reliable as serial amniocentesis in detecting moderate to severe fetal anaemia, without the potential complications associated with the invasive procedure (Nishie, Brizot et al. 2003; Oepkes and Adama van Scheltema 2007). Doppler ultrasound has now largely replaced serial amniocentesis for the detection of fetal anaemia (Moise and Argoti 2012).

1.4.5.5 Detection of free fetal DNA in maternal circulation
At the same time as developments in the use of ultrasound, research into noninvasive techniques to accurately determine the rhesus status of the fetus was under way. This was already possible via direct serology from a fetal blood sample or from DNA extracted from amniotic fluid cells amplified using the polymerase chain reaction (Bennett, Kim et al. 1993; Urbaniak and Greiss 2000). However these techniques required the invasive procedures of amniocentesis or cordocentesis, with the associated risks for mother and fetus.

The isolation of free fetal DNA (ffDNA) from maternal plasma in the 1990s paved the way for noninvasive prenatal diagnosis of fetal rhesus status (Lo, Bowell et al. 1993; Lo, Hjelm et al. 1998). The enormous benefits of a non-invasive approach were rapidly apparent and considerable research to refine the process of ffDNA detection ensued (Moise 2005). The establishment of the SAFE (Special Non-invasive Advances in Fetal and Neonatal Evaluation) network in Europe in 2004 underscored the perceived importance of this technology and fostered collaborative work to develop and implement routine and cost-effective noninvasive prenatal diagnosis techniques (Maddocks, Alberry et al. 2009). The initial focus was identification of fetal rhesus status, particularly for rhesus negative women whose partners were heterozygous, or for whom paternal rhesus status was not known, as accurate identification of rhesus negative fetuses obviates the need for fetal monitoring and anti-D immunoprophylaxis (Moise and Argoti 2012).

The development of ffDNA detection has been complicated by the existence of rhesus gene variations in different ethnic groups, necessitating the use of polymerase chain reaction primers targeting multiple exons of the rhesus gene (Moise 2005). However a recent meta-analysis established that the diagnostic accuracy of noninvasive fetal rhesus genotyping from maternal blood is 94.8% when used in both sensitised and unsensitised rhesus negative women (Geifman-Holtzman, Grotegut et al. 2006). Detection of ffDNA for fetal rhesus genotyping in rhesus negative alloimmunised pregnancies is now used in several centres in
Europe (Freeman, Szczepura et al. 2009) and two recently published reports from Sweden and Denmark confirm the ability of this method to accurately detect fetal rhesus status and thus determine the requirement for anti-D prophylaxis (Clausen, Christiansen et al. 2012; Tiblad, Taune Wikman et al. 2013). However, the cost-effectiveness of ffDNA detection for this purpose has been questioned, with marginal or no cost reduction demonstrated in studies performed in England and Wales, France and the United States (Szczepura, Osipenko et al. 2011; Benachi, Delahaye et al. 2012; Hawk, Chang et al. 2013). Thus, routine antenatal testing of ffDNA for rhesus negative mothers has not yet been widely implemented (Moise and Argoti 2012).

1.4.6 Clinical Features of Rhesus Haemolytic Disease in the Fetus

1.4.6.1 Spectrum of disease
A wide spectrum of disease severity exists for fetuses at risk of RhHD, from minimal or no evidence of anaemia to severe anaemia, jaundice, compensatory hyperplasia of erythropoietic tissue, widespread oedema (hydrops fetalis) and possibly intrauterine death (Stoll and Kliegman 2000).

Of those infants affected by RhHD, approximately half require no treatment. Of those remaining, approximately half are born near term and are clinically well. However they are at risk of becoming severely jaundiced in the first few days and if left untreated, may develop kernicterus leading to death or severe neurodisability with deafness and spastic choreoathetosis. The remaining 25% of affected infants become severely anaemic and hydropic in utero (Stockman 2001). However disease severe enough to require IUT prior to 34 weeks occurs in only 10% of rhesus positive fetuses of sensitised rhesus negative mothers (Mari, Deter et al. 2000).

1.4.6.2 Fetal anaemia
The pathological consequences of RhHD in the fetus are a direct result of red cell destruction secondary to placental transfer of maternally derived anti-D antibody (Whittle 1992). The severity of disease is therefore determined by the severity of the haemolytic process (Urbaniak and Greiss 2000). Moderate anaemia stimulates marrow hyperactivity which may be adequate to compensate for red cell destruction. However, when the compensatory capacity of the marrow is exceeded by severe haemolysis, extramedullary haematopoiesis occurs in the liver, spleen, kidneys and adrenal glands (Urbaniak and Greiss 2000). Reticulocytes and immature nucleated red blood cells are released into the
circulation and increased levels of erythropoietin are found (Ramasethu and Luban 2006). The nucleated red cell count reflects the extent of extramedullary erythropoiesis and is directly correlated to the severity of fetal anaemia. Hepatosplenomegaly results and other functions of the liver are obtunded as red cell production supervenes (Nicolini, Nicolaidis et al. 1991). Jaundice occurs when the capacity of fetomaternal placental exchange and the fetal liver to excrete bilirubin are exceeded (Urbaniak and Greiss 2000). Hypoalbuminaemia occurs as a result of reduced albumin production by the liver, leading to a fall in plasma oncotic pressure (Whittle 1992).

1.4.6.3 Hydrops fetalis
Hydrops fetalis refers to the accumulation of excessive interstitial fluid in the fetus (Apkon 1995). RhHD is the most common cause of immune hydrops fetalis, which is usually defined as abnormal accumulation of fluid in two or more fetal compartments (Stoll and Kliegman 2000). However, the definition of fetal hydrops is not universally standardised and some consider the collection of fluid in a single body compartment to be sufficient for the diagnosis (van Kamp, Klumper et al. 2001).

The occurrence of hydrops in RhHD is directly related to the degree of fetal anaemia. For hydrops to occur, fetal haemoglobin is usually at least 70 g.l⁻¹ below the mean for gestational age or haematocrit is less than 15% (Nicolini, Nicolaidis et al. 1991). The earliest detectable manifestations of hydrops in a fetus affected by RhHD are ascites and pericardial effusion, followed by skin oedema and placental thickening. Pleural effusions are a late clinical manifestation (van Kamp, Klumper et al. 2001; Oepkes and Adama van Scheltema 2007). Without treatment, severe RhHD in the fetus may result in the final common pathway of high output cardiac failure, metabolic acidosis and intrauterine death (Lindenburg, van Klink et al. 2013).

1.4.7 Management of Rhesus Haemolytic Disease in the Fetus
1.4.7.1 Monitoring of fetuses affected by rhesus haemolytic disease
Survival following IUT is significantly worse for a hydropic fetus than a nonhydropic fetus (van Kamp, Klumper et al. 2001). Therefore, the aim of serial monitoring is to identify the fetus with moderate to severe anaemia before the development of hydrops (Brennand and Cameron 2008).
Current recommendations involve initiation of serial MCA-PSV Doppler ultrasound studies at one to two week intervals from 24 weeks’ gestation in a first affected pregnancy and 18 weeks in women with a history of a previously affected fetus. A finding of MCA-PSV greater than 1.5 multiples of the median (MoM) at any time before 35 weeks’ gestation indicates the need for fetal blood sampling, with progression to IUT if the fetal haematocrit is $\leq 30\%$ (Moise 2008a). Although there is no relationship between the decline in MCA-PSV and rise in haemoglobin concentration immediately following IUT (Grubbs, Korst et al. 2013), MCA-PSV monitoring can be used to assist with the timing of the second IUT, using a modified MoM threshold of 1.32. However, MCA-PSV monitoring thereafter is affected by changes in blood flow due to the increased viscosity of adult donor red blood cells (Welch, Rampling et al. 1994), thus third and subsequent transfusions are usually performed at fixed intervals of two to three weeks until a gestational age of 35 to 36 weeks (Papantoniou, Sifakis et al. 2012).

1.4.7.2 Intrauterine transfusion

IUT technique has been refined since Liley’s pioneering work in the 1960s, and is now routinely performed by the intravascular route with ultrasound guidance. Intravascular IUT results in faster restoration of fetal haematocrit and is especially beneficial for the hydropic fetus in whom absorption of red cells from the peritoneal cavity is impaired (Oepkes and Adama van Scheltema 2007). However some centres use a combination of intravascular and intraperitoneal IUT, with the aim of prolonging the interval between transfusions due to the slow absorption of red cells from the peritoneal cavity (Oepkes and Adama van Scheltema 2007; Moise 2008a).

The usual site of access to the fetal circulation is the placental insertion of the umbilical vein (Oepkes and Adama van Scheltema 2007; Moise 2008a). At the start of the procedure a sample of fetal blood is taken to check fetal haematocrit and the volume of blood to be transfused is determined using a formula incorporating the fetal and donor blood haematocrits and the fetoplacental blood volume. Target haematocrit post transfusion is 40 to 50% (Brennand and Cameron 2008; Moise 2008a). Transfusion is performed using irradiated type O rhesus negative blood, which is less than 72 hours old and has been screened for cytomegalovirus, hepatitis B and human immunodeficiency virus. In order to prevent sensitisation to other red cell antigens, donor blood is also cross-matched with maternal blood (Papantoniou, Sifakis et al. 2012). Using donor blood with a haematocrit of at least 75% reduces the risk of volume overload of the fetus (Kumar and Regan 2005;
The anticipated decline in fetal haematocrit following IUT is 1% per day. (Jones, Lynch et al. 1986; MacGregor, Socol et al. 1989)

The most common complication of IUT is transient fetal bradycardia, which occurs in 8% of procedures (Oepkes and Adama van Scheltema 2007). This is thought to result from arterial vasospasm secondary to the development of cord haematoma following umbilical vein puncture (Brennand and Cameron 2008). Excluding fetal bradycardia, the overall procedure related complication rate following intravascular IUT was 3.1% in a cohort study of 254 fetuses treated with 740 transfusions (van Kamp, Klumper et al. 2005). Serious complications encountered in this cohort included rupture of membranes, chorioamnionitis and premature or emergency delivery. The reported procedure related death rate was 1.6% per procedure. These findings are similar to complication rates reported by others (Schumacher and Moise 1996; Oepkes and Adama van Scheltema 2007).

1.4.7.3 Other emerging antenatal treatment modalities
The treatment of severely affected fetuses prior to 20 weeks’ gestation remains a challenge as intravascular IUT is technically difficult and MCA-PSV monitoring is less reliable (Papantoniou, Sifakis et al. 2012). Intraperitoneal transfusion has been used with some success in this situation (Gallot, Boiret et al. 2004; Howe and Michailidis 2007). A less invasive approach, using maternally administered intravenous immunoglobulin (IVIG) and/or regular plasmapheresis to reduce maternal antibody titre and delay the time to first IUT has also been described, with 8 isolated case reports or small case series comprising a total of 27 patients published since 2006 (Palfi, Hilden et al. 2006; Kriplani, Malhotra Singh et al. 2007; Ruma, Moise et al. 2007; Fox, Martin et al. 2008; Novak, Tyler et al. 2008; Connan, Kornman et al. 2009; Isojima, Hisano et al. 2011; Lakhwani, Machado et al. 2011). However, treatment with IVIG is expensive, and can result in severe allergic reactions. Furthermore, given the limited reports of its use, it is possible that the available evidence is distorted by publication bias. Thus, although the use of IVIG and/or plasmapheresis appears promising, especially for the management of affected fetuses early in gestation, much is still to be elucidated regarding optimal treatment protocols and the clinical utility of this approach (Papantoniou, Sifakis et al. 2012).

1.4.7.4 Timing of delivery
In optimal circumstances, fetal interventions are planned to enable continuation of pregnancy until 37 to 38 weeks’ gestation, thereby avoiding the potential risks of premature delivery (Moise 2002). However for the severely affected fetus in whom IUT has been
unsuccessful or is not possible, premature delivery may be necessary. In this situation the risks of premature delivery must be weighed against the risks of remaining in utero. Improved survival has been demonstrated for infants treated with IUT beyond 32 weeks’ gestation compared to infants in whom IUT was discontinued at 32 weeks in favour of premature delivery (Klumper, van Kamp et al. 2000).

1.4.8 Clinical Features of Rhesus Haemolytic Disease in the Newborn
1.4.8.1 Spectrum of disease
As in the fetus, a wide spectrum of disease severity exists for newborns affected by RhHD (Stoll and Kliegman 2000). The original description by Louis Diamond in 1932 of a syndrome of jaundice, generalised oedema and anaemia represents the severe end of the clinical spectrum (Naiman 2001). However, with timely and successful fetal therapy with IUT and modern obstetric care, the neonatal manifestations of RhHD can be mitigated (Whittle 1992). IUT corrects fetal anaemia, thereby ameliorating circulatory compromise and potentially reversing hydrops fetalis (van Kamp, Klumper et al. 2001). In addition, replacement of rhesus positive fetal red blood cells with rhesus negative adult red blood cells slows the haemolytic process. Therefore, the lifespan of red cells in the fetal and subsequently neonatal circulation is prolonged and the production of bilirubin is decreased (Whittle 1992).

However in some circumstances there may be inadequate opportunity for intervention with fetal therapy and RhHD may remain untreated or partially treated. In unmonitored pregnancies fetal compromise may go undetected, especially if RhHD was not anticipated through previous maternal obstetric history. Alternatively, a fetus may be severely affected at the time of diagnosis, in which case IUT is less likely to reverse hydrops fetalis. In other cases fetal therapy may not be possible for technical reasons, for example due to unfavourable fetal position or lack of availability of appropriate technology and expertise. In these situations anaemia, hydrops and jaundice frequently complicate the neonatal course, often in conjunction with the challenges presented by premature birth.

1.4.8.2 Neonatal anaemia and haematological findings
A moderate to severely anaemic newborn will be pale on delivery and may have signs of cardiac failure with cardiomegaly, tachycardia and respiratory distress (Stoll and Kliegman 2000). Compensatory hyperplasia of erythropoietic tissues may result in hepatosplenomegaly (Urbaaniak and Greiss 2000). Infants with severe haemolysis may have
cutaneous signs of haematopoiesis. Thrombocytopenia may result in petechiae or purpura (Petricc 1995). Cord haemoglobin concentration is frequently normal, especially those treated with IUT (Petricc 1995; Weisz, Rosenbaum et al. 2009). Increased reticulocyte and nucleated red cell counts reflect the severity of haemolysis and degree of extramedullary erythropoiesis (Nicolini, Nicolaides et al. 1991).

Persistent anaemia may occur. In the first few days following delivery, this is usually due to ongoing haemolysis from passively acquired maternal antibody (Kumar and Regan 2005). More commonly, late hyporegenerative anaemia may occur from 7 days of age, particularly in infants treated with IUT (Smits-Wintjens, Walther et al. 2008). Late anaemia is thought to be due to suppression of haematopoiesis following transfusion (Farrant, Battin et al. 2001; McGlone, Simpson et al. 2009). A reduction in survival of transfused red cells may also contribute (Smits-Wintjens, Walther et al. 2008). The reticulocyte count in infants with late anaemia is low despite marked anaemia (Petricc 1995; De Boer, Zeestraten et al. 2008). Late anaemia may persist for up to three months post delivery (Farrant, Battin et al. 2001).

1.4.8.3 Hydrops fetalis and cardiorespiratory compromise
The hydropic newborn may have subcutaneous and visceral oedema in addition to fluid in the pericardial, peritoneal and pleural cavities (Urbaniak and Greiss 2000). These features may significantly compromise respiratory and cardiovascular status. Resuscitation may be difficult due to circulatory compromise and the presence of pulmonary oedema or effusions (Stoll and Kliegman 2000).

Cardiorespiratory compromise is frequently multifactorial in the newborn with RhHD. Congestive cardiac failure from severe anaemia and pleural and pericardial effusions due to hydrops often coexist. Blood pressure and perfusion may be further compromised by perinatal asphyxia (Petricc 1995). Respiratory distress may be exacerbated by pulmonary hypoplasia due to hydrops or premature delivery. Hypoxia may result from, or contribute to cardiovascular instability (Petricc 1995). Hepatosplenomegaly may be due to congestive cardiac failure and/or extramedullary haematopoiesis and may contribute to ventilatory difficulties.

1.4.8.4 Jaundice, bilirubin encephalopathy and cholestasis
Elevated levels of bilirubin in the neonate cause jaundice. In severely affected newborns jaundice may be present at birth and the amniotic fluid, vernix and cord may be visibly stained with bilirubin (Stoll and Kliegman 2000). Other infants become rapidly jaundiced in
the first few hours due to loss of placental clearance of unconjugated bilirubin (Stoll and Kliegman 2000; Roberts 2008). Jaundice develops in a cephalocaudal direction with increasing bilirubin concentration (Peterec 1995).

If unconjugated hyperbilirubinaemia remains untreated, acute bilirubin encephalopathy may develop, the early phase of which is characterised by jaundice, lethargy, hypotonia and a poor suck. As the disease process progresses, the infant becomes drowsy and irritable and may alternate between hyper- and hypotonia. Fever and a high-pitched cry may develop. In advanced acute bilirubin encephalopathy the infant may display opithotonus-retrocollis, apnoea, fever, no feeding, coma and seizures. Without treatment, permanent neurological damage or death may result from the toxic effect of bilirubin on the basal ganglia and brainstem nuclei (American Academy of Pediatrics 2004; Smits-Wintjens, Walther et al. 2008). The term “kernicterus” is used to describe the chronic clinical sequelae of bilirubin toxicity (American Academy of Pediatrics 2004).

The occurrence of acute bilirubin encephalopathy and kernicterus is dependent upon interaction between the level of unbound unconjugated bilirubin and the integrity of the blood brain barrier (Rennie, Sehgal et al. 2009). Unconjugated bilirubin bound to albumin does not cross the blood brain barrier as readily as unbound bilirubin (American Academy of Pediatrics 2004). Therefore infants with lower serum albumin levels are at greater risk of neurological damage secondary to hyperbilirubinaemia (Urbaniak and Greiss 2000). In addition, infants with ongoing haemolysis are at greater risk of neurological damage as haem may displace bilirubin from albumin binding sites and other products of haemolysis may potentiate the toxicity of bilirubin (Greenough 1999). The integrity of the blood brain barrier may be affected by factors including prematurity, hypoxia, hypercarbia, acidosis, sepsis and hypothermia (Peterec 1995). Therefore the serum bilirubin level in isolation is a poor predictor of short-term neurological manifestations or long-term neurodevelopmental outcome (American Academy of Pediatrics 2004).

Cholestatic liver disease and elevated levels of conjugated bilirubin may also occur in RhHD as a result of hyperferritinaemia and hepatic iron overload following multiple intrauterine and/or postnatal blood transfusions (Smits-Wintjens, Walther et al. 2008). Infants with high conjugated bilirubin levels who receive phototherapy may develop brown or bronze skin discolouration (Bertini, Dani et al. 2005). Lower plasma albumin concentrations in infants with Bronze Baby Syndrome may result in the development of
acute bilirubin encephalopathy at lower total bilirubin concentrations in these infants (Smits-Wintjens, Walther et al. 2008).

1.4.8.5 Thrombocytopenia
Thrombocytopenia has been described in two small studies of moderate to severely affected infants with RhHD, with platelet counts of less than $50 \times 10^9\text{ l}^{-1}$ associated with increased risk of intracranial haemorrhage in the first week after birth (Koenig and Christensen 1989; van den Akker, de Haan et al. 2008). Suppression of platelet production due to increased erythropoiesis is the likely cause, although increased destruction may also occur in utero or secondary to exchange transfusion (Rath, Smits-Wintjens et al. 2011).

1.4.8.6 Other clinical findings
Prematurity may complicate the clinical course of the newborn with RhHD. IUT is an invasive procedure and carries with it a risk of premature delivery (McGlone, Simpson et al. 2009). However in a recent cohort study, only one infant out of 54 treated with IUT required delivery prior to 32 weeks’ gestation (Weisz, Rosenbaum et al. 2009). For those infants in whom premature delivery is necessary, the known cardiac, respiratory, gastrointestinal, metabolic and neurologic complications associated with prematurity may complicate their clinical course.

Surfactant deficiency may also occur in hydropic infants (Peterec 1995), resulting in lung immaturity and respiratory distress independent of gestational age (Naeye 1975). The possibility of an association between IUT and necrotising enterocolitis has also been noted (Musemeche and Reynolds 1991). However, neither of these findings has been confirmed in subsequent reports (Rao, Patole et al. 2004; Weisz, Rosenbaum et al. 2009).

Hypoglycaemia frequently occurs in newborns suffering severe RhHD secondary to pancreatic islet cell hypertrophy and consequent hyperinsulinaemia (Stoll and Kliegman 2000). Increased glucagon suppression by insulin has also been shown in these infants (Fallucca, Gerlini et al. 1977). These findings occur despite maternal normoglycaemia (Fallucca, Sabbatini et al. 2000). The cause of fetal islet cell hyperplasia in RhHD is unknown. However it may be related to intrauterine hypoxia secondary to fetal anaemia (Fallucca, Sabbatini et al. 2000). Alternatively, it has been postulated that products of haemolysis may inactivate or destroy circulating fetal insulin, leading to compensatory pancreatic cell hyperplasia (Steinke, Gries et al. 1967).
1.4.9 Management of Rhesus Haemolytic Disease in the Newborn

1.4.9.1 Immediate stabilisation and management of hydrops fetalis
Hydropic infants often require immediate resuscitation. Immediate drainage of pleural effusions and ascites may be necessary (Greenough 1999; Smits-Wintjens, Walther et al. 2008) and ventilation may be difficult (Smits-Wintjens, Walther et al. 2008). Rapid establishment of venous access via the umbilical route allows administration of drugs of resuscitation, monitoring of central venous pressure, and provision of inotropic support if required (Petroe 1995). As hydropic infants often demonstrate cardiovascular instability despite normovolaemia, immediate correction of anaemia should occur by partial exchange transfusion rather than simple red blood cell administration (Petroe 1995; Greenough 1999; Smits-Wintjens, Walther et al. 2008).

Hypoalbuminaemia occurs frequently in hydropic infants and correlates with disease severity. However, there is no evidence to support the administration of albumin to the hydropic infant in the immediate postnatal period. Diuretics, peritoneal dialysis and digoxin have also not been found to be beneficial (Petroe 1995).

1.4.9.2 Management of hyperbilirubinaemia
1.4.9.2.1 Phototherapy
Phototherapy was first used to treat neonatal hyperbilirubinaemia due to ABO incompatibility in 1971 (Sisson, Kendall et al. 1971). Since then, its use has become standard practice in the management of infants with hyperbilirubinaemia. Phototherapy induces photo-isomerisation and photo-oxidation of bilirubin, rendering it more water soluble and thus more readily excreted in urine and bile (Petroe 1995). Delivery of optimal phototherapy is dependent on a number of factors including the intensity and spectral qualities of the delivered light, distance from the light, surface area covered, and duration of exposure (Smits-Wintjens, Walther et al. 2008; Bhutani 2011). Clinically significant complications from the use of phototherapy are exceptionally rare (American Academy of Pediatrics 2004).

Infants with severe RhHD usually commence intensive phototherapy as soon as possible after birth as prompt initiation of phototherapy may prevent the need for exchange transfusion (Smits-Wintjens, Walther et al. 2008). Treatment with IUT may reduce the time for which phototherapy is required as replacement of fetal rhesus positive red blood cells with donor rhesus negative red blood cells reduces the rate of haemolysis (De Boer, Zeestraten et al. 2008).
1.4.9.2.2 Exchange transfusion

Both bilirubin and rhesus positive fetal red blood cells coated with maternal antibody are directly removed from the circulation by exchange transfusion. Exchange transfusion may also establish a normal haematocrit for infants who are anaemic (Peterec 1995; Smits-Wintjens, Walther et al. 2008).

Exchange transfusion is indicated for an infant with RhHD when intensive phototherapy has failed to lower, or halt the rise, of serum bilirubin, or if signs of acute bilirubin toxicity are evident regardless of bilirubin level (American Academy of Pediatrics 2004; Bhutani, Maisels et al. 2008). Exchange transfusion reduces serum bilirubin concentration to approximately 60% of the pre-exchange value (Greenough 1999). However, it is an invasive procedure, and although mortality associated with exchange transfusion is rare (0.3%), the overall morbidity rate is up to 24%, with potential complications including apnoea, bradycardia, cyanosis, vasospasm, thrombosis, haemorrhage, infection, portal hypertension and necrotising enterocolitis (Smits-Wintjens, Walther et al. 2008). As fewer exchange transfusions are now performed due to the decreasing incidence of RhHD, relative inexperience with the technique of exchange transfusion may result in an increased frequency of procedure related complications (American Academy of Pediatrics 2004; Smits-Wintjens, Walther et al. 2008).

1.4.9.2.3 Intravenous immunoglobulin

IVIG reduces the need for exchange transfusion when used in conjunction with phototherapy compared to treatment of hyperbilirubinaemia by phototherapy alone (Gottstein and Cooke 2003; American Academy of Pediatrics 2004; Smits-Wintjens, Walther et al. 2008; Walsh, Yao et al. 2008). IVIG is thought to reduce haemolysis by binding to the Fc receptor of reticuloendothelial cells, thereby preventing destruction of neonatal red blood cells sensitised by anti-D (Gottstein and Cooke 2003; Smits-Wintjens, Walther et al. 2008; Walsh, Yao et al. 2008). The American Academy of Pediatrics guideline on the management of hyperbilirubinaemia in the neonate recommends administration of 0.5 to 1 g.kg$^{-1}$ of IVIG if the serum bilirubin is rising despite intensive phototherapy or is within 34 to 51 µmol.l$^{-1}$ of the exchange level (American Academy of Pediatrics 2004).

However, clinical trials investigating the use of IVIG for the management of hyperbilirubinaemia have been small, with varying inclusion criteria and thresholds for
intervention with exchange transfusion (Smits-Wintjens, Walther et al. 2008). A recent investigation of the efficacy of early IVIG compared to conventional treatment for the management of infants with hyperbilirubinaemia due to RhHD found that duration of phototherapy and hospital stay were shortened following IVIG, but the requirement for exchange transfusion was unchanged (Elalfy, Elbarbary et al. 2011). Furthermore, a randomised controlled trial comparing IVIG versus placebo in infants with RhHD demonstrated no reduction in requirement for exchange transfusion, duration of phototherapy, peak serum bilirubin concentration or requirement for top up transfusion (Smits-Wintjens, Walther et al. 2011). Thus, the available evidence does not support the use of IVIG in these infants, and the most recent Cochrane review on this subject concluded “further well designed studies are needed before routine use of IVIG can be recommended for the treatment of isoimmune haemolytic jaundice” (Alcock and Liley 2002).

1.4.9.2.4 Metalloporphyrins
Metalloporphyrins have the potential to reduce the concentration of unconjugated bilirubin by inhibition of haem oxygenase, the enzyme responsible for the catabolism of haem to bilirubin (Smits-Wintjens, Walther et al. 2008). While there is reasonable evidence of efficacy in animal studies, experience in humans is limited and concerns remain regarding the potential for toxicity (Schulz, Wong et al. 2012). At present metalloporphyrins are used only for infants at particularly high risk of bilirubin encephalopathy or those participating in clinical trials (Wong, Bhutani et al. 2007).

1.4.9.3 Management of anaemia
Early anaemia and severe hyperbilirubinaemia frequently occur concurrently and exchange transfusion is a useful treatment modality for both (Peterec 1995). However, anaemia can occur up to 3 months after birth in infants with RhHD, and at least one top up transfusion is required for the management of late anaemia in 80% of infants who received IUT, and 65% of those who did not (Rath, Smits-Wintjens et al. 2011). Consensus regarding the haemoglobin concentration or haematocrit at which top up transfusion should occur is lacking. The infant’s clinical condition, feeding status and oxygen requirement are important factors to consider (Smits-Wintjens, Walther et al. 2008; Rath, Smits-Wintjens et al. 2011).

Given that late anaemia in infants with RhHD is characterised by a reduced reticulocyte count and is thought to reflect a hyporegenerative bone marrow, the use of erythropoietin has been investigated in these infants (Ohls, Wirkus et al. 1992; Scaradavou, Inglis et al.
1993; Ovali, Samanci et al. 1996; Zuppa, Maragliano et al. 1999; Nicaise, Gire et al. 2002; Zuppa, Alighieri et al. 2010; Erduran and Bahadir 2011; Zuppa, Alighieri et al. 2012). Most of these studies show reduced need for top up transfusion following erythropoietin therapy. However, the majority of these reports are small case series, and the protocols used vary considerably, as does the gestational age of infants included. Thus, there is no clear consensus regarding the use of erythropoietin in RhHD infants (Rath, Smits-Wintjens et al. 2011).

1.4.10 Outcome of Rhesus Haemolytic Disease

1.4.10.1 Short-term outcome
Survival following IUT for RhHD has improved steadily over the last forty-five years, and most centres now report that over 90% of infants treated with IUT survive to discharge (Pattison, Roberts et al. 1992; Smits-Wintjens, Walther et al. 2008; Papantoniou, Sifakis et al. 2012; Lindenburg, van Klink et al. 2013). However, survival is less favourable for fetuses with severe hydrops and those requiring first transfusion prior to 24 weeks’ gestation (Pattison, Roberts et al. 1992; van Kamp, Klumper et al. 2001; van Kamp, Klumper et al. 2005; Weisz, Rosenbaum et al. 2009).

Infants with RhHD treated with IUT require fewer days of phototherapy but more top up red blood cell transfusions than those infants who did not receive IUT (Janssens, de Haan et al. 1997; De Boer, Zeestraten et al. 2008). However, the requirement for exchange transfusion is similar in the two groups (De Boer, Zeestraten et al. 2008).

Prior to the widespread use of IUT, birth weight was lower in infants with RhHD than age matched unaffected controls, with the size of this effect dependent on the severity of fetal affliction (Schumacher and Moise 1996). However, more recent studies have shown that transfused fetuses undergo catch up growth in utero, with similar birth weights to unaffected matched controls (Utter, Socol et al. 1990; Roberts, Grannum et al. 1993).

Given the increased risk of preterm delivery for infants with RhHD, short-term outcome may be affected by morbidities associated with prematurity. One third of neonates in a large Scottish cohort of IUT recipients required some sort of respiratory support (McGlone, Simpson et al. 2009). An association between RhHD and necrotising enterocolitis has been previously suggested (Musemeche and Reynolds 1991). However more recent experience
does not support this, with reported rates of necrotising enterocolitis similar to that seen in association with prematurity (Rao, Patole et al. 2004; Weisz, Rosenbaum et al. 2009).

1.4.10.2 Long-term outcome
There is relatively little information on the long-term outcome of individuals who suffered RhHD and were treated with IUT (Greenough 1999; Smits-Wintjens, Walther et al. 2008). Theoretically, neurodevelopmental outcome of infants affected by RhHD may be compromised by anaemia and consequent hypoxia in utero (Harper, Swingle et al. 2006). Therefore, the few studies that have been conducted have focused on assessment of neurodevelopmental outcome in childhood (White, Goplerud et al. 1978; Hardyment, Salvador et al. 1979; Janssens, de Haan et al. 1997; Hudon, Moise et al. 1998; Grab, Paulus et al. 1999; Harper, Swingle et al. 2006; Weisz, Rosenbaum et al. 2009). None of these found any statistically significant differences when neurodevelopmental outcome of IUT recipients was compared to that of an unaffected sibling or age matched control. In children who were hydropic in utero, hence most severely affected, neuropsychological outcomes were within the normal range (Harper, Swingle et al. 2006). Similarly, neurodevelopmental outcomes were similar in children who suffered mild to moderate fetal anaemia and those who suffered severe fetal anaemia (Weisz, Rosenbaum et al. 2009).

Although this evidence is reassuring and has led several authors to conclude that fetuses with severe isoimmune anaemia are not at increased risk of neurodevelopmental abnormalities if optimal treatment is received, these studies have several limitations. They are all small, with participant numbers ranging from 15 to 69 (Smits-Wintjens, Walther et al. 2008), and duration of follow up was short, with a mean age of only 10 years in the oldest cohort studied (Harper, Swingle et al. 2006). Comparison between studies is also difficult because the children were assessed using different psychometric and neurological tests.

However, a follow up study of neurodevelopmental outcome in a large cohort of children with RhHD treated with IUT in The Netherlands between 1992 and 2007 has been reported (Lindenburg, Smits-Wintjens et al. 2011). A total of 291 children were assessed at a median age of 8.2 years (range 2 to 17 years). Median gestational age of participants at birth was 36 weeks, 75 (26%) were hydropic at some stage, and 168 (58%) required exchange transfusion. Neurodevelopmental impairment (least one of: cerebral palsy, severe developmental delay (< 2 SD below the mean for age), bilateral deafness, or blindness) was diagnosed in 14 children (4.8%) and was significantly associated with a history of hydrops,
haemoglobin concentration at first IUT, gestation at birth less than 32 weeks, and severe neonatal morbidity. Similar rates of severe developmental delay, blindness and deafness were found in the Dutch population, but the rate of cerebral palsy was higher in IUT recipients (2.1% versus 0.7%). However, findings were not compared to unaffected siblings or controls matched for age, sex or gestation at birth. Thus, while this study is the largest and most recent investigation of neurodevelopmental outcome following IUT for RhHD, a number of factors may have confounded the reported outcomes. Nevertheless, the fact that neurodevelopment was favourable in over 95% of the cohort led the authors to conclude that there is an excellent chance of successful recovery following IUT for RhHD with appropriate antenatal treatment (Lindenburg, Smits-Wintjens et al. 2011). More subtle neurodevelopmental difficulties, such as speech delay, learning difficulties and behavioural problems are currently being evaluated in this cohort (Lindenburg, van Klink et al. 2013).

The observation that IUT with non-irradiated non-leukocyte depleted blood can lead to graft-versus-host disease was made in the late 1960s (Naiman, Punnett et al. 1969). Subsequently, only irradiated and leukocyte depleted blood has been used for IUT (Vietor, Hallensleben et al. 2000). However, the cohort of individuals who received IUT before this time forms a unique group for genetic analysis. Donor leukocytes have been detected in the circulation of these individuals up to 5 years after birth (Hutchinson, Turner et al. 1971; Turner, Hutchinson et al. 1973). In addition, Y-chromosome specific sequences have been detected by polymerase chain reaction from the blood of female IUT recipients given non-irradiated blood from male donors more than 20 years earlier (Vietor, Hallensleben et al. 2000). This provides evidence that IUT with nonirradiated unrelated donor blood can result in long-term microchimerism. The significance of this finding with regard to long-term health outcomes is unknown, but microchimerism with fetal cells in the maternal circulation may be involved in the pathogenesis of systemic sclerosis (Vietor, Hallensleben et al. 2000).

Apart from the study of cardiac function in childhood following fetal anaemia and IUT mentioned previously (section 1.3.5) (Dickinson, Sharpe et al. 2010), no investigations of other long-term health outcomes of recipients of IUT have been reported to date.

1.5 Nonimmune Causes of Fetal Anaemia

1.5.1 Definition
Nonimmune causes of fetal anaemia encompass a wide spectrum of disease including infection, haemorrhage, red blood cell disorders and haemoglobinopathies. Although
individually rare conditions, given the decline in incidence of RhHD following the implementation of anti-D prophylaxis, up to 60% of cases of fetal anaemia are now attributable to a nonimmune cause (Abrams, Meredith et al. 2007; Amann, Geipel et al. 2011). IUT may be used to treat nonimmune fetal anaemia, regardless of the underlying aetiology (Brennand and Cameron 2008; Roberts 2008; Habli, Lim et al. 2009; Bonvicini, Puccetti et al. 2011).

1.5.2 Infectious Causes of Fetal Anaemia

Human parvovirus (hPV) B19 is the most common infectious cause of fetal anaemia (Brennand and Cameron 2008). hPV B19 is a small DNA virus that is transmitted via respiratory secretions or transplacentally from mother to fetus. Only 1 to 2% of women susceptible to hPV B19 become infected during pregnancy and transplacental transmission occurs in approximately 30% of cases (Lamont, Sobel et al. 2011). Most infants suffer no adverse effects in utero and are born healthy. However, 40% of fetuses develop signs of congenital infection, usually following infection in the second trimester (Bonvicini, Puccetti et al. 2011). Exposure to hPV B19 at this gestation can result in infection of the liver, the main site of fetal haematopoiesis, leading to apoptosis of precursor red blood cells and development of severe fetal anaemia. Without treatment, cardiac failure, hydrops and fetal death can result (Bonvicini, Puccetti et al. 2011; Lamont, Sobel et al. 2011).

Other causes of congenital infection, including toxoplasmosis, cytomegalovirus, rubella and Treponema pallidum may also result in fetal anaemia albeit of milder severity than that seen with hPV B19 (Hernandez-Andrade, Scheier et al. 2004; Yinon, Farine et al.). Fetal anaemia may also result from malarial infection in areas in which this organism is endemic (Steketee, Nahlen et al. 2001). Malarial infection is more frequent and severe in pregnant women due to pregnancy-related depression of cell-mediated immunity. An estimated 24 million pregnant women are infected with malaria each year in Sub-Saharan Africa, with between 75,000 and 200,000 infant deaths annually attributed to malarial infection during pregnancy (Steketee, Nahlen et al. 2001; Uneke 2007).

1.5.3 Haemorrhagic Causes of Fetal Anaemia

Fetal anaemia may be caused by acute or chronic loss of blood from the fetal to the maternal circulation via a breach in the placental circulation. Although leakage of a small amount of fetal blood is thought to occur in almost all pregnancies, fetomaternal haemorrhage severe enough to cause fetal anaemia is thought to be a rare event (Ahmed and Abdullatif 2011).
The antenatal diagnosis of fetomaternal haemorrhage relies upon maternal reporting of decreased or absent fetal movements, detection of a sinusoidal trace on cardiotocograph and evidence of hydrops fetalis on ultrasound. In the setting of chronic fetomaternal haemorrhage, these are late clinical manifestations indicative of severe fetal anaemia (Giacoia 1997). The presence of fetal red blood cells in the maternal circulation can be confirmed with a Kleihauer test and methods exist to allow estimation of the volume of fetal blood lost to the maternal circulation (Fliegner, Fortune et al. 1987). IUT has been used with success to treat fetomaternal haemorrhage (Giacoia 1997).

Twin to twin transfusion syndrome is a serious complication which affects 10 to 20% of monochorionic twin gestations and accounts for 17% of perinatal mortality associated with twin pregnancies (Crombleholme 2003; Habli, Lim et al. 2009). Twin to twin transfusion syndrome occurs when there is unequal sharing of fetal blood through vascular anastomoses, resulting in an anaemic, hypoperfused “donor” fetus and hypervolaemic, polyuric “recipient” fetus. These haemodynamic alterations result in complex hormonal and biochemical changes as each fetus attempts to restore euvoalaemia (Habli, Lim et al. 2009). Twin to twin transfusion syndrome is managed using techniques such as amnioreduction, fetal surgery and fetoscopic laser photocoagulation. A recent Cochrane review recommended that this latter technique be considered for the management of all cases of twin to twin transfusion syndrome, to optimise perinatal outcome (Roberts, Neilson et al. 2008). While it is not the mainstay of management, in certain circumstances IUT of the anaemic twin may also be performed (Quarello, Stirnemann et al. 2008; Herway, Johnson et al. 2009).

1.5.4 Haematological Causes of Fetal Anaemia
Fetal anaemia may be caused by disorders of the red blood cell which lead to either excessive loss or underproduction of erythrocytes (Arcasoy and Gallagher 1995). The most common haematological cause of excessive erythrocyte loss leading to anaemia in the fetus is α-thalassaemia. This genetic disorder involves deletion of α-globin genes from the short arm of chromosome 16 and occurs mainly in individuals of South-East Asian, Middle Eastern and Mediterranean descent. Loss of three of the four α-globin genes leads to Haemoglobin H disease, which causes moderate fetal anaemia and significant neonatal hyperbilirubinaemia. Homozygous α-Thalassaemia occurs when there is loss of all four α-globin genes, leading to severe intrauterine anaemia and hydrops fetalis. These infants may
survive with early diagnosis and institution of IUT plus ongoing postnatal transfusion, although usually the outlook is bleak (Steiner and Gallagher 2007). Other disorders which lead to excessive loss of red blood cells include cell membrane defects such as hereditary spherocytosis and hereditary elliptocytosis, although most infants with these conditions present with hyperbilirubinaemia in the neonatal period rather than fetal anaemia (Roberts 2008). Similarly, red blood cell enzyme defects such as glucose-6-phosphate dehydrogenase deficiency and pyruvate kinase deficiency frequently cause haemolysis in the neonate but only rarely lead to fetal anaemia (Arcasoy and Gallagher 1995).

Disorders of red blood cell underproduction which may lead to fetal anaemia are uncommon and may be associated with other somatic abnormalities. For example, Diamond-Blackfan Anaemia, Shwachman-Diamond Syndrome and Fanconi’s Anaemia are rare genetic conditions associated with pure red cell aplasia or bone marrow failure plus a variety of dysmorphic features and abnormalities of other organ systems. These conditions usually become apparent in the neonatal period although they have been reported to result in fetal anaemia and hydrops fetalis (Steiner and Gallagher 2007). Rarely, red cell underproduction may also occur due to conditions which cause replacement of fetal liver and bone marrow, for example, transient myeloproliferative disorder and true congenital leukaemias (Arcasoy and Gallagher 1995).

1.6 Anaemia of Prematurity
1.6.1 Background
All infants experience a decline in haemoglobin concentration in the first few weeks after birth. However, this decline is more rapid and severe in infants born preterm and of low birth weight (Luban 2008; Widness 2008; Whyte and Kirpalani 2011). Although changing management practices in recent years have resulted in a reduction in the number of red blood cell transfusions given to preterm infants, at least 90% of infants of birth weight less than 1000 g require transfusion during their neonatal course (Strauss 2010). The aetiology of anaemia in preterm infants is multifactorial, including physiologic factors such as rapid postnatal growth, impaired erythropoietin response, reduction in maternally derived iron stores and decreased red blood cell survival. Nonphysiologic factors also contribute significantly, particularly blood loss due to repeated phlebotomy and clinical problems such as sepsis and inadequate nutrition (Widness 2008; Strauss 2010).
1.6.2 Consequences of Anaemia of Prematurity

Anaemia may have metabolic and haemodynamic consequences in preterm infants. Decreased haemoglobin concentration leads to a reduction in oxygen carrying capacity which may, in turn, compromise oxygen supply to tissues. Lactic acidaemia may occur in response to tissue hypoxia (Bard, Fouron et al. 1998; Fredrickson, Bell et al. 2011). Compensatory haemodynamic changes have also been documented in anaemic preterm infants, including increased cardiac output manifest by elevated heart rate and stroke volume, increased left ventricular end systolic and diastolic diameters and higher myocardial contractile state (Lachance, Chessex et al. 1994; Alkalay, Galvis et al. 2003; Cambonie, Matecki et al. 2007; Fredrickson, Bell et al. 2011). However, co-existing cardiac or respiratory difficulties are common in the preterm infant and may not only compromise this compensatory response but also increase oxygen consumption (Bard, Fouron et al. 1998). Therefore, it is not possible to define a haemoglobin concentration below which oxygen carrying capacity may be inadequate. However, clinical problems such as apnoea, poor weight gain and congestive cardiac failure result when haemoglobin concentration falls beyond the point at which compensatory responses can maintain adequate cardiorespiratory function (Whyte and Kirpalani 2011).

No long-term consequences of anaemia of prematurity have been documented (Whyte and Kirpalani 2011). However, anaemia of prematurity occurs at a time when corrected gestational age is similar to that of fetuses who become anaemic due to RhHD. Furthermore, the short-term cardiovascular adaptations that occur in the anaemic preterm infant are similar to those seen in the anaemic fetus (Copel, Grannum et al. 1989). Therefore, it is plausible that there may be long-term cardiovascular consequences for preterm infants exposed to anaemia in the first few weeks after birth.

1.6.3 Red Blood Cell Transfusion for Anaemia of Prematurity

Red blood cell transfusion is the mainstay of treatment for anaemia of prematurity. Several studies have documented improvement in haemodynamic parameters of preterm infants following transfusion, including reduction in cardiac output and improved myocardial contractility, with the degree of change dependent upon the severity of anaemia (Lachance, Chessex et al. 1994; Bard, Fouron et al. 1998; Alkalay, Galvis et al. 2003; Cambonie, Matecki et al. 2007; Fredrickson, Bell et al. 2011). However, there are conflicting reports regarding the utility of transfusion for improvement in clinical outcomes, such as reduction in apnoea frequency and weight gain (Stockman and Clark 1984; Joshi, Gerhardt et al.
The risks of transfusion to the preterm infant must also be considered. Transfusion related infection is now rare due to rigorous testing of donated blood (Luban 2008). However, transfusion of preterm infants has been associated with iron overload, increased rates of retinopathy of prematurity, bronchopulmonary dysplasia and necrotising enterocolitis, as well as increased overall mortality (Englert, Saunders et al. 2001; Hirano, Morinobu et al. 2001; Mally, Golombek et al. 2006; Valieva, Strandjord et al. 2009; Dos Santos, Guinsburg et al. 2011). Transfusion may also suppress production of erythropoietin, thereby compromising the innate haematological response of the infant to anaemia (Keyes, Donohue et al. 1989).

Due to these concerns, a more restrictive approach to transfusion of the preterm infant has been adopted in recent years (Crowley and Kirpalani 2010). However, as there is no defined threshold of haemoglobin concentration at which transfusion is required, transfusion practices among neonatal units continue to vary (Alkalay, Galvis et al. 2003; Cambonie, Matecki et al. 2007). A recent Cochrane review assessed the evidence for the use of a low versus high haemoglobin transfusion thresholds in very low birth weight infants (Whyte and Kirpalani 2011). Four trials comparing restrictive (low) to liberal (high) transfusion strategies were assessed, including a total of 614 infants (Connelly, Stone et al. 1998; Bell, Strauss et al. 2005; Kirpalani, Whyte et al. 2006; Chen, Tseng et al. 2009). A further trial of 56 infants which compared transfusion for clinical signs of anaemia with transfusion at a predetermined haemoglobin concentration was also assessed (Blank, Sheagren et al. 1984). The authors concluded that although use of a restrictive transfusion strategy was associated with a modest reduction in transfusion episodes, there was no clear evidence of risk or benefit to either strategy for any clinical outcome. However, they cautioned that the risk of neurosensory deficit at 18 months was based on a single trial powered for short-term outcomes only, thus the possibility exists that this risk was underestimated (Whyte and Kirpalani 2011). In addition, post-hoc analysis of this data suggested a significantly increased risk of cognitive delay with use of the restrictive transfusion strategy (Whyte, Kirpalani et al. 2009). Conversely, a recently published study comparing neurocognitive outcomes of 11 and 12 year old children born preterm suggested worse outcome for those treated with a liberal transfusion strategy (McCoy, Conrad et al. 2011). Given the uncertainties that remain, the Cochrane review authors recommend that haemoglobin concentration in preterm infants should not be allowed to fall below those tested within the
current literature and that further studies investigating the impact of transfusion strategies on long-term outcome are required (Whyte and Kirpalani 2011).

Thus, the available evidence regarding short-term outcomes provides no clear guidance as to the threshold at which preterm infants should be transfused. Whether exposure to anaemia in the early neonatal period results in increased cardiovascular risk in the long-term may have important implications for transfusion thresholds for the preterm infant.

1.7 Cardiovascular Magnetic Resonance Imaging
1.7.1 Historical Background
Nuclear magnetic resonance first emerged as a medical imaging tool in the 1970s. Paul Lauterber and Peter Mansfield laid the foundations for Magnetic Resonance Imaging (MRI) in 1973 when they independently demonstrated that magnetic field gradients could be used for the spatial localisation of nuclear magnetic resonance signals (Geva 2006). However, it took more than a decade before the technique had been refined adequately to allow widespread clinical application. The potential use of MRI for cardiac imaging was recognised early, with a considerable amount of work in the 1980s focused on improving image quality, reducing imaging time and developing new MRI techniques for measuring blood flow and assessing myocardial viability. First pass myocardial perfusion imaging, stress imaging and assessment of coronary flow became possible in the 1990s (Geva 2006; Pohost 2008). Cardiovascular magnetic resonance imaging (CMR) continues to undergo rapid technological advancement and can now be used to assess ventricular function, cardiac morphology, coronary perfusion, non-coronary vascular anatomy, myocardial viability and cardiac metabolism, prompting some authors to refer to it as the “one-stop-shop” for cardiac imaging (Pohost 2008; Macovski 2009).

1.7.2 Physics of Magnetic Resonance Imaging
MRI is based on the detection of protons within the body using very strong magnets, with fields up to 60,000 times the strength of the earth’s magnetic field. When a patient enters an MRI scanner, protons within hydrogen nuclei align with the axis of the magnetic field. Small magnetic field pulse sequences are then applied to alter this alignment, resulting in signals which are detected by the scanner and processed to produce an image. The type of pulse sequence applied is dependent upon the anatomical site to be imaged and the purpose of the imaging (Hundley, Bluemke et al. 2010).
The magnetic field strength of commercially available MRI scanners is typically from 1.5 to 3 Tesla (T). The use of a higher magnetic field strength results in stronger signals, which produce images of greater spatial resolution with reduced signal to noise ratio. However, inhomogeneity of the area being imaged can be exacerbated by the use of higher field strengths, leading to excessive artefact which may negate the advantage of higher spatial resolution (Bandettini and Arai 2008; Hundley, Bluemke et al. 2010).

1.7.3 Uses of Cardiovascular Magnetic Resonance Imaging
CMR is used in the diagnosis and assessment of a variety of cardiovascular conditions including cardiac failure, cardiomyopathy, myocarditis, valvular heart disease, congenital heart disease, pericardial disease and abnormalities of non-coronary thoracic vascular structures (Pilz, Heer et al. 2009; Hundley, Bluemke et al. 2010). In addition, a single CMR investigation has the unique ability to noninvasively assess the so-called coronary artery disease “diagnostic triad” of ventricular function, myocardial viability, and myocardial perfusion (Pilz, Heer et al. 2009).

1.7.4 Assessment of Ventricular Function
CMR evaluation of ventricular function involves analysis of a number of parameters, including qualitative assessment of ventricular wall thickness and quantitative assessment of ventricular volumes, ejection fraction and mass. End diastolic and end systolic volumes are determined at the phase of the cardiac cycle with maximum and minimum ventricular volumes respectively, with stroke volume calculated as the difference between these two values. Ejection fraction is then derived by dividing stroke volume by end diastolic volume. These parameters can be ascertained for both the left and right ventricles (Sarwar, Shapiro et al. 2008).

In a clinical setting, this information is usually obtained by the use of transthoracic echocardiography, which is widely available, portable and does not require exposure to ionising radiation (Pilz, Heer et al. 2009). However, echocardiography is limited by dependence upon a number of assumptions regarding the geometry of the left ventricle. Significant variability in ventricular measurements can also occur through intra- and interobserver variability or from anatomical limitations resulting in difficulties obtaining images (Sarwar, Shapiro et al. 2008; Pilz, Heer et al. 2009).
By comparison, CMR has been shown in both animal and human models to be a highly accurate tool for the assessment of ventricular volume and ejection fraction, with excellent reproducibility and low intra- and interobserver variability (Longmore, Klipstein et al. 1985; Stratemeier, Thompson et al. 1986; Markiewicz, Sechtem et al. 1987; Sechtem, Pflugfelder et al. 1987; Semelka, Tomei et al. 1990; Mogelvang, Stokholm et al. 1992; Lorenz, Walker et al. 1999; Koch, Poll et al. 2000; Grothues, Smith et al. 2002). CMR acquires three-dimensional images of the ventricles that do not rely on geometric assumptions. In addition, CMR provides improved spatial resolution in comparison to echocardiography, with clear definition of myocardial borders (Sarwar, Shapiro et al. 2008; Pilz, Heer et al. 2009). These factors result in precise determination of ventricular size and function, allowing small alterations in cardiac mass or volume to be detected (Grothues, Smith et al. 2002; Natori, Lai et al. 2006). Thus, smaller sample sizes can be used in research studies due to the improved accuracy and reproducibility of these measurements (Bellenger, Davies et al. 2000; Sarwar, Shapiro et al. 2008; Hundley, Bluemke et al. 2010). Taken together, these factors have led to the use of CMR as the current reference standard for assessment of ventricular function (Sarwar, Shapiro et al. 2008; Pilz, Heer et al. 2009; Heydari, Jerosch-Herold et al. 2011).

1.7.5 Assessment of Myocardial Viability

Myocardial viability is assessed through analysis of images taken 10 to 20 minutes after the administration of gadolinium. As gadolinium diffuses more slowly from infarcted tissue than healthy myocardium, these so-called “late gadolinium enhancement” (LGE) images can be used to detect the extent and location of infarcted myocardial tissue (Kim, Fieno et al. 1999; Kwong, Schussheim et al. 2003; Ingkanisorn, Rhoads et al. 2004; Hundley, Bluemke et al. 2010). Infarct related myocardial changes can be seen as early as one hour following onset of acute myocardial infarction and allows distinction between ischaemic but viable myocardial tissue and nonviable infarcted tissue, thereby assisting decision making regarding the use of reperfusion therapy (Pilz, Heer et al. 2009; Hundley, Bluemke et al. 2010). Assessment of the location of scar tissue within the myocardial wall by LGE imaging can also help distinguish myocardial damage due to coronary artery disease from other causes, particularly myocarditis (Arai 2007; Assomull, Lyne et al. 2007). In a nonacute setting, the presence of LGE on CMR imaging provides evidence of prior myocardial infarction and has important prognostic implications (Heydari, Jerosch-Herold et al. 2011).
1.7.6 Myocardial Perfusion Imaging

1.7.6.1 Comparison of cardiac magnetic resonance perfusion assessment with other imaging modalities

The first measurements of myocardial blood flow (MBF) in man were made over 60 years ago by Bing et al, who plotted nitrous oxide clearance from simultaneous arterial and coronary sinus blood samples following nitrous oxide inhalation (Bing, Hammond et al. 1949). Subsequent improvements were made to this method over the ensuing 20 years, including use of a variety of molecular tracers (Klocke 1976). However, poor temporal resolution meant that it was not possible to measure rapid changes in flow with this technique and its use was poorly validated in the presence of coronary artery disease (Marcus, Wilson et al. 1987). In 1971, Ganz and Swan proposed measurement of myocardial blood flow by thermodilution (Ganz, Tamura et al. 1971). This technique was easier, cheaper and more widely available than gas clearance methods, but it also performed poorly in validation studies under clinically relevant conditions, especially in severe coronary disease (Marcus, Wilson et al. 1987). Intracoronary Doppler flow probes have also been used to assess changes in MBF velocity, although this technique does not allow absolute quantification of myocardial flow (Marcus, Wright et al. 1981). A further important drawback to these techniques is that they all require cardiac catheterisation (Marcus, Wilson et al. 1987).

More recently, efforts have focused on the development of noninvasive techniques that allow blood flow to be assessed qualitatively, using visual analysis of areas of relative hypoperfusion, and/or semi-quantitatively, by assessment of the mean signal intensity within a region of interest over time. Some also allow absolute quantification of MBF in ml.min\(^{-1}\).g\(^{-1}\) (Lee and Johnson 2009). Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are both well validated for qualitative and semi-quantitative assessment of MBF (Hutchins, Schwaiger et al. 1993; He, Iskandrian et al. 1997). Quantitative assessment is also possible with PET and is commonly regarded as the gold standard for quantification of MBF (Kaufmann and Camici 2005). However, both these techniques involve exposure to ionising radiation and have relatively poor spatial resolution, limiting their ability to detect subendocardial defects (Salerno and Beller 2009).

Contrast echocardiography offered promise as an alternative noninvasive technique for measurement of MBF, using contrast agents containing small, gas-filled microbubbles which can be detected in an acoustic field. MBF is related to the speed of replenishment of
microbubbles following administration of a high mechanical index pulse which destroys the bubbles in the imaging plane (Salerno and Beller 2009). This real-time, bedside technique involves no exposure to ionising radiation, has improved spatial resolution compared with SPECT and PET, and can be used for absolute quantification of MBF (Vogel, Indermuhle et al. 2005; Lee and Johnson 2009). However, the quality of images obtained is operator dependent and can be affected by body habitus, respiratory motion and lung disease. Furthermore, there are currently no approved contrast echocardiography agents available, thus clinical application of this technique is substantially limited (Salerno and Beller 2009).

Computed tomography (CT) angiography can also be used to produce high-resolution qualitative perfusion analysis. This technique has the advantage that assessment of ventricular function and cardiac anatomy can be undertaken with MBF analysis in a single study (Salerno and Beller 2009). Quantitative assessment of MBF using dynamic CT studies has also been achieved in animals, although this technology has not yet been applied to humans (George, Jerosch-Herold et al. 2007). However, use of CT for assessment of MBF is limited by exposure to high doses of ionising radiation, especially if images are obtained both at rest and with stress (Lee and Johnson 2009).

In recent years, first-pass CMR perfusion imaging has emerged as an alternative tool for the assessment MBF, demonstrating several important advantages over other noninvasive techniques. Firstly, CMR perfusion imaging has higher spatial resolution, allowing assessment of transmural blood flow and detection of subendocardial defects (Muehling, Jerosch-Herold et al. 2004). Secondly, this technique is reproducible, largely operator independent, and good intra- and interobserver agreement has been reported for semi-quantitative and quantitative analysis (Muhleng, Dickson et al. 2001; Larghat, Maredia et al. 2013). Thirdly, the entire study, including rest and stress perfusion, together with ventricular function, anatomy and viability analysis, can be performed in less than 60 minutes, thus providing rapid and comprehensive cardiovascular assessment with a single investigation without exposure to ionising radiation (Salerno and Beller 2009).

Several studies have compared the diagnostic performance of CMR perfusion analysis with other imaging modalities. CMR perfusion analysis compares favourably with invasive coronary angiography for detection of significant coronary artery disease, with overall sensitivity of 89% and specificity of 81% (Gerber, Raman et al. 2008). In addition, CMR perfusion assessment of coronary flow reserve shows good correlation with the gold standard of fractional flow reserve assessed by coronary angiography (Rieber, Huber et al.
2006). Compared to PET, semi-quantitative method CMR perfusion analysis tended to underestimate coronary flow reserve, but diagnostic accuracy was acceptable for localisation of coronary artery disease (Ibrahim, Nekolla et al. 2002). A more recent comparison of PET using quantitative methods has demonstrated equivalent findings to MBF analysis by CMR, with minimal intra-subject variability when studies were repeated one year apart (Jerosch-Herold, Vazquez et al. 2008). Furthermore, in a large multi-centre, multi-operator trial, CMR perfusion analysis performed at least as well as SPECT for the detection of coronary artery disease (Schwitter, Wacker et al. 2008).

1.7.6.2 First-pass imaging technique
First-pass myocardial perfusion imaging by CMR involves rapid dynamic imaging to observe the transit of contrast agent through the heart in the first few seconds after bolus contrast injection (Gerber, Raman et al. 2008). Gadolinium is the contrast agent of choice. Following rapid intravenous injection, contrast is first delivered to the right heart, followed by the left heart before finally perfusing the myocardium. The intensity of contrast signal achieved within the myocardium is directly proportional to myocardial perfusion, which is in turn directly proportional to coronary blood flow. Therefore, delayed diffusion of contrast indicates myocardial ischaemia (Pilz, Heer et al. 2009).

For the purpose of myocardial perfusion imaging, the heart is typically imaged in the short axis view using three to five T1-weighted slices for complete coverage of the ventricles (Pilz, Heer et al. 2009; Hundley, Bluemke et al. 2010). Problems associated with cardiac and respiratory motion are overcome by the use of short image acquisition times and the technique of gating, which synchronises image acquisition with the cardiac cycle (Jerosch-Herold and Kwong 2008).

1.7.6.3 Stress imaging
At rest, coronary autoregulation maintains MBF even in the face of significant coronary artery stenosis. However, under conditions of physiological stress with associated coronary vasodilation, the ability to compensate for the presence of coronary stenosis may be exhausted. Therefore, assessment of myocardial perfusion by CMR requires imaging to be performed under conditions of both rest and stress (Jerosch-Herold and Muehling 2008; Hundley, Bluemke et al. 2010). Stress is usually induced by infusion of adenosine or dipyridamole (Heydari, Jerosch-Herold et al. 2011). Adenosine acts directly on adenosine receptors in the coronary vasculature, and dipyridamole blocks reuptake of adenosine into endothelial cells, increasing extracellular concentrations. In non-ischaemic myocardium the
Adenosine is the most widely used agent for induction of stress for CMR perfusion studies. Both adenosine and dipyridamole commonly result in similar side effects, but adenosine has a half-life of less than ten seconds whereas dipyridamole acts over 30 minutes. Therefore, if significant side effects occur, termination of the infusion will usually suffice if adenosine has been used, whereas reversal of drug action with aminophylline is often required if dipyridamole has been used. In addition, use of adenosine results in reduced drug administration and monitoring costs compared to dipyridamole (Pennell 2004; Miyamoto, Vemotico et al. 2007). Most protocols involve intravenous infusion of adenosine at a rate of 0.14 mg.kg.\(^{-1}\)min\(^{-1}\) for a minimum of three minutes in order to induce maximal vasodilation, with contrast injection and imaging performed towards the end of the infusion (Gerber, Raman et al. 2008; Pilz, Heer et al. 2009). The use of separate intravenous lines for the administration of contrast and adenosine is usually recommended to avoid delivery of a small bolus of adenosine that may precipitate transient atrioventricular (AV) block (Gerber, Raman et al. 2008; Jerosch-Herold and Muehling 2008). However, if this occurs it is usually short-lived and self-limiting. Therefore, the use of a single intravenous line has been validated as a safe alternative and is now widely used, particularly for outpatient or day stay scanning (Latus and Underwood 2001; Kawai and Kishino 2006; Miyamoto, Vemotico et al. 2007; Jerosch-Herold, Vazquez et al. 2008; Henzlova, Cerqueira et al. 2009). Patients are monitored with continuous three lead electrocardiogram (ECG) and blood pressure is recorded every minute throughout the infusion (Henzlova, Cerqueira et al. 2009).

As the effect of adenosine is competitively inhibited by methylxanthines, including caffeine, aminophylline and theophylline, these substances must be withheld for at least 24 hours prior to stress perfusion scanning. If required, adverse effects of adenosine can be managed by administration of aminophylline (Henzlova, Cerqueira et al. 2009).

1.7.6.4 Adverse effects and safety of adenosine
Adenosine exerts its vasodilatory effect on the coronary blood vessels by binding to A2a receptors in the coronary endothelium. However, several other adenosine receptor subtypes are present in other tissues throughout the body and activation of these receptors may lead
to unwanted adverse effects. The most common are due to peripheral vasodilation caused by activation of the A2b receptor and occur in up to 80% of patients (Cerqueira, Verani et al. 1994). Peripheral vasodilation leads to a small increase in heart rate and fall in systolic blood pressure, often associated with dizziness, flushing, headache, nausea, shortness of breath and non-specific chest discomfort. These adverse effects are well tolerated and resolve within a few seconds of discontinuation of the infusion (Latus and Underwood 2001; Henzlova, Cerqueira et al. 2009). They are so common that the complete absence of vasodilator symptoms should raise the possibility of unreported caffeine intake prior to scanning or failure of adenosine administration (Miyamoto, Vemotico et al. 2007).

More significant but less common adverse effects of adenosine infusion are AV block and bronchospasm, which result from stimulation of A1 and A3 receptors. In the Adenoscan trial, a multicentre study of 9,256 subjects conducted to determine the safety of adenosine infusion in conjunction with radionuclide imaging, significant bronchospasm occurred in 12 (0.1%) and AV block in 706 (7.6%) of patients. Seven episodes of bronchospasm were classified as severe and required administration of aminophylline. All episodes of AV block terminated spontaneously or with reduction or cessation of adenosine infusion. No deaths occurred in association with adenosine infusion. These authors concluded that adenosine infusion is safe and although mild adverse effects are common, they are generally well tolerated. They also noted that serious adverse effects are rare and can usually be managed by termination of adenosine infusion (Cerqueira, Verani et al. 1994). An earlier study by Lee and colleagues noted that second or third degree AV block occurred in 6.4% of patients undergoing adenosine infusion for thallium imaging and was transient and well tolerated in almost all patients. In keeping with the findings from the Adenoscan trial, there were no differences in the occurrence of ischaemia or myocardial perfusion defects on imaging between patients who experienced AV block and those who did not, suggesting that AV block occurs due to the effect of adenosine on the AV node rather than as a manifestation of ischaemia (Lee, Heo et al. 1992; Cerqueira, Verani et al. 1994).

Thus, the available evidence suggests that serious adverse effects as a result of adenosine infusion are rare and usually easily managed by discontinuation of the infusion. However, both these studies excluded patients with active asthma or second or third degree AV block. Therefore, most centres continue to exclude patients with these pre-existing conditions from adenosine infusion protocols. However, two recent studies have investigated the use of a step-wise adenosine infusion protocol in conjunction with inhaled salbutamol administered
immediately prior to commencing adenosine infusion. Patients with mild to moderate asthma requiring regular inhaled medication with or without oral steroids or with mild chronic obstructive airways disease were included. Both studies found that adenosine infusion was well tolerated in these patients with no increase in the incidence of bronchospasm or other adverse events compared to controls. The authors concluded that adenosine can be safely administered to patients with asthma if a modified infusion protocol is used (Reyes, Loong et al. 2007; Sundram, Notghi et al. 2009).

In an attempt to decrease the incidence of adverse effects from the administration of adenosine, attention has focused on development of a specific A2a adenosine receptor agonist. The agent with most promise is Regadenoson, which produces a comparable haemodynamic response in coronary arteries with a simplified fixed-dose bolus administration protocol and significant reduction in adverse effects compared to adenosine (Botvinick 2009; Henzlova, Cerqueira et al. 2009; Mekkaoui, Jadabaie et al. 2009). However, the safety of this agent in patients with bronchospasm, the effect of caffeine and the incidence of AV block are still unknown (Botvinick 2009). Although approved by the Federal Drug Agency in the United States of America, Regadenoson is not currently available in New Zealand.

1.7.6.5 Qualitative versus quantitative analysis
Assessment of MBF from CMR images can be performed using qualitative or quantitative techniques (Heydari, Jerosch-Herold et al. 2011). Qualitative analysis involves detection of areas of hypoperfused myocardium by an experienced observer, with concomitant assessment for the presence of myocardial scars. Although potentially subjective, qualitative analysis is the standard technique for interpretation of myocardial perfusion CMR images in a clinical setting for detection of myocardial ischaemia with or without evidence of infarction (Gerber, Raman et al. 2008).

By comparison, quantitative analysis attempts to objectively quantify MBF at rest and at maximal vasodilation. This involves assessment of changes in myocardial tissue contrast enhancement using arterial contrast enhancement as a reference, measured either from the left ventricle or proximal aorta (Jerosch-Herold and Kwong 2008). In animals, this method has been shown to correlate well with measurements using microspheres (Jerosch-Herold, Swingen et al. 2002; Christian, Rettmann et al. 2004; Jerosch-Herold 2010).
of MBF by this technique has also been validated in humans (Rosen, Lima et al. 2006; Jerosch-Herold, Vazquez et al. 2008).

However, there are a number of difficulties associated with quantitative analysis of MBF. Enhancement of myocardial tissue may be influenced by variations in the rate, amount and timing of delivery of contrast agent in blood, factors collectively referred to as the arterial input function. Complex mathematical “deconvolution” models have been devised to account for the effect of these factors on tissue contrast enhancement, in order to estimate the mean transit time of contrast through the coronary vessels (Gerber, Raman et al. 2008). In addition, quantitative analysis of MBF requires identification of a specific “region of interest” in the myocardium, to allow the dynamic changes in MRI signal intensity to be followed. Movement artefact from breathing and the presence of premature ventricular beats can make reliable positioning of the region of interest difficult (Gerber, Raman et al. 2008). Furthermore, the relationship between contrast concentration and signal intensity is nonlinear, such that when arterial contrast concentration is high, the signal intensity response may be saturated. When this occurs arterial contrast enhancement may be underestimated and consequently myocardial contrast enhancement overestimated. Several methods have attempted to overcome this difficulty, for example using lower contrast doses, dual contrast sequences or dual contrast bolus protocols or by correcting retrospectively for signal saturation (Jerosch-Herold and Kwong 2008; Jerosch-Herold 2010).

Myocardial perfusion can be quantified using a number of different mathematical models, which all involve the use of various mathematical assumptions and simplifications of vascular spaces and haemodynamics that can adversely influence the accuracy of blood flow estimation. The four most commonly used methods (Fermi function modelling, two-compartment modelling, model-independent analysis and Patlak one-compartment plot analysis) have been validated by comparison with PET or Doppler ultrasound measurements of MBF (Koskenvuo, Sakuma et al. 2001; Parkka, Niemi et al. 2006; Fritz-Hansen, Hove et al. 2008; Kurita, Sakuma et al. 2009), but due to the different mathematical assumptions inherent within them, quantitative MBF results from one model may not be directly comparable with those from another, especially when analysis is undertaken by different research groups (Pack and DiBella 2010). Thus, a standardised protocol for the quantitative assessment of myocardial perfusion is yet to be achieved, and consequently quantitative analysis of myocardial perfusion has not been adopted into mainstream clinical practice. However, there is widespread acceptance and use of these techniques in research settings.
and work is ongoing towards optimisation and standardisation of analysis models (Rosen, Lima et al. 2006; Wang, Jerosch-Herold et al. 2006a; Knaapen, Camici et al. 2009; Jerosch-Herold 2010).

1.7.7 Prognostic Utility of Cardiac Magnetic Resonance Imaging

In the setting of suspected coronary artery disease, CMR has been shown to have powerful prognostic utility (Heydari, Jerosch-Herold et al. 2011). For example, in a study of 135 patients with acute chest pain in whom myocardial infarction had been excluded by serial cardiac enzymes, CMR stress perfusion imaging predicted a diagnosis of coronary artery disease or major cardiovascular event at one year with a sensitivity of 100% and specificity of 93%, and a negative predictive value of 100% (Ingkanisorn, Kwong et al. 2006). Similarly, in a study of 218 patients presenting with suspected coronary artery disease, normal CMR stress perfusion imaging had a negative predictive value of 99.1% for major adverse cardiac events at one year. Thus CMR is a reliable noninvasive alternative to coronary angiography (Pilz, Jeske et al. 2008), and is as effective and reliable for stratifying disease risk in women as it is in men presenting with suspected coronary artery disease (Coelho-Filho, Seabra et al. 2011).

Late gadolinium enhancement (LGE) in 195 patients presenting with chest pain but no history of myocardial infarction was associated with an 8-fold increase in risk of major cardiac event and a 11-fold increased risk of cardiac death at a median follow up of 16 months. LGE was the strongest predictor of major cardiac event or cardiac mortality, independent of ventricular function or the presence of coronary stenosis on angiography (Kwong, Chan et al. 2006). Furthermore, in 1200 patients undergoing CMR for investigation of suspected coronary artery disease, both myocardial ischaemia on CMR stress perfusion imaging and the presence of LGE were predictors of cardiac death or myocardial infarction (Krittayaphong, Chaithiraphan et al. 2011).

1.7.8 Safety Issues and Limitations of Cardiac Magnetic Resonance Imaging

1.7.8.1 Safety concerns

1.7.8.1.1 Ferromagnetic objects

As the magnetic field of an MRI scanner is always “on”, any ferromagnetic object which enters the static magnetic field of the device will be attracted to the magnetic core. Thus, rapidly moving metallic projectiles may pose a potentially lethal danger to patients or staff
within the scanning room. To avoid this, all MRI units have strict safety guidelines to prevent the entry of ferromagnetic objects into the magnetic field of the scanner (Hundley, Bluemke et al. 2010). There is also the risk that the static magnetic field generated by the scanner will cause any ferromagnetic object within the patient’s body to move. This includes ferromagnetic components of surgically implanted devices or items which may have entered the body accidentally, such as intraocular foreign bodies, of which the patient may be unaware (Lagouros, Langer et al. 1987; Zhang, Cheng et al. 2009).

1.7.8.1.2 Implantable cardiac devices
Patients with implantable cardiac devices, such as pacemakers or defibrillators, may be vulnerable to safety risks in addition to mechanical forces on ferromagnetic components described above. Small and rapidly fluctuating gradient magnetic fields are generated during scanning and may produce electrical currents in the wires of these devices, which may in turn lead to cardiac arrhythmias. In addition, radiofrequency waves emitted by the scanner may cause heating of wires or interfere with electronic components within the device. This may cause pacemakers to reprogramme, induce over- or under-sensing of cardiac activity or lead to complete inhibition of pacemaker activity. Thus, CMR is relatively contraindicated in patients with these devices. So-called “MRI-safe” implantable cardiac devices are currently in development and initial reports are promising (Jung, Zvereva et al. 2011).

1.7.8.1.3 Gadolinium based contrast agents
Acute adverse reactions to gadolinium, including shortness of breath and allergic skin reactions, have been reported to occur in approximately 1 in 250 patients, with more than 95% mild and transient. More severe allergic reactions occur in approximately 1 in 10,000 patients (Li, Wong et al. 2006).

Nephrogenic systemic fibrosis is a rare but serious complication specifically associated with the use of gadolinium based contrast agents. It was first described in 2006 and occurs almost exclusively in patients with serious acute or chronic renal impairment (Grobner 2006; Heydari, Jerosch-Herold et al. 2011). Nephrogenic systemic fibrosis is characterised by fibrosis of the skin, typically involving the limbs, leading to flexion contractures and significant impairment of function. Fibrosis may also occur in the pleura, pericardium, lungs, joints and striated muscle (Bardin and Richette 2010). Nephrogenic systemic fibrosis may occur up to three months after exposure in gadolinium in susceptible individuals. The progression of the disease is usually relentless and may be associated with a decreased life expectancy. In susceptible patients, the incidence of nephrogenic systemic fibrosis is
estimated to be between 1% and 4%, although since first described the incidence has declined sharply due to a wider understanding of risk factors and avoidance of the use of gadolinium in patients with known renal impairment (Hellman 2011; Heydari, Jerosch-Herold et al. 2011).

The use of gadolinium based contrast agents in breast-feeding mothers raises the potential for toxicity to the infant from contrast excreted into breast milk. However, the dose of contrast an infant may absorb in this way is extremely low, with less than 0.04% of the total dose detected in breast milk in the 24 hours following administration and less than 1% of the ingested dose absorbed systemically by the infant from the gastrointestinal tract (Kubik-Huch, Gottstein-Aalame et al. 2000). The European guidelines on the use of gadolinium based contrast agents in pregnancy and lactation conclude that the extremely low risk does not warrant the disruption or cessation of breast feeding following gadolinium administration to a breast-feeding mother (Webb, Thomsen et al. 2005).

1.7.8.2 Claustrophobia
Acquisition of CMR images may be hampered by claustrophobia, which affects approximately 2.3% of all patients undergoing MRI scans (Dewey, Schink et al. 2007). Newer “short-bore” and “open” scanners do not reduce the rate of claustrophobia reported by patients (Enders, Zimmermann et al. 2011).

1.7.8.3 Image artefact
CMR images may be distorted by artefact, which does not have an anatomical basis but results from poor spatial or temporal resolution, technical factors which cause image blurring or mis-triggering of image acquisition, or motion from patient movement, breathing or heart pulsation (Stadler, Schima et al. 2007). In particular, CMR perfusion imaging can be impaired by so-called “dark rim” artifact, which appears transiently when the gadolinium contrast bolus enters the left ventricle. The resulting endocardial dark rim is thought to be due to myocardial motion, limited spatial resolution and/or changes in the magnetic field associated with gadolinium. This artefact is easily detected on qualitative analysis as it disappears with the contrast bolus, but it can cause difficulties with semi-quantitative or quantitative analysis (Di Bella, Parker et al. 2005). Thus the quality of images acquired by CMR is dependent upon the ability of those undertaking the examination to detect and eliminate potential sources of artefact (Stadler, Schima et al. 2007).
1.7.8.4 Cost and availability
CMR is an expensive investigation which requires not only access to specialised MRI devices but also staff with appropriate skill and training to operate the equipment and interpret the images obtained (Sarwar, Shapiro et al. 2008). Quantitative interpretation of myocardial perfusion data remains largely within the realm of research studies or the few centres around the world with experience in this analysis (Bandettini and Arai 2008; Knaapen, Camici et al. 2009).

1.8 Heart Rate Variability for Assessment of Cardiac Autonomic Control

1.8.1 Autonomic Control of Heart Rate
In health, heart rate is characterised by significant beat-to-beat variability, as well as fluctuations which occur over a longer time period (Sandercock, Bromley et al. 2005). Under normal physiological conditions, the main determinants of heart rate are the parasympathetic and sympathetic arms of the autonomic nervous system, which operate in a coordinated fashion. Parasympathetic activation slows heart rate through release of acetylcholine from the vagus nerve, primarily in response to changes in ventilation. Acetylcholine acts rapidly and has a short half-life, thus beat-to-beat variation in heart rate is controlled by parasympathetic influence. Conversely, sympathetic activation accelerates heart rate through release of noradrenaline, which is synthesised and metabolised more slowly. Thus fluctuations in heart rate modulated by sympathetic activity have a slower time course (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology 1996). Due to the differences in onset and duration of action of these two neurotransmitters, the two arms of the autonomic system operate at different frequencies, with parasympathetic activity mediating high frequency fluctuations in heart rate and sympathetic activity primarily mediating low frequency fluctuations (Pumprla, Howorka et al. 2002). Therefore, analysis of heart rate variability allows the opportunity to examine the state and integrity of the autonomic nervous system (Bilchick and Berger 2006).

1.8.2 Heart Rate Variability: Definition and Historical Perspective
The term Heart Rate Variability (HRV) is used to describe the oscillation in intervals between consecutive normal heart beats (Lahiri, Kannankeril et al. 2008). HRV is well established as a noninvasive and reliable measure of cardiac autonomic control (Schaffer, Burkhardt et al. 2008). High HRV suggests the control mechanisms of the autonomic nervous system are functioning well, providing good adaptability in a healthy individual.
Reduced HRV suggests that autonomic modulation is unable to adequately adapt to physiological perturbations, implying compromised health (Pumprla, Howorka et al. 2002).

HRV was first utilised clinically in 1963 when Hon and Lee noted that fetal distress was associated with reduced beat-to-beat variability, which preceded any detectable change in heart rate (Hon and Lee 1963). Simple and reliable techniques to assess HRV were explored in the 1970s (Luczak and Laurig 1973; Sayers 1973; Ewing, Martyn et al. 1985), and in 1978 a reduction in HRV was correlated with increased mortality following myocardial infarction (Wolf, Varigos et al. 1978); a finding subsequently confirmed by others (Kleiger, Miller et al. 1987; Malik, Farrell et al. 1989; Bigger, Fleiss et al. 1992). Reduced HRV has also been identified in patients with diabetic neuropathy, cardiac failure and hypertension (Pagani, Malfatto et al. 1988; Casolo, Balli et al. 1989; Freeman, Saul et al. 1991; Kienzle, Ferguson et al. 1992; Nolan, Flapan et al. 1992; Singh, Larson et al. 1998; Greenwood, Stoker et al. 1999; Lucini, Mela et al. 2002).

Measurement of HRV is simple and noninvasive. Two different techniques can be used to obtain the ECG traces required. Twenty-four hour ambulatory recordings can be used to generate all time and frequency domain measures of HRV (Sandercock, Bromley et al. 2005). Short-term recordings taken over 5 to 15 minutes have gained popularity due to ease of data collection and editing, as well as the ability to collect data under controlled laboratory conditions, but their re-test reliability has been questioned (Sandercock, Bromley et al. 2005), and it is not possible to accurately measure the very slow fluctuations in heart rate observed in longer recordings. Time domain measures of HRV may also be less reliable when assessed from short-term ECG recordings (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology 1996; Kleiger, Stein et al. 2005; Sandercock, Bromley et al. 2005). Despite these concerns, most authors consider short-term recording to be an accurate technique for the assessment of HRV (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology 1996; Carter, Banister et al. 2003; Kleiger, Stein et al. 2005; Lahiri, Kannankeril et al. 2008).

1.8.3 Time Domain Measures of Heart Rate Variability

Time domain analysis is based on mathematical manipulation of the R-R interval and is most commonly performed on 24-hour recordings. Accurate identification of consecutive normal QRS complexes is required to determine the interval between normal beats (NN
interval) (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology 1996). Therefore, HRV traces must be carefully edited to remove non-sinus beats and artefact. Time domain variables can then be calculated directly from the NN intervals or derived from the differences between the intervals. SDNN (the standard deviation of all NN intervals) is the measure most commonly used as an estimate of overall HRV. As SDNN increases with the length of analysed recording, it is imperative that calculations of SDNN are made from recordings of the same duration (Lahiri, Kannankeril et al. 2008). Other frequently used time domain measures include SD\(\delta\)NN (standard deviation of difference between adjacent NN intervals); RMSSD (square root of the mean of the squares of successive NN interval differences) and pNN50 (percentage of NN intervals that differ by >50 (ms) from the preceding interval) (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology 1996; Kleiger, Stein et al. 2005). As these variables are based on comparison between consecutive beats, they estimate short-term (high frequency) variation in the NN interval. Therefore, time domain measures primarily reflect parasympathetic modulation of heart rate (Bilchick and Berger 2006; Lahiri, Kannankeril et al. 2008).

1.8.4 Frequency Domain Measures of Heart Rate Variability

Frequency domain measures of HRV allow quantification of the cyclical fluctuations in NN intervals by describing how power is distributed as a function of frequency. Using the Fast Fourier Transformation algorithm, HRV data from short-term recordings yield three main spectral components: a very low frequency (VLF) band (0.003 to 0.04 Hz), a low frequency (LF) band (0.04 to 0.15 Hz) and a high frequency (HF) band (0.15 to 0.4 Hz). Analysis of longer recordings incorporating circadian rhythms allows measurement of a further ultra low frequency (ULF) spectral band. Total power is calculated from the sum of all spectral bands. The HF band is governed almost completely by parasympathetic effects and the LF band by a combination of parasympathetic and sympathetic effects (Akselrod, Gordon et al. 1981). The significance of the VLF and ULF spectra remain unclear, although they have been shown to have prognostic utility in cardiovascular disease (Bigger, Fleiss et al. 1992).

To minimise the effect of changes in total power on the LF and HF, these values are often reported as normalised units (LF\(_{nu}\) and HF\(_{nu}\)), calculated by dividing the power in each spectra by the total power minus the VLF component. The ratio of LF to HF power has been used as a measure of sympathovagal balance (Pagani, Lombardi et al. 1986; Eckberg 1997).
1.8.5 Clinical Utility of Heart Rate Variability

1.8.5.1 Myocardial infarction

Numerous studies have reported an association between reduced HRV and increased mortality following acute myocardial infarction (Kleiger, Miller et al. 1987; Bigger, Fleiss et al. 1992; La Rovere, Bigger et al. 1998; Lanza, Guido et al. 1998; Nolan, Batin et al. 1998; La Rovere, Pinna et al. 2003; Buccelletti, Gilardi et al. 2009). Large epidemiological studies of otherwise healthy subjects have linked reduced HRV to increased risk of coronary heart disease, cardiac mortality and all-cause mortality (Tsuji, Larson et al. 1996; Liao, Cai et al. 1997; Dekker, Crow et al. 2000).

Although the mechanisms underlying these relationships are unclear, it is postulated that reduced vagal tone and increased sympathetic activity lead to instability of the cardiac conducting system (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology 1996). Predominate sympathetic activity is associated with reduced HRV following acute myocardial infarction (Lombardi, Sandrone et al. 1996; Singh, Mironov et al. 1996), and diminution of sympathetic activity by β-blockade or potentiation of parasympathetic activity using low dose atropine or scopolamine increases HRV in both animals and humans (Vybiral, Bryg et al. 1990; Molgaard, Mickley et al. 1993; Adamson, Huang et al. 1994; Keeley, Page et al. 1996; Wang, Zhang et al. 2002). Assessment of HRV can be used as a predictor of mortality and arrhythmic complications following myocardial infarction (Odemuyiwa, Malik et al. 1991; Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology 1996; Vanoli, Adamson et al. 2008). However, improvement in HRV does not necessarily translate into improved survival and a causal link between reduced HRV and death has not been demonstrated (Hull, Vanoli et al. 1995; Lahiri, Kannankeril et al. 2008).

1.8.5.2 Hypertension

Reduced HRV with predominance of sympathetic activity has been demonstrated in individuals with essential hypertension, and is correlated with the severity of hypertension (Guzzetti, Piccaluga et al. 1988; Singh, Larson et al. 1998; Lucini, Mela et al. 2002). Similar changes have been detected in normotensive populations preceding the onset of overt hypertension (Singh, Larson et al. 1998). Individuals with systolic blood pressure at the upper limit of the normal range have increased LF and decreased HF components of HRV spectral power, indicating alterations in sympathovagal balance (Lucini, Mela et al. 2002).
Furthermore, young normotensive individuals with a history of hypertension in one parent demonstrated greater fluctuation in HRV markers of sympathetic modulation and reduced parasympathetic activity than age matched controls from two normotensive parents (Davrath, Goren et al. 2003). Taken together, these data suggest that autonomic function is altered prior to the onset of overt hypertension and that HRV assessment may assist with the early identification of those at risk (Malliani 2005).

1.8.5.3 Diabetic autonomic neuropathy
Autonomic neuropathy is a common complication of diabetes mellitus. It is characterised by early and widespread degradation of both sympathetic and parasympathetic function, resulting in a variety of clinical symptoms. Once clinical signs of diabetic neuropathy are apparent, 5-year mortality is approximately 50% (Ewing, Campbell et al. 1980). Thus, early detection provides an opportunity to stratify risk and potentially alter disease progression (Vinik, Maser et al. 2003). HRV analysis has been used for the assessment of autonomic function in diabetic patients for nearly 20 years, with a reduction in time and/or frequency HRV domain parameters indicative of autonomic neuropathy with cardiovascular involvement (Ewing, Neilson et al. 1984; Pagani, Malfatto et al. 1988; Freeman, Saul et al. 1991). Reduced HRV in the diabetic patient confers a two-fold relative risk of silent myocardial infarction and cardiac death (Vinik, Maser et al. 2003). Alterations in HRV also precede the onset of clinical signs of autonomic neuropathy in diabetic patients (Malpas and Maling 1990; Bellavere, Balzani et al. 1992).

1.8.6 Cardiac Autonomic Control and the Developmental Origins Hypothesis
As regulation of the cardiovascular system is dependent upon autonomic control, changes in autonomic function in response to unfavourable intrauterine conditions may be involved in the programming of adult cardiovascular disease (Galland, Taylor et al. 2006; Schaffer, Burkhardt et al. 2008).

1.8.6.1 Influence of growth restriction
Elevated levels of circulating catecholamines, indicating increased sympathetic activity, have been demonstrated in growth restricted fetuses in both animal models and human studies (Okamura, Watanabe et al. 1990; Simonetta, Rourke et al. 1997). In addition, changes to sympathovagal balance following exposure to an unfavourable intrauterine environment have been shown to persist into postnatal life. For example, increased
sympathetic nervous system activity has been demonstrated in adult rats subjected to growth restriction *in utero* (Shaul, Cha et al. 1989; Jansson and Lambert 1999).

In humans, HRV in term neonates with a birth weight <3rd centile (n=10) was indicative of alterations in both sympathetic and parasympathetic function in comparison to appropriately grown infants (n=16). (Spassov, Curzi-Dascalova et al. 1994) Increased resting sympathetic tone and reduced tachycardic reflex response to tilting were also demonstrated in small for gestational age term infants at 1 and 3 months (Galland, Taylor et al. 2006). However, in another cohort of late preterm and term infants, there was no association between size at birth and HRV. Of note, these authors also reported a significant effect of gender and gestational age on LF/HF ratio, although the direction of this effect was not described (Schaffer, Burkhardt et al. 2008).

In adult twins, low birth weight was associated with increased cardiac sympathetic activity (Ijzerman, Stehouwer et al. 2003). In addition, in a large cohort (n=179) at mean age of 26 years, small size at birth was associated with increased sympathetic and decreased parasympathetic activity only in women, suggesting a sex-specific effect on cardiac autonomic control (Jones, Beda et al. 2007).

### 1.8.6.2 Influence of prematurity

Maturation of sympathetic and parasympathetic control of the cardiovascular system continues throughout the latter half of pregnancy, with a progressive decrease in heart rate and increase in HRV with increasing gestational age. In the human fetus, the rate of change of HRV slows from the 32nd week of gestation, but spectral densities continue to increase throughout the third trimester, indicating ongoing maturation of cardiac autonomic control over this time (Van Leeuwen, Geue et al. 2003; David, Hirsch et al. 2007).

In keeping with fetal data, reduced HRV has been demonstrated in preterm infants at birth compared to term infants (Sahni, Schulze et al. 2000; Longin, Gerstner et al. 2006; Khattak, Padhye et al. 2007; Patural, Pichot et al. 2008). HRV increases from birth to term equivalent age (TEA) in preterm infants (Khattak, Padhye et al. 2007), with power increases most marked in the HF band, indicating predominant parasympathetic maturation (Sahni, Schulze et al. 2000; Longin, Gerstner et al. 2006), in keeping with the reduction in heart rate with increasing gestational age. However, others have found no significant increase in HRV in preterm infants from birth to TEA (Patural, Barthelemy et al. 2004; Patural, Pichot et al. 2008).
2008). Nonetheless, reduced HRV with relatively greater sympathetic activity and higher heart rate has been consistently documented in preterm infants at TEA, in comparison to term infants (Eiselt, Curzi-Dascalova et al. 1993; Henslee, Schechtman et al. 1997; Longin, Gerstner et al. 2006; De Rogalski Landrot, Roche et al. 2007; Patural, Pichot et al. 2008). Reduced HRV persists up to 6 months post TEA in infants born preterm (Henslee, Schechtman et al. 1997; Yiallourou, Witcombe et al. 2013). However, by 2 to 3 years of age HRV during quiet sleep is not related to gestational age at birth, suggesting that there is a phase of accelerated maturation of cardiac autonomic control in those born preterm, although differences in HRV under conditions of stress may persist in later life (De Rogalski Landrot, Roche et al. 2007).

No follow up studies of HRV in adulthood have been reported following preterm birth. However, at 9 years of age, children born preterm have higher heart rate and urinary catecholamine concentrations in response to stress than term born controls, suggesting increased sympathoadrenal activity in these individuals (Johansson, Norman et al. 2007). Moreover, juvenile sheep born preterm have increased sympathetic activity and decreased parasympathetic activity compared to term controls (Berry et al, unpublished data).

1.8.6.3 **Influence of anaemia**

No studies of HRV have been reported in the anaemic fetus or infant exposed to anaemia prior to birth. However, reduced heart reactivity on cardiotocography has been described in anaemic fetuses, with decelerations and a sinusoidal trace in severe cases (Sadovsky, Visser et al. 1988; Nicolaides, Sadovsky et al. 1989; Olofsson, Stangenberg et al. 1990; Reddy, Moulden et al. 2009). Furthermore, adult sheep exposed to intrauterine anaemia have increased baseline heart rate compared to controls (Yang, Hohimer et al. 2008). Thus, it is plausible that exposure to intrauterine anaemia may influence the maturation of cardiac autonomic control in the perinatal period.
Chapter 2: Methods

2.1 Fetal Anaemia Study

In this thesis the term Fetal Anaemia Study is used to refer to the follow up study of recipients of IUT, including physiological and cardiac MRI assessments. This study attempted to trace and recruit all individuals who received IUT at National Women’s Hospital, Auckland and were born between 1963 and 1992.

An important consideration in planning the study was selection of an appropriate control group with whom to compare the IUT recipients. We chose to recruit unaffected siblings of the IUT recipients (affected participants) in order to minimise the impact of social, familial and genetic diversity that may have confounded the outcomes of interest. An alternative comparator group could have comprised age, sex and gestation-matched individuals, which would have the advantage of ensuring that differences between groups for these variables did not confound the outcomes of interest. However, this approach would have been more complex and also more expensive, due to the much greater efforts needed to identify, trace and recruit participants who did not have a particular reason to be interested in our research question. Transport and accommodation costs would also have been more expensive, as these could frequently be shared between siblings. Further, given the relatively invasive nature of the investigations undertaken in this study, and the time required to participate, we felt that siblings would be more motivated to participate than unrelated individuals, due to their potential interest in our study question and willingness to support their affected sibling.

2.1.1 Tracing and Recruitment

2.1.1.1 Pilot study

Between February 2007 and February 2008 a pilot study was conducted by Professor Jane Harding, Drs Stuart Dalziel and Mariam Buskh, and medical student Iris Grooten. The primary aims of this study were to:

1. Assess whether adults who had received IUT for rhesus disease 20 to 40 years ago at National Women’s Hospital, and their unaffected siblings, could be located and would be willing to be involved in a follow up study;
2. Assess the practicality and cost of locating these adults;
3. Estimate the time and resources required for the main study;
4. Develop and test an MRI protocol that would provide appropriate data for the main study.
The first ever IUT for rhesus disease was performed by Sir William Liley at National Women’s Hospital in 1963. Since then, apart from a very small number of procedures performed in Christchurch and Wellington in the late 1960s, all IUTs in New Zealand have been undertaken at National Women’s Hospital. The pilot study established that from 1977, the details of all fetal interventions occurring at National Women’s Hospital were recorded in a central logbook, known as the “Special Procedures Book”, and that babies who received IUT prior to this date could be identified from the neonatal intensive care unit admission books. From this information it was possible to determine the name and date of birth of mothers of babies who had received IUTs, as well as the date of birth, sex and sometimes the name of the baby. Other databases, including the National Health Index, Electoral Roll and Births, Deaths and Marriages, were then used to obtain current contact details.

Due to resource constraints, the pilot study was limited to babies born between 1977 and 1990 and revealed that 110 fetuses received IUT for rhesus disease in this time period. Record tracing was attempted for 61 individuals; 3 (5%) had no records, 8 (13%) had died in the perinatal period, and 50 (82%) had records available. Of those 50 potential participants, 24 living in the greater Auckland area were approached to undergo an MRI scan; 7 (29%) did not respond within the available timeframe, 4 (17%) declined, and 13 (54%) were willing to participate, of whom 11 (46%) had appropriately matched and willing unaffected siblings. It was estimated that at least another 110 fetuses were treated between 1963 and 1976, before the introduction of the anti-D immunoglobulin prophylaxis programme reduced the incidence of rhesus disease. Thus, even allowing for the higher mortality of affected babies in this earlier period, the pilot study suggested that there were at least another 70 individuals with an unaffected sibling who would be willing to participate in a follow up study.

In total, 13 individuals underwent MRI for the pilot study. Those scanned included two healthy controls to finalise scan protocols, four pairs of affected and unaffected siblings, and one sibling group comprised of two affected participants and one unaffected sibling. These scans allowed development and testing of an appropriate MRI protocol in collaboration with colleagues at the Centre for Advanced MRI, University of Auckland, and the Heart Research Center, Oregon Health Sciences University, Portland, Oregon, USA. The data produced were of high quality and suitable for analysis. Participants were extremely positive about the study and tolerated the scans without difficulty. There were no negative
comments from possible participants or their mothers about being contacted up to 30 years after the original treatment.

From this pilot study it was concluded that a follow up study of IUT recipients was feasible, that approximately 80 sibling pairs were likely to take part, and that possible participants could be successfully traced, had hospital records available, were willing to participate and found the study protocol acceptable.

2.1.1.2 Tracing and recruitment protocol
Between October 2009 and February 2012 attempts were made to identify, locate and recruit to the Fetal Anaemia Study all adult survivors of IUT using the following protocol (Figure 2.1):

Figure 2.1: Summary of Fetal Anaemia Study tracing protocol

1. The mothers of fetuses who received IUT from 1977 to 1992 were identified from the Special Procedures Book. 1992 was chosen as the final year to ensure that participants would be at least 18 years of age at the time of study.
2. For babies born prior to 1977, NICU admission books were searched to identify all those with an admission diagnosis pertaining to any aspect of rhesus disease or its management, such as rhesus sensitisation, jaundice, exchange transfusion, anaemia,
and hydrops. Archived hospital records for these babies were then reviewed to identify any who received an IUT.

3. For babies identified as IUT recipients, hospital records were reviewed to confirm the baby’s date of birth, sex, and if possible, full name. Hospital records of the mothers of these babies were also reviewed to identify mother’s date of birth and full name, and dates of birth, sex and if possible names of other children.

4. If the hospital records of the baby or mother could not be located, the archived card index records of admissions to National Women’s Hospital were searched to:
   a. Confirm the mother’s full name and date of birth.
   b. Confirm the baby’s sex and date of birth and if possible, ascertain the baby’s name.
   c. Determine the sex and date of birth of any possible siblings of the baby.

5. Using the mother’s name and the sex and date of birth of the baby, possible names of IUT recipients were identified from the National Birth Register, accessed via the New Zealand Public Library network. If the baby’s surname was uncommon, this process often resulted in only one possible individual. However, if the surname was common, a number of possibilities could be generated.

6. The National Health Index database (New Zealand Health Information Service, Wellington, accessed at Auckland District Health Board) was then searched. From its inception in 1980, this database has recorded the name, date of birth, address, date of last admission and, if relevant, date of death of all individuals who have utilised a public health service in New Zealand. The database was searched using the names and dates of birth of mothers and babies. If the baby’s first name was not known, an alphabetical search was performed, looking for individuals with the relevant surname and date of birth and of the appropriate sex. Identification of an individual on this database allowed confirmation of their full name, including details of any name changes or aliases and the date they were last at the documented address.

7. Further information regarding residential and postal addresses of mothers and babies was sought through an electronic search of the New Zealand electoral roll.

8. Further contact information for mothers and babies was sought through an electronic search of the New Zealand white pages (www.whitepages.co.nz).

9. If necessary, further contact information was sought through social networking Internet sites, in particular Facebook (www.facebook.com).
10. The IUT recipients for whom an address was found were sent an introductory letter inviting them to take part in the Fetal Anaemia Study (Appendix 1). If there was no response to this letter within two to four weeks, a second letter was sent to the same address, or to a second address if it was uncertain if the first address was accurate and an alternative address was available.

11. IUT recipients were contacted by telephone or email if no response was received to the second letter after a further two weeks or if this was the only contact information available.

12. If no contact information for the IUT recipient was found or if contact had not been made, an introductory letter was sent to mothers for whom an address was known. This letter reminded them of the treatment their baby had received before birth and asked them to provide contact details for their child(ren) (Appendix 1). If there was no response to this letter within two to four weeks, a second letter was sent to the same address, or to a second address if it was uncertain if the first address was accurate and an alternative address was available.

13. Mothers were contacted by telephone or email if no response was received to the second letter after a further two weeks or if this was the only contact information available.

14. If contact had still not been made, steps 5 to 8 were repeated to identify possible names and contact information for siblings of the IUT recipient.

15. Siblings were sent an introductory letter, providing details about the Fetal Anaemia Study and asking them to provide contact information for the IUT recipient (Appendix 1). If there was no response to this letter within two to four weeks, a second letter was sent to the same address, or to a second address if it was uncertain if the first address was accurate and if an alternative address was available.

16. Siblings were contacted by telephone or email if no response was received to the second letter after a further two weeks or if this was the only contact information available.

17. If contact had still not been made, the National Health Index database and white pages were searched again three to six months later to identify new contact information. In addition, an updated version of the electoral roll acquired prior to the 2011 general election was searched for all IUT recipients, and mothers for whom contact had not been made.

18. If a new address was identified for an IUT recipient or their mother, appropriate letters were sent to the new address.
19. The National Deaths Register, accessed via the New Zealand Public Library network, was searched for all IUT recipients who could not be located and who may have died prior to the inception of the National Health Index database in 1980.

Information explaining the Fetal Anaemia Study (Appendix 2) was sent to IUT recipients with the introductory letter, together with a reply form. Individuals were asked to confirm that the information we had about them was correct (name, sex, date of birth, hospital of birth, mother’s name), state birth order if they were a twin or a triplet and indicate if they wished to be involved in the study, had questions to discuss with the study investigators or did not wish to be contacted again. They were also asked to provide current contact details, including postal address, phone numbers and an email address. A self-addressed free post envelope was included to facilitate return of the reply form. A free phone number was also provided to allow the individual to contact the study investigators. A second copy of the study information sheet was enclosed for the unaffected sibling(s) of the IUT recipient.

Mothers and siblings contacted were also sent the study information sheet (Appendix 2) and a reply form with their introductory letter. They were asked to indicate if they knew how to contact the person we were trying to find and if so, whether they were willing to pass on the contact details for that individual. A self-addressed free post envelope was included to facilitate return of the reply form.

Given the unusual nature of this study in that the identity of potential index cases was not known at the outset, a media statement was released soon after commencement of tracing and recruitment. This resulted in publication of an article in the New Zealand Listener magazine and a number of articles in local community newspapers, which included contact details for the study investigators. This publicity generated a number of queries from IUT recipients and mothers.

2.1.1.3 Inclusion and exclusion criteria
All IUT recipients born in New Zealand between 1963 and 1992 with whom contact was successfully made were invited to participate in the Fetal Anaemia Study. Possible participants were excluded for the following reasons:

1. Residence outside of New Zealand and with no intention to return to New Zealand within the study timeframe;
2. No unaffected sibling available;
3. Pregnant;
4. Known cardiac disease;
5. Allergy or contraindication to gadolinium contrast (eg renal impairment);
6. Unsuitable for MRI (eg presence of cerebral aneurysm clips or pacemaker, significant claustrophobia or any other medical condition that would have made participation difficult).

In addition, participants with active asthma requiring regular preventive medication did not undergo CMR perfusion imaging with adenosine due to the potential for bronchospasm to occur as a side effect of adenosine infusion. However, these participants completed the remainder of the study protocol.

2.1.1.4 Sample size and power calculations
Sample size calculations were based on the findings of the pilot study and power calculations were performed using JMP Statistical Discovery software, version 10.0.0 (SAS Institute, Cary, USA). Assuming 80 sibling pairs were available to participate, the study had the power to detect a difference of 0.45 standard deviations between study groups (alpha=0.05, beta=0.2). This compares well with the sheep studies, where one week of fetal anaemia resulted in maximal coronary conductance in adulthood that was almost twice that of controls, an increase of 2.8 standard deviations (Davis, Roullet et al. 2003).

2.1.1.5 Ethical approval
Ethical approval was given by the Multi-Region Ethics Committee (MEC/09/04/037).

2.1.2 Collection of Perinatal and Maternal Data
Perinatal data of IUT recipients and maternal obstetric data were collected from archived hospital records; most from National Women’s Hospital, but a few babies were born at regional hospitals elsewhere in New Zealand and these records were requested from the relevant hospitals. Given the length of time since the original treatment, it was anticipated that hospital records would be difficult to locate. However, over 90% of the required records were eventually located. From October 2009 to July 2012 these records were reviewed and the following clinical data extracted.

2.1.2.1 Perinatal data
2.1.2.1.1 IUT details
- Number IUTs received
- Date and gestation at which IUT was given
- Volume of blood and amount of haemoglobin transfused
- Pre and post transfusion fetal haemoglobin
- Presence of ascites and/or oedema
- Imaging method used (ultrasound or X-ray)

2.1.2.1.2 Postnatal details
- Date and time of delivery
- Gestation at delivery
- Mode of delivery
- Birth weight, length and head circumference
- Apgar scores
- Steroid exposure prior to delivery
- Presence of hydrops at birth
- Umbilical cord blood haemoglobin and bilirubin
- Highest bilirubin concentration and date
- Exchange transfusion requirement and details
- Phototherapy requirement
- Number of top up transfusions received and date given
- Lowest haemoglobin concentration and date
- Ventilatory support required in the first 24 hours after birth
- Date of death (if applicable), classified as neonatal death (0 to 28 days after birth) and infant death (29 to 365 days)

2.1.2.2 Maternal obstetric data
- Gravidity (total number of pregnancies) and parity (number of pregnancies reaching ≥ 24 weeks’ gestation)
- Last menstrual period date, estimated date of delivery, sex, gestation, date of birth, mode of delivery, place of birth, birth weight and birth outcome (live birth, neonatal death, fetal death > 20 weeks; fetal death < 20 weeks) for each pregnancy
- Rhesus disease status for each child and if required, treatment each child received

Given the age of the records reviewed and the variability inherent in written hospital records, it was not uncommon for some of these data to be missing. In addition, it was not possible to collect some variables for some babies. For example, although Apgar scores were devised in 1952, they were not routinely recorded at National Women’s Hospital until the late 1960s.
2.1.3 Questionnaire

The study questionnaire (Appendix 3) was based on large national or internationally validated questionnaires including the New Zealand Census, New Zealand Blood Donors’ Health Study (Ameratunga, Norton et al. 2002), Fletcher Challenge-University of Auckland Heart and Health Study (MacMahon, Norton et al. 1995), the Motherwell Study (Scotland) (Shiell, Campbell-Brown et al. 2001), and the European Respiratory Health Survey (worldwide) (Burney, Luczynska et al. 1994). A similar questionnaire was used in the Steroid Follow-up Study (Dalziel, Walker et al. 2005), conducted from February 2002 to December 2003 by Prof Harding and Dr Dalziel, in which it proved acceptable to patients and provided reliable data. It was designed specifically to gather information about:

- Socioeconomic status and ethnicity;
- Participant and parental medical history, particularly with regard to cardiovascular disease risk factors;
- Respiratory symptoms;
- Alcohol and drug use;
- Exercise frequency and intensity;
- Reproductive history;
- Birth weight and gestation;
- Rhesus disease history including requirement for IUT and/or postnatal management of complications of rhesus disease.

All participants completed the questionnaire prior to the study appointment. The questionnaire was checked at the appointment to answer any queries raised by participants and to ensure completeness. Responses to questions regarding wheeze and a history of asthma or allergy were also checked carefully prior to MRI scanning.

2.1.3.1 Derivation of variables from study questionnaire

The following variables were derived from the study questionnaire:

2.1.3.1.1 Participant variables

*Ethnicity*

Prioritised ethnicity was determined by the response to question 4 “Which ethnic group do you belong to?” This question allowed participants to identify with multiple ethnic groups. Responses were interpreted using a hierarchical definition in which participants were classified as “Maori” if they identified themselves as “Maori” regardless of identification with any other ethnic group. Remaining participants were classified as “Pacific Island” if
they identified themselves as “Samoan”, “Cook Island Maori”, “Tongan”, “Niuean”, or “Other” if this was determined to be a Pacific community. Remaining participants were classified as “Other” if they identified themselves as “Chinese”, “Indian” or “Other” if this was not determined to be a Pacific community. Remaining participants identified themselves as “New Zealand European” and were classified as such. This classification is consistent with the method used for determination of ethnicity for New Zealand Census data.

**Socio-economic variables**

The following variables were used to assess socio-economic status:

- Marital status, determined by responses to question 5 “What is your marital status?”
- Education, determined by responses to question 6 “What is the highest level of education you have received?” This question allowed participants to indicate the number of years they had spent at secondary school, if this was their highest educational attainment. Responses were classified as “≤ 3 years secondary schooling” if highest educational attainment was “primary/intermediate school” or “high school/secondary school” with attendance of ≤ 3 years; “> 3 years secondary schooling” if highest educational attainment was “high school/secondary school” with attendance > 3 years; and “post secondary school education” if highest educational attainment was “polytech or similar” or “university”. Sensitivity analyses were also conducted using the categories stated in question 6 and by placing all those without tertiary education into a category of “school only”. These analyses did not change the results and are not reported.
- Occupation, determined by responses to question 9 “which of the following categories best describes your current work situation?” Participants were asked to indicate whether they were a worker for pay, self-employed, a homemaker (with questions regarding previous occupation and partner’s occupation), a student, unemployed, or other (with space for explanation). Responses were used to classify participants’ occupations into the following groups: professional, trade, administrative or sales work, manual or unskilled labour, beneficiary, or student.
- Income, determined by responses to question 11 “What was your total personal income and your household’s income before tax during the past 12 months?” Personal income was determined to be household income if a participant lived alone or in a flating situation in which income was not shared (question 10). Household income was categorised to “<$40,000”, “$40,000 to $70,000”, and “>$70,000”.

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Socio-economic status was also assessed for each participant using New Zealand Deprivation Index scores, obtained from 2006 New Zealand Index of Deprivation scores. This scale measures socio-economic deprivation for small geographical areas in deciles, according to 2006 Census results for the following nine variables: household income, receipt of a means-tested benefit, household crowding, home ownership, unemployment, qualifications, single-parent families and access to a car and telephone. Decile 1 represents the 10% of households in the least deprived areas, and decile 10 the 10% in the most deprived areas (Tobias, Bhattacharya et al. 2008). Since its inception in the early 1990s, the New Zealand Index of Deprivation has been shown to be a valid and reliable tool for assessing social inequalities across a wide range of health outcomes (Salmond and Crampton 2001; Salmond and Crampton 2002).

**Lifestyle variables**

The following questions were assessed as lifestyle variables:

- **Smoking exposure** was determined by answers to questions 12 to 25 which included information regarding current or previous cigarette, pipe and cigar use; duration and frequency of use; type of tobacco smoked; and passive smoke exposure. Participants were classified as “current smoker”, “ex-smoker”, or “non-smoker”.

- **Alcohol use** was determined by responses to questions 29 to 33 regarding frequency and quantity of alcohol consumption. A participant was classified as a “non-drinker” if they had never consumed alcohol or currently did not consume alcohol once a month or more. The Alcohol Advisory Council of New Zealand criteria were applied to categorise a participant as a “heavy drinker” if a male had consumed > 5 standard units of alcohol in one session, or on average > 15 standard units per week or > 3 standard units of alcohol per day in the previous 3 months, or if a female had consumed > 4 standard units of alcohol in one session, or on average > 10 standard units per week or > 2 standard units of alcohol per day in the previous 3 months (www.alac.org.nz/alcohol-you/your-drinking-okay/low-risk-alcohol-drinking-advice). Remaining participants were classified as “social drinkers”.

- **Recreational drug use** was determined from responses to questions 34 and 35. If marijuana or other illegal drugs had been used at any stage in the last 12 months, recreational drug use was classified as “present”.

- **Level of physical activity** was determined by responses to questions 36 to 41 regarding intensity, frequency and duration of exercise. Physical activity was categorised as “no exercise” if no moderate or vigorous exercise was undertaken,
“exercises but does not fulfil CVD prevention criteria” if less than 150 minutes of moderate exercise or less than 60 minutes of vigorous exercise was undertaken in total over less than 3 days per week, or “exercises to level at or above CVS disease prevention criteria” if these limits were exceeded and exercise was undertaken on 4 or more days per week (Perk, De Backer et al. 2012).

**Past medical history**

The following questions were used to assess past medical history:

- A response of “yes” to question 42 “Has a doctor ever told you that you have high blood pressure?” or question 137 (females only) “During your pregnancy did a doctor ever tell you that your blood pressure was high?” was used determine a history of hypertension.
- A response of “yes” to question 45 “Has a doctor ever told you that you have high cholesterol?” was used to determine a history of hyperlipidaemia.
- A response of “yes” to question 46 “Has a doctor ever told you that you have had a heart attack or angina?” was used to determine a history of coronary heart disease.
- A response of “yes” to question 47 “Has a doctor ever told you that you have had a stroke?” was used to determine a history of cerebrovascular disease.
- A response of “yes to question 48 “Has a doctor ever told you that you have diabetes?” or question 139 (females only) “During your pregnancy did a doctor ever tell you that you had developed diabetes as a result of your pregnancy?” was used to determine a history of diabetes.
- The European Community Health Survey questions were used to determine a history of asthma (Burney, Luczynska et al. 1994). A response of “yes” to question 55 “Have you ever had wheezing or whistling in the chest at any time on the last 12 months?” was used to determine a history of wheeze. The following questions were used to determine a history of current asthma: waking with shortness of breath in the last 12 months (question 61); attack of asthma in the last 12 months (question 80); current use of asthma medications (questions 54 and 82). A response of “yes to questions 75 “Have you ever had asthma” and 76 “Was this confirmed by a doctor” was used to determine a lifetime history of asthma.
- A response of “no” to question 151 “If you did not receive blood transfusions before birth, were you still affected by rhesus disease?” was used to confirm that participants who did not receive IUTs before birth were not affected by rhesus
disease in any way. Blood group including rhesus status was also checked to further confirm this.

- Response to the open-ended question 52 regarding other medical conditions was used to determine if there was any other medical history of note.

2.1.3.1.2 Maternal variables

The following questions were used to assess maternal health:

- A response of “no” to question 90 “Is your mother still alive” was used to determine maternal death.
- A response of “coronary heart disease” or “cerebrovascular disease” to question 92 “What did she die of?” was used to determine a maternal history of cardiovascular death.
- Completion of question 93 “If she died of cancer, please specify the main site of cancer” was used to determine a maternal history of cancer death.
- A maternal history of hypertension was determined to be present if the response to question 94 “Was your mother ever told by a doctor that she had high blood pressure?” was “yes, but it was unrelated to any pregnancies” or “yes, but I’m not sure if it was related to any pregnancies or not.”
- A response of “yes” to question 95 “Was your mother ever told by a doctor that she had had a stroke?” was used to determine a maternal history of cerebrovascular disease.
- A response of “yes” to question 96 “Was your mother ever told by a doctor that she had had a heart attack or angina?” was used to determine a maternal history of coronary heart disease.
- A response of “yes” to question 97 “Was your mother ever told by a doctor that she had high cholesterol?” was used to determine a maternal history of hyperlipidaemia.
- A maternal history of diabetes unrelated to pregnancy was determined to be present if the response to question 99 was “yes, but it was unrelated to any pregnancies” or “yes, but I’m not sure if it was related to any pregnancies or not.”
- If any sibling gave a response of “yes” to questions regarding maternal history of coronary heart disease, cerebrovascular disease, hypertension, hyperlipidaemia or diabetes, the maternal history was recorded as positive for the relevant diagnosis.
• A response of “yes” to question 27 “Did your mother ever smoke regularly during your childhood, or before you were born?” was used to determine a maternal history of smoking during participants’ childhood.
• A maternal history of smoking whilst pregnant with the participant was determined to be present if the response to question 28 “When your mother was pregnant, in particular with you, did she” was “cut down or stop during pregnancy?” or “smoke as usual during pregnancy?”

2.1.3.1.3 Paternal variables
Paternal variables were classified in the same way as maternal variables, with the following exceptions:
• A response of “yes” to question 108 “Was your father ever told by a doctor that he had high blood pressure?” was used to determine a paternal history of hypertension.
• A response of “yes” to question 113 “Was your father ever told by a doctor that he had diabetes?” was used to determine a paternal history of diabetes.

2.1.4 Clinical Assessment
2.1.4.1 Location and transportation
All MRI scans were performed on a Siemens 1.5 Tesla MAGNETOM Avanto scanner with Sygno MR VB11-17 software (Siemens, Erlangen, Germany) at the Centre for Advanced MRI, Auckland; initially at the Faculty of Medical and Health Sciences campus in Grafton, and from October 2010, when the scanner was relocated, in the Radiology Department at Auckland City Hospital.

Transport and accommodation were funded for all participants residing outside of Auckland. Once the appointment and travel plans had been made, a letter confirming these details was sent to participants, together with directions to the Centre for Advanced MRI, the study questionnaire and the MRI safety questionnaire.

2.1.4.2 Assessment sequence
In most cases, siblings attended for assessment on the same day. As MRI scans could be performed on only one participant at a time, the assessment proceeded by one of two sequences, with Participant A undergoing MRI scan first (Figure 2.2).

2.1.4.3 Consent
Written informed consent was obtained from all participants, with particular emphasis on potential side effects from adenosine and gadolinium.
2.1.4.4 Anthropometric and blood pressure assessment

A study nurse measured and recorded participants’ height using a stadiometer and weight using digital scales accurate to the nearest 0.1 kg. Body mass index was calculated as:

\[
\text{BMI} = \frac{\text{weight} (\text{kg})}{\text{height}^2 (\text{m})}
\]

Body surface area was calculated using the Mosteller formula (Mosteller 1987):

\[
\text{BSA} = \sqrt{\frac{\text{weight} (\text{kg}) \times \text{height} (\text{cm})}{3600}}
\]

Maximum occipito-frontal diameter was measured three times using a paper tape measure. An average of these measurements was recorded as head circumference.

Following anthropometric assessment, three measurements of blood pressure, resting heart rate and oxygen saturations were recorded using a GE Dash 4000 patient monitor (GE Healthcare, Wisconsin, USA) with the participant sitting.

2.1.4.5 Fasting blood samples

Blood samples were taken after an overnight fast from an intravenous cannula placed in the antecubital fossa, and analysed for plasma lipids, glucose, insulin, electrolytes, urea,
creatinine, liver function tests, full blood count and blood group including rhesus status. Participants then underwent a standard 75 g oral glucose tolerance test, with blood samples taken at 30 minutes and 120 minutes.

All blood samples were delivered to the laboratory within 4 hours of collection. Analyses were undertaken by LabPLUS (Auckland District Health Board) and the New Zealand Blood Service reference laboratory (Great South Road, Auckland). All staff processing and analysing blood samples were blinded to the fetal anaemia status of the participants.

2.1.4.6 Electrocardiogram
A standard 12-lead electrocardiogram (ECG) was then performed at the Clinical Physiology Department, Auckland City Hospital, a short distance from the Centre for Advanced MRI. If the oral glucose tolerance test had been commenced, the participant was transported to the Clinical Physiology Department by wheelchair to minimise physical activity which may affect glucose utilisation. The ECG was assessed by the study doctor prior to commencement of the MRI scan, noting in particular the PR and QT intervals to ensure there was no contraindication to administration of adenosine.

2.1.4.7 Heart rate variability
Heart rate and rhythm were recorded using a PowerLab 4/25T data acquisition system (ADInstruments, Dunedin, NZ). With the participant supine, adhesive electrodes were attached to both wrists and ankles and recording was commenced from lead II at amplitude 2mV. Participants were asked to maintain a state of quiet wakefulness for ten to fifteen minutes during the recording.

Five minutes of heart rate variability traces were analysed using Labchart 7 Pro analysis software (ADInstruments, Dunedin, NZ). Sections of the trace distorted by movement artefact were excluded. Analysis was performed using the derivative of the signal with filter applied. The trace was inverted if this was required for R wave capture, and ectopic beats were excluded. Spectrum band widths were set as follows: very low frequency < 0.04 Hz; low frequency 0.04 to 0.15 Hz; high frequency 0.15 to 0.4 Hz. The variation threshold, which describes the difference in duration between successive normal R-R intervals, was set at 50 ms (Lahiri, Kannankeril et al. 2008). R-R interval limits defining artefacts, ectopics and normal beats were according to appearance of beats within each recording. The following variables were recorded for each trace: SDNN, SDNN, RMSSD, pNN50, total power, LF power, LFnu, HF power, HFnu, LF/HF ratio.
2.1.4.8 MRI scans

2.1.4.8.1 Protocol development
The MRI protocol was developed prior to the pilot study in conjunction with Prof Kent Thornburg and Drs Michael Jerosch-Herold and Craig Broberg, at the Oregon Health and Sciences University, Portland, Oregon. Two healthy volunteers were scanned in November 2007 to assist with finalisation of the protocol. Further refinements were made following the pilot study, including changes to the formulation of the adenosine infusion and addition of late gadolinium enhancement imaging following perfusion imaging.

2.1.4.8.2 Left ventricular volume and flow imaging
Following positioning of the participant with ECG gating on the MRI table and acquisition of scout images, standard long and short axis cardiac cines were obtained using a steady state free-precession sequence. The participant was asked to hold their breath for approximately 10 heart beats for each of the following views: two, three and four chamber long axis views and a stack of true short-axis views from base to apex. The short axis stack comprised 12 slices, with a slice thickness of 7 mm, and inter-slice gap of 1 to 3 mm to ensure coverage of the entire heart from base to apex. Phase contrast velocity flow mapping with appropriate velocity encoding was performed in the proximal aorta and pulmonary artery.

2.1.4.8.3 Perfusion imaging
Rest, cold pressor and adenosine imaging
Images were acquired for assessment of myocardial blood flow at rest, during cold pressor challenge and at maximal vasodilation with adenosine infusion. For each assessment intravenous gadolinium contrast (Omniscan, GE Healthcare, Wisconsin, USA) was given at 0.04 mmol.kg\(^{-1}\) using a Sonic Shot 50 MRI power injector (Nemoto Kyorindo, Tokyo, Japan). Image acquisition commenced at the time of contrast injection and was ECG-gated. Ten minutes was allowed between perfusion scans for wash out of the gadolinium. Myocardial blood flow was measured in all three states with a T1 weighted single-shot gradient echo sequence with a saturation recovery magnetisation preparation. Three slices at basal, mid ventricular and apical levels were obtained in each heart beat for rest and cold pressor perfusion imaging. For adenosine perfusion imaging, three slices per heart beat were obtained if the time between heart beats was long enough to allow this; otherwise only the basal and mid-level slices were acquired.
Cold pressor challenge, used to measure flow-dependent coronary vasodilation, was achieved by placing the participant’s hand in a bag of ice for three minutes prior to the start of the perfusion scan.

Measurement of coronary flow at maximal vasodilation was achieved by administration of intravenous adenosine (Adenocor, Sanofi-Aventis Ltd, Paris, France) at 0.14 mg.kg$^{-1}$.min$^{-1}$. As caffeine is a pharmacological antagonist of adenosine, participants were asked to abstain from consuming beverages containing caffeine for 24 hours prior to the assessment. To ensure administration of an equivalent volume of adenosine to each participant, a predetermined dose chart was used to formulate a 50 ml infusion of appropriate concentration for the participant’s body weight. The infusion was administered using a Medfusion 3500 infusion pump (Medfusion Inc, Cary, USA) via a double lumen extension. Gadolinium contrast was administered through the second lumen, thus negating the need for insertion of a second intravenous cannula. Perfusion imaging commenced three minutes after the start of the adenosine infusion, which continued for a further twenty seconds to ensure maximal vasodilation was maintained for the first pass of contrast agent. Imaging continued for approximately 45 seconds in total.

Concurrent administration of gadolinium and adenosine
Gadolinium must be injected rapidly during MRI scans, at a rate of 4 ml.s$^{-1}$. By comparison, the infusion rate for the adenosine was 0.17 ml.s$^{-1}$. Therefore, it was possible that the flow of adenosine would be interrupted at the time of gadolinium administration, if both substances were given via the same intravenous cannula. We therefore measured the actual volumes delivered when adenosine and gadolinium were infused separately and concurrently. The experiment was designed to mimic the methods of administration during MRI scanning as closely as possible, using the same fluids, infusion pumps, cannulae and extension tubing, and accurate weighing to determine the volumes delivered. We found that adenosine was delivered reliably, but the power injector delivered less than the pre-programmed volume of gadolinium (Table 2.1). The power injector was therefore checked and serviced to ensure accuracy of contrast administration. With concurrent administration of adenosine and gadolinium via a single intravenous cannula, the volume of fluid delivered was the same as the volume delivered when each infusion was administered separately. However, on one occasion the pump administering the adenosine terminated the infusion prematurely due to detection of high upstream pressure at the time of gadolinium injection. To overcome this problem, the pressure detection limits on the adenosine infusion pump
were set to the highest level. In addition, the pump was watched carefully at the time of gadolinium injection and was restarted immediately if an interruption to flow occurred.

Table 2.1: Volumes expected and delivered for separate and concurrent administration of adenosine and gadolinium/flush

<table>
<thead>
<tr>
<th></th>
<th>Volumes expected and delivered (ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenosine</td>
<td>Gadolinium</td>
<td>Adenosine + Gadolinium</td>
</tr>
<tr>
<td>Expected volume</td>
<td>33.33</td>
<td>26.00</td>
<td>59.33</td>
</tr>
<tr>
<td>Iteration 1</td>
<td>33.83</td>
<td>20.64</td>
<td>54.47</td>
</tr>
<tr>
<td>Iteration 2</td>
<td>30.43†</td>
<td>22.06</td>
<td>52.49</td>
</tr>
<tr>
<td>Iteration 3</td>
<td>33.35</td>
<td>21.84</td>
<td>55.19</td>
</tr>
<tr>
<td>Iteration 4</td>
<td>33.26</td>
<td>21.69</td>
<td>54.95</td>
</tr>
<tr>
<td>Iteration 5</td>
<td>33.05</td>
<td>22.96</td>
<td>56.01</td>
</tr>
<tr>
<td>Mean volume: (±SD)</td>
<td>33.37 ± 0.33†</td>
<td>22.45 ± 2.06</td>
<td>55.16 ± 0.64‡</td>
</tr>
</tbody>
</table>

*Adenosine infusion terminated prematurely during iteration 2 due to detection of high upstream pressure secondary to concurrent contrast infusion. † Calculated with data from iteration 2 excluded.

Monitoring of participant during adenosine infusion
Blood pressure, heart rate and oxygen saturations were recorded at the start of the MRI, prior to the administration of adenosine, one minute into the adenosine infusion and one minute after completion of the adenosine infusion. In addition, verbal communication was maintained with the participant to monitor their tolerance of potential side effects. Common side effects experienced included nausea, dizziness, headache, flushing, abdominal discomfort, chest and neck tightness and shortness of breath. If a patient complained of shortness of breath, they were monitored closely for signs of bronchospasm. The ECG trace was also watched carefully for signs of atrioventricular block. Algorithms were devised to assist with management in the event of significant bradycardia or bronchospasm. If a patient developed intolerable side effects, significant bronchospasm or sustained bradycardia with heart rate ≤ 40 beats per minute, the adenosine infusion was discontinued.

2.1.4.8.4 Late enhancement imaging
Short axis late enhancement images were acquired during a single breath hold ten minutes after completion of perfusion imaging. If any abnormalities were noted, long axis slices were acquired through the region of interest.

2.1.4.8.5 MRI data analysis
Analysis of all MRI data was performed by the specialised core laboratory at the Auckland MRI Research Group (AMRG).
Analysis occurred in two parts. Firstly, measurement of cardiac volumes to assess cardiac size and contractile function were determined by segmenting the endo- and epicardial borders of all cine images using guide-point modelling to create a 4 dimensional finite-element model of the left ventricle, followed by summation of the cavity and myocardial volumes by numerical integration. End systole and end diastole were identified by the minimum and maximum on the volume versus time curve. The flow images were analysed by defining a region of interest and summing the velocity in each pixel, and across all phases.

The second aspect of MRI data analysis was the computationally more challenging myocardial perfusion analysis. When this study was originally planned, all the myocardial perfusion analyses were to be undertaken by one of our collaborators in the United States, Dr Michael Jerosch-Herold, who is a world authority in this field. Unfortunately, following the pilot study Dr Jerosch-Herold changed institutions and was unable to complete any further analyses due to lack of time and support in his new institution. Fortunately, our colleagues at AMRG were willing to develop the protocols required to perform the remaining myocardial perfusion analyses. This was a multi-step process:

1. **Database:** The images were assembled into a database for analysis. Each slice position and the stress level was identified and attached to the image data.

2. **Registration:** As the heart moves with diaphragmatic motion during breathing, it was necessary to register the position of the heart on every MRI slice so that each anatomical feature occurred in the same place on every image. This was performed using non-rigid registration.

3. **Segmentation:** The endo- and epicardial borders were defined on the first few images using 2 dimensional guide-point modelling, and copied to all other images in the slice. Fiducial markers (such as the insertion of the right ventricle) were defined to allow definition of the American Heart Association regions.

4. **Data extraction:** The grey scale of all pixels in each region on every slice was averaged to provide a single number for each image in the cine. These were formatted as a time series of data into a spreadsheet reflecting the perfusion of contrast through the myocardium.

5. **Parameters:** From the time curves for each region, the final parameters were extracted using a model based approach analogous to fitting a straight line to data and reporting the slope and intercept.
The most challenging aspect of this process was the last step of extracting the parameters from the time curves. This final step is an area of active research and it was important that results were analysed in a way that was acceptable to the cardiac MRI reviewing community. Therefore, AMRG used a technique compatible with that published by Dr Jerosch-Herold and verified their findings by comparison with results from scans he had analysed from the pilot study. MRI scan results were entered into Microsoft Excel spreadsheets by AMRG and checked prior to release to the study investigators.

All staff involved in the acquisition and analysis of MRI scans were blinded to the fetal anaemia status of the participants.

### 2.1.5 Data Management

All tracing and contacting information, perinatal and maternal obstetric data and clinical assessment data were recorded in a Microsoft Access database developed specifically for this study. Participants were assigned an individual study code number and all data were identified only using this code. The study database was password protected and available only to staff directly involved with the study. Data were easily and reliably exported from the database for the purpose of statistical analysis. Prior to statistical analysis, body size data and MRI scan results for every participant and blood test and blood pressure data for every fifth participant were checked to assess data entry errors. These were found to be < 0.5%. All data were also examined for errors and anomalies during analysis.

All participant information will be stored for at least ten years in locked filing cabinets at the Liggins Institute, University of Auckland, under the responsibility of the study investigators.

### 2.1.6 Intra- and Interobserver Variability

The calibration of all equipment (scales, sphygmomanometers, stadiometer) was regularly checked to minimise intraobserver variability. Clinical assessments were undertaken by the same study nurse and one of two study doctors, with the same routine adhered to for each assessment. MRI scans were undertaken by two of four MRI radiographers, all of whom had received training on magnetic resonance imaging of myocardial perfusion and were very familiar with the study protocol. All MRI scans were analysed by the same Radiologists at AMRG and all blood samples were analysed by the same laboratories.
2.1.7 Dissemination of Results to Participants

2.1.7.1 Body size, blood pressure and blood test results
All participants were asked at the clinical assessment if they would like a copy of their body size, blood pressure and blood test results to be sent to them and/or their General Practitioner. Without exception, all participants wished to receive these results and most also wished for them to be sent to their General Practitioner. Results for each participant were collated onto a single form, with normal ranges quoted for reference if appropriate (Appendix 4). Results were posted or emailed to participants, according to the individual’s preference, together with a letter thanking them for their participation. Results were posted to General Practitioners, together with a letter briefly explaining the study and asking the General Practitioner to follow up any abnormal findings as required (Appendix 4). If clinically significant incidental findings were present, the study investigator contacted participants by telephone to inform them of the abnormal finding(s) and advise them to see their General Practitioner for further assessment and follow up.

2.1.7.2 MRI scan results
MRI scan results were not routinely provided to participants. However, the study investigator notified participants by telephone of clinically significant incidental findings as soon as these were identified and advised them to see their General Practitioner for further assessment and follow up. Offer was also made to notify the participant’s General Practitioner of the abnormal finding(s). Following contact by the study investigator, a letter was sent to the participant by the Centre for Advanced MRI together with an electronic copy of the MRI scan.

2.1.7.3 Overall results of study
All participants were asked at the clinical assessment if they would like to receive a copy of the final results of the study. Without exception, all participants wished to receive these results.

2.2 Preterm Anaemia Pilot Study
In this thesis the term Preterm Anaemia Study is used to refer to the pilot study of cardiovascular outcome in sheep following preterm delivery and induction of anaemia prior to term equivalent age (TEA). The specific aims of this study were to determine:

- The severity of anaemia that can be tolerated by preterm sheep;
- The time frame over which anaemia can be safely induced;
The normal pattern of changes in haematocrit after birth in both preterm and term sheep;
Practical methods for assessing cardiovascular outcome, particularly coronary flow, in postnatal sheep.

All aspects of this study were undertaken at the Liggins Institute’s Ngapouri Animal Laboratory, apart from the terminal cardiovascular assessments, which were undertaken at the Large Animal Facility, Vernon Jansen Unit, Faculty of Medical and Health Sciences, University of Auckland.

2.2.1 Study Design

2.2.1.1 Study groups and haematocrit targets
Romney-Dorset cross ewes carrying twin pregnancies were used to provide lambs for this study. Three breed groups of 8 to 10 ewes delivered preterm lambs at gestational age (GA) 138 days and term lambs at GA 147 days from June to August 2011. Lambs were randomly allocated to one of four groups: preterm lambs in which anaemia was induced by daily venesection until reaching a target haematocrit of (a) 50% or (b) 70% of baseline, non-anaemic preterm controls, and non-anaemic term controls, with the aim of achieving 8 lambs per group.

Haematocrit monitoring of preterm control lambs in the first breed group revealed a spontaneous reduction to 75% of baseline from birth to TEA, hence it was felt that a haematocrit target of 70% would not result in anaemia. Therefore for subsequent breed groups, the haematocrit target of the second group of experimental preterm lambs was modified to 30% of baseline. As no lambs with a haematocrit target of 70% had resulted from the first breed group, the final study groups were:

1. Preterm lambs in which anaemia was induced by daily venesection until reaching a target haematocrit of 50% of baseline (PT50%);
2. Preterm lambs in which anaemia was induced by daily venesection until reaching a target haematocrit of 30% of baseline (PT30%);
3. Non-anaemic preterm controls (PC);
2.2.1.2 Inclusion and exclusion criteria

2.2.1.2.1 Ewe selection

Ewe mating was closely controlled to ensure that all ewes in a group were mated on one day only. Ultrasound scan of pregnant ewes was performed at GA 42 and 56 days to confirm twin pregnancy. All ewes had liver function tests checked at GA 110 days to look for evidence of cholestasis as an indication of exposure to sporidesmin spores, the causative agent of facial eczema. Ewes with a gamma-glutamyl transferase (GGT) of \( > 150 \text{ IU} \cdot \text{l}^{-1} \) (normal \( \leq 60 \text{ IU} \cdot \text{l}^{-1} \)) were excluded from the study.

2.2.1.2.2 Lamb selection

Antenatally, all lambs from twin bearing ewes allocated to the study were deemed eligible for inclusion.

Postnatally, lambs were excluded from the study for the following reasons:

- Birth weight less than 3 kg (approximately 2 SD below the mean in our flock). These lambs were excluded to avoid any confounding effects of fetal polycythaemia due to intrauterine growth restriction on the outcomes of interest;
- Delivery more than 24 hours after the expected date of preterm or term birth;
- Respiratory difficulties related to preterm delivery and consistent with poor prognostic outcome within the first 6 hours after birth;
- Maternal problems interfering with ability to feed lambs prior to weaning (eg poor milk supply secondary to fibrous udder).

2.2.1.3 Sample size and randomisation

Twenty-six ewes with twin pregnancies were allocated to this study, resulting in 52 lambs. The target for this study was to achieve 8 lambs per group, 32 lambs in total. It was estimated there would be a 40 to 50% lamb loss in the preterm groups, with the largest losses expected in the lambs made more anaemic. Therefore, to ensure target numbers were achieved by the end of the study, it was determined before the study commenced that 14 lambs would be randomly assigned to the PT50% group, 16 to PT30%, 12 to PC and 10 to TC.

Ewes were randomised to preterm or term delivery by withdrawing animal identification numbers from one hat and preterm or term delivery from a second hat. Preterm lamb twin pairs were randomised to one of the three preterm groups within the first six hours after birth by withdrawing ewe numbers from one hat and PC, PT50% or PT30% from a second
hat, with both lambs from the same ewe allocated to the same experimental group. The study investigator who performed randomisation of all ewes and lambs was blinded to the condition of the animals. Once the previously determined number of lambs required for a particular group had been achieved, no further lambs were allocated to that group. Lambs of ewes assigned to delivery at term became term controls. Lambs born at term after failure of induction of preterm labour were also allocated to the term control group.

2.2.1.4 Ethics approval
Ethical approval was given by the Animal Ethics Committee, University of Auckland (R890). Approval for amendment of the target haematocrit to 30% of baseline for the second experimental group of preterm lambs was sought after delivery of the first breed group. Provisional approval was granted for two PT30% lambs to be generated from the second breed group. As these two lambs did well, approval was then granted for further PT30% lambs from the third breed group.

2.2.2 Perinatal Care and Interventions
2.2.2.1 Induction of labour and delivery
Starting on gestational day 135, ewes randomised to preterm delivery received 0.25 mg.kg\(^{-1}\) dexamethasone sodium phosphate (Dexa 0.2, 2 mg.ml\(^{-1}\), Southern Veterinary Supplies, Hamilton, New Zealand) by intramuscular injection between 1700 to 1900 hours and a second dose of 0.25 mg.kg\(^{-1}\) 12 hours later. Starting on day 145, ewes randomised to term delivery had labour induced using the same protocol. Ewes were housed in individual pens following administration of dexamethasone and closely monitored.

Vaginal delivery was expected 48 hours after the administration of the first dose of dexamethasone. At least two study investigators were present for all preterm deliveries and the majority of term deliveries were also attended. Assistance was given only if delivery was protracted or the ewe was in distress.

2.2.2.2 Maintenance of temperature and housing
Body temperature of preterm lambs was maintained through the use of woollen jackets, heat lamps and straw lining of pens. Lambs were housed with their mothers in individual pens until 10 days post TEA. Thereafter, lambs and ewes were moved to a communal indoor pen for a further 7 to 10 days before rejoining the flock outside. Routine stock management including vaccination and drenching was undertaken according to the standard operating procedures of the farm.
2.2.2.3 Feeding
All preterm lambs received one feed of artificial colostrum within the first 12 hours after birth to help protect against infection. Feeding and weight gain was monitored closely in all lambs in the first few days after birth. Expressed sheep milk or lamb formula milk was given via bottle or oro-gastric feeding tube if a lamb was too weak to suckle or mother’s milk supply was poor.

2.2.2.4 Care of unwell animals
In the immediate postnatal period, all lambs were observed for signs of respiratory distress (tachypnoea, use of accessory muscles, flaring of the alar nasae). Lambs that were apnoeic or developed respiratory distress at or soon after birth were given assistance to initiate and maintain respiratory effort. If significant respiratory distress requiring oxygen or respiratory support occurred the lamb was euthanised with 2 to 5 mg.kg\(^{-1}\) propofol (Diprivan 2%, Astra Zeneca, London, United Kingdom).

Lambs or ewes that became unwell postnatally were reviewed by a study investigator and, if required, a veterinarian. Antibiotics were administered if appropriate, benzylpenicillin 25 mg.kg\(^{-1}\), (Crystapen 100 mg.ml\(^{-1}\), Sandoz, Holzkirchen, Germany) or gentamicin 4 mg.kg\(^{-1}\) loading dose, 2 mg.kg\(^{-1}\) thereafter (40mg.ml\(^{-1}\), Pfizer, New York, United States). If the animal did not improve or deteriorated further after institution of appropriate treatment it was euthanised.

2.2.2.5 Venesection and haematocrit assessment
2.2.2.5.1 Induction of anaemia
Anaemia was induced in preterm lambs by daily venesection, commencing not less than 6 hours after birth. Venesection was performed via jugular venepuncture using a butterfly needle with extension tubing connected to a 3-way tap. The volume of blood removed was replaced ml for ml with warmed normal saline. Venesection volume for the first 2 days after birth was based on birth weight (Table 2.2). From day 3 onwards, venesection volumes were adjusted based on haematocrit results (Table 2.3).

Table 2.2: Day 1 and 2 venesection volumes by birth weight

<table>
<thead>
<tr>
<th>Birth weight (kg)</th>
<th>Day 1 and 2 venesection volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 – 3.4</td>
<td>35</td>
</tr>
<tr>
<td>3.5 – 3.9</td>
<td>40</td>
</tr>
<tr>
<td>4.0 – 4.4</td>
<td>45</td>
</tr>
<tr>
<td>4.5 – 4.9</td>
<td>50</td>
</tr>
<tr>
<td>5.0 – 5.4</td>
<td>55</td>
</tr>
</tbody>
</table>
Table 2.3: Venesection volume adjustment by haematocrit from day 3

<table>
<thead>
<tr>
<th>Haematocrit progress</th>
<th>Venesection volume adjustment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematocrit declining by 10 – 20% per day</td>
<td>Continue to remove same volume</td>
</tr>
<tr>
<td>Haematocrit declining by 0 – 10% per day</td>
<td>Increase volume removed by 20ml</td>
</tr>
<tr>
<td>Haematocrit increasing</td>
<td>Increase volume removed by 40ml</td>
</tr>
<tr>
<td>Haematocrit declining by &gt;20% per day</td>
<td>Decrease volume removed by 10ml</td>
</tr>
</tbody>
</table>

Daily venesection continued until target haematocrit was reached, with the aim of achieving this on day 5 in PT50% lambs and day 7 in PT30% lambs. Daily monitoring of haematocrit continued once the target was reached. If haematocrit rose above target prior to TEA, venesection was recommenced. If target haematocrit was not reached, venesection was discontinued at TEA. To aid haematological recovery following induction of anaemia, PT50% and PT30% lambs received intramuscular iron 100 mg (Ferrosig 50 mg.ml\(^{-1}\), Healthcare Logistics, Auckland) at TEA (Figure 2.3).

Figure 2.3: Timing of interventions for all lambs from birth to 12 months of age
2.2.2.5.2 Haematocrit monitoring and blood sampling

Haematocrit was monitored daily in all preterm lambs from birth to TEA, on day 1 in term lambs and for all lambs on day 5 post TEA, every two weeks from day 14 to 3 months of age and thereafter every 4 weeks to 12 months of age (Figure 2.3). Blood for haematocrit assessment was collected from an ear vein. The ear was shaved, a small puncture made in a vein using a lancet and a drop of blood collected into a capillary tube. Two capillary samples were taken from each lamb, with an approximate volume of 0.2 ml each. The samples were then spun in a Centurion 1010D centrifuge (Centurion Scientific Ltd, West Sussex, United Kingdom) and the haematocrit read using a Centurion haematocrit reader.

Blood samples were also taken from all lambs at birth, TEA (preterm lambs only), 3 months, 8 months and 12 months of age for laboratory analysis of complete blood count, iron stores (TEA onwards), electrolytes, glucose, lactate and thyroxine (Figure 2.3). Additional samples were taken if clinically indicated. Samples required on day 1 of life in PT50% and PT30% lambs were taken from blood removed at the time of venesection for induction of anaemia. All other blood samples in both anaemic and nonanaemic lambs were taken by jugular venepuncture using a butterfly needle.

Lambs were restrained in a box to allow safe collection of blood. Lambs were placed into the box in a comfortable sitting position, covered with a blanket and restrained using straps. One study investigator ensured the lamb remained still, while a second investigator performed the venesection.

2.2.2.5.3 Blood sample handling and analysis

Blood for electrolytes, glucose, lactate and thyroxine analysis was transferred to heparinised specimen tubes and stored on ice immediately after collection. Within an hour of collection, plasma was extracted from the whole blood samples and transferred to eppendorf tubes which were stored in a -20°C freezer at the Ngapouri Animal Laboratory. Analysis of these samples was performed at the Liggins Institute laboratory, University of Auckland on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan): glucose by enzymatic colorimetric assay (Roche Diagnostic, Mannheim, Germany); electrolytes by indirect potentiometry (Roche), thyroxine by homogeneous cloned enzyme donor immunoassay (Roche) and lactate by enzymatic colorimetric assay (Randox Laboratories Ltd, Ardmore, United Kingdom).
Complete blood count and iron store analyses were performed by New Zealand Veterinary Pathology Limited (Hamilton). Samples were transported to the laboratory within 24 hours. As inaccuracies in iron store analysis may occur if whole blood samples are not tested within 24 hours, plasma was extracted and transferred to eppendorf tubes prior to transportation.

### 2.2.2.6 Anthropometric assessment

All lambs were weighed and measured on day 1, day 5 and TEA (preterm only), then every 4 weeks to 8 months, and at 10 and 12 months of age (Figure 2.3). Weight was recorded to the nearest 0.1 kg using Wedderburn DS-520 DIGI scales (Wedderburn, Auckland) for lambs up to 4 weeks of age and Tru-Test DR-3000 scales (Tru-Test, Auckland) thereafter. In addition to the planned weights, PT50% and PT30% lambs were weighed as required during the venesection period to help assess fluid status and adequacy of feeding. Weight was also recorded if concerns arose regarding the well-being of a lamb at any time.

Measurements taken included crown-rump length, biparietal diameter, hock-hoof length, hindlimb length, chest girth and abdominal girth. For measurements up to 4 weeks of age, lambs were placed in a purpose built cradle. Thereafter, lambs and ewes were brought into the shed as a flock and measurements were taken with lambs in the stock race. Measurements were recorded to the nearest 0.1 cm and using manual calipers (biparietal diameter) or a retractable tape measure (all other measurements).

### 2.2.2.7 Intra- and interobserver variability

All equipment required for haematocrit assessments, weights and measures of lambs was checked and calibrated regularly. All haematocrits, venesections, weights and measures were performed by the same three study investigators.

### 2.2.3 Cardiovascular Assessments

Assessment of cardiovascular function and coronary artery flow in adulthood following exposure to anaemia in the preterm period requires significant surgical expertise and access to technical equipment capable of monitoring and recording the physiological data of interest. The aim of this aspect of the Preterm Anaemia Study was to pilot a protocol for these assessments in collaboration with our colleagues, Associate Professor Ian LeGrice, Professor Bruce Smaill, Dr Greg Sands, Dr Nigel Lever and Mrs Linley Nisbet from the Department of Physiology, University of Auckland.
2.2.3.1 Animal selection and care prior to experiments
In order to minimise ethical cost, the animals used for these experiments were mothers of lambs included in the Preterm Anaemia Study. Five ewes, 4 to 5 years old, were selected randomly from this group. Prior to the experiments, the selected ewes were housed indoors for 7 days at the Ngapouri Animal Laboratory to accustom them to indoor housing and pelleted food, before transport to the Large Animal Facility, Faculty of Medical and Health Sciences, University of Auckland, where the experiments were undertaken. The animals were fasted for 18 to 24 hours prior to the procedure, with water freely available.

2.2.3.2 Surgical procedure
Anaesthesia was induced using intravenous alfaxalone 0.25 ml.kg$^{-1}$ (Alfaxan 10 mg.ml$^{-1}$, Jurox Pty Ltd, Rutherford, Australia), and maintained with 2.5 to 5% isoflurane (Baxter International, Illinois, United States) via endotracheal tube and a Harvard Apparatus 607 Respiration Pump (Harvard Apparatus, Massachusetts, USA). Isoflurane concentration was adjusted according to the depth of anaesthesia, monitored by regular assessment of palpebral and pinch reflexes, heart rate and blood pressure and tidal volume and respiratory rate were adjusted to maintain end tidal carbon dioxide between 37 and 42 mmHg. ECG monitoring used a PowerLab 8/35 data acquisition system and LabChart 7 Pro software (ADInstruments, Dunedin, NZ).

A pigtail catheter (Impulse Diagnostic Catheter 6 French, 100 cm, Boston Scientific, Massachusetts, USA) connected to a pressure transducer (P23XL, Grass Technologies, Rhode Island, USA) was advanced via an introducer sheath (Avanti+ 8 French, 11 cm, Cordis Corporation, New Jersey, USA) from the right carotid to the aortic root to allow continuous monitoring of aortic pressure. A second catheter was advanced from the left internal carotid artery into the left ventricle for monitoring of left ventricular pressure. A third catheter was advanced from the right internal jugular vein to the right atrium for monitoring of right atrial pressure. Correct positioning of these catheters was confirmed by measurement of pressure at the time of catheter placement. All catheters were flushed with heparinised saline to preserve patency and baseline heart rate, mean aortic and right atrial pressures were measured. A further introducer sheath inserted into the left internal jugular vein provided access for maintenance of fluid volume using 6% hydroxyethyl starch in 0.9% sodium chloride (Voluven, Fresenius Kabi, Bad Homburg, Germany). The fluid infusion rate was titrated to maintain mean aortic pressure between 80 and 90 mmHg throughout the procedure.
The heart was then accessed through an intercostal thoracotomy. A catheter was inserted directly into the left atrium for systemic drug administration. An ultrasonic Doppler flow 3S probe (Transonic Systems Inc, New York, USA) was placed around the circumflex artery in the coronary sulcus for measurement of coronary flow. Cotton ties were placed around the proximal aorta and inferior vena cava to allow extrinsic occlusion during pressure-flow measurements. An ultrasound probe (Philips Sonos 5500 s8 cardiac probe, Eindhoven, Netherlands) was placed on the anterior left ventricular wall for assessment of left ventricular volumes, ejection fraction and fractional shortening.

2.2.3.3 Measurement of coronary blood flow
Intravenous propranolol 2 mg (Global RX, Carolina, USA) and atropine 2 mg (Phoenix Pharm Distributors Ltd, Auckland) were given to minimize baroreceptor-induced heart rate changes. Mean coronary blood flow was then measured at basal pressure and at a range of mean arterial pressures from 50 to 120 mmHg. Arterial pressures above or below basal levels were induced by variable extrinsic occlusion of the aorta or inferior vena cava. Following recovery of mean arterial pressure, heart rate and coronary blood flow to baseline levels, intravenous adenosine infusion 0.14 mg.kg\(^{-1}\).min\(^{-1}\) (Adenocor, Sanofi-Aventis Ltd, Paris, France) was commenced via the left atrial catheter using a Medfusion 3500 infusion pump (Medfusion Inc, Cary, USA) to achieve maximal steady state vasodilation. Pressure-flow measurements were then repeated at basal pressure and at a range of mean arterial pressures from 50 to 120 mmHg. Arterial blood gases were measured before each set of pressure-flow measurements and at the conclusion of the experiment.

2.2.3.4 Post mortem procedures
Once all recordings were complete, systemic heparin 100 IU.kg\(^{-1}\) (Multiparin 5000 IU.ml\(^{-1}\), CP Pharmaceuticals, Wrexham, United Kingdom) was administered to prevent post mortem occlusion of coronary vasculature. The heart was then arrested in diastole by injection of 50 ml of 15% potassium citrate (May and Baker Ltd, London, United Kingdom) into the left ventricle while cross-clamping the aorta. The heart was excised, rinsed in cold saline and weighed. Cannulae were placed in the coronary arteries and the heart perfused with standard Krebs solution to clear the coronary circulation of blood. A cannula was then placed in the circumflex artery at the point at which coronary blood flow had been assessed. The artery was tied off at this point and perfused with ink to mark the left ventricular territory supplied by this vessel. The area of left ventricle marked by ink was dissected away from the remaining left ventricle and the resulting tissue weighed in order to quantify the mass of myocardium supplied by the vessel from which flow was measured.
2.2.3.5 Quantification of coronary conductance and perfusion reserve
Coronary blood flow, calculated as flow per 100 grams of perfused myocardium (ml.min⁻¹.(100g)⁻¹) was plotted against perfusion pressure, calculated as mean aortic minus mean right atrial pressure (mmHg) to display the linear pressure-flow relationship at rest and during maximal coronary vasodilation with adenosine. Coronary conductance was calculated as the slope of these lines. Coronary perfusion reserve, defined as the difference between coronary blood flow under resting conditions and maximal vasodilation, was interpolated at an assumed perfusion pressure of 90 mmHg.

2.2.3.6 Intra- and interobserver variability
All equipment required for the pilot cardiovascular assessments (pressure transducers, flow probes, anaesthetic and ventilation equipment) was checked and calibrated prior to each procedure. All surgery was performed by Assoc Prof LeGrice and Dr Lever. As the purpose of these assessments was to trial and refine the surgical and coronary flow measurement techniques, the procedure was altered with each performance after team discussion and assessment of the quality of the data produced. The above method describes the final protocol for cardiovascular assessment.

2.3 General Statistical Methods
Continuous data were assessed for normal distribution using the Shapiro-Wilk Test and skewed data were log-transformed to approximate normal distribution when possible. Continuous variables were compared with one-way analysis of variance or Wilcoxon test (Kruskal-Wallis when more than two groups), with Tukey HSD or Wilcoxon All Pairs post-hoc corrections for multiple comparisons as appropriate. Categorical data were compared with Chi squared tests.

Multiple regression analyses were performed with participants nested within their sibling groups, and adjusted for age, sex and birth weight z-score, as these variables were all independent predictors of the outcomes of interest.

Analyses were conducted using JMP Statistical Discovery software, version 10.0.0 (SAS Institute, Cary, USA).

As a large number of comparisons were made in the analysis of this data set, we considered applying correction for multiple comparisons with Family Wise Error Rate or False
Discovery Rate techniques, in order to reduce the possibility that some of the statistically significant findings reported occurred by chance. However, the studies described in this thesis were not designed to test or confirm previously identified associations, but rather were exploratory, hypothesis generating investigations. Thus, we felt that correction for multiple comparisons was both inappropriate and unhelpful in the analysis of these data, as the purpose of this work was to identify potentially important associations within the data set that could provide the basis for future investigations.

Data are presented as mean (SD) for unadjusted data, mean (95% CI) for adjusted data, antilog-transformed geometric mean (95% CI), median (range), number (percentage), difference between means (95% CI) and ratio of geometric means (95% CI). If the 95% CI for a ratio of geometric means includes 1, there is no significant difference between groups (Bland and Altman 1996).

Analyses included all study participants, apart from glucose tolerance test data presented in chapter 4, in which the two participants with a previous diagnosis of diabetes mellitus were excluded. Their exclusion did not change the results.
Chapter 3: Perinatal Characteristics of the Cohort

3.1 Summary of Chapter Contents
This chapter reports on recruitment of participants to the Fetal Anaemia Study, and the survival statistics and perinatal characteristics of IUT recipients born in New Zealand from 1963 to 1992.

3.2 Introduction
Until the middle of the twentieth century, 50% of infants affected by rhesus haemolytic disease (RhHD) died in the perinatal period, and 10% of neonatal deaths were due to RhHD (Bowman 1998). The development of exchange transfusion in the late 1940s led to a significant reduction in neonatal mortality from RhHD in the ensuing decade (Lowenstein and Sabin 1957). However, it was not until 1963, when Liley pioneered the technique of intraperitoneal IUT, that treatment of the fetus prior to delivery became possible (Liley 1963). Liley’s technique became the standard of care worldwide for fetuses with severe RhHD and remained largely unchanged for nearly two decades (Schumacher and Moise 1996). Significant advances in neonatal care also occurred over this time, including improvements in ventilation techniques, and the introduction of antenatal corticosteroids and phototherapy, the latter being of particular importance to the postnatal management of RhHD (Sisson, Kendall et al. 1971; Liggins and Howie 1972). In addition, the use of antenatal and postpartum anti-D immunoprophylaxis led to a considerable reduction in the clinical burden of RhHD (Roberts 2008). The development of ultrasound guided intravascular IUT in the mid 1980s was a further turning point for the course of RhHD, with survival rates in New Zealand improving from 60% for fetuses receiving intraperitoneal IUT prior to 1986, to 90% for fetuses receiving intravascular IUT from 1986 to 1990 (Pattison, Roberts et al. 1992). Thus, analysis of the perinatal characteristics of this cohort of IUT recipients reflects changes in both the management of RhHD and the care of preterm neonates that occurred over this time, and provides useful insight into the effect of these changes on perinatal outcome.

3.3 Results
3.3.1 Recruitment
From 1963 to the end of 1990, 459 fetuses received IUT(s) at National Women’s Hospital (Pattison, Roberts et al. 1992). A further 15 received IUT(s) in 1991 and 1992, giving a total
of 474 IUT recipients between 1963 and the end of 1992. One IUT was performed for the treatment of twin to twin transfusion, with the remaining 473 fetuses receiving IUT for RhHD. Of these, 242 (51%) survived the perinatal period and 228 (48%) survived to adulthood (Figure 3.1).

Figure 3.1: Participant flow for Fetal Anaemia Study follow-up

* Percentages calculated using total in preceding box as denominator. Neonatal death = death from birth to 28 days post term equivalent age. Infant death = death from 28 days to 12 months post term equivalent age.

Of the 242 perinatal survivors, 213 (88%) were successfully traced (Table 3.1). One hundred and twenty four (58%) were traced in the pilot study or by initial search of archived National Women’s Hospital records, National Birth Register microfiche, National Health Index database, electoral roll or telephone directory. A further 73 (34%) were traced by first locating their mother or siblings.
Table 3.1: Successful tracing methods of IUT recipient perinatal survivors

<table>
<thead>
<tr>
<th>Tracing Method</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilot study</td>
<td>23 (11)</td>
</tr>
<tr>
<td>Initial search of databases</td>
<td>101 (47)</td>
</tr>
<tr>
<td>Search of databases for mother’s contact details</td>
<td>59 (28)</td>
</tr>
<tr>
<td>Search of databases for sibling’s contact details</td>
<td>14 (7)</td>
</tr>
<tr>
<td>Media release</td>
<td>11 (5)</td>
</tr>
<tr>
<td>Internet search</td>
<td>2 (1)</td>
</tr>
<tr>
<td>National Death Register search</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Second search of databases</td>
<td>2 (1)</td>
</tr>
<tr>
<td><strong>Total number of perinatal survivors successfully traced</strong></td>
<td>213</td>
</tr>
</tbody>
</table>

* Search included archived National Women’s Hospital NICU admission books, Special Procedures Books, daily birth records and card index records of all admissions, National Birth Register microfiche, New Zealand National Health Index database, New Zealand electoral roll and New Zealand telephone directory.

Ninety five of 242 (39%) perinatal survivors were subsequently enrolled in the Fetal Anaemia Study (42% of those presumed alive and 48% of those located and presumed alive) together with 92 unaffected siblings (Figure 3.1). Of the 187 participants, 176 (94%) completed the full study protocol. One hundred and seventy five (94%) participants resided in New Zealand; 68 (39%) in the greater Auckland area, 76 (43%) in the remainder of the North Island and 31 (18%) in the South Island (Figure 3.2). Of those who resided overseas, 8 (4%) lived in Australia and the remaining 4 (2%) in the United States, United Kingdom, Dubai and Hong Kong.

Fourteen of 242 (6%) perinatal survivors died between 28 days of age and commencement of the study. A further 29 (12%) were unable to be contacted and considered lost to follow up. Further reasons for nonparticipation included: 56 (23%) having no unaffected sibling available to participate, 25 (10%) residing overseas, 12 (5%) declining participation and 11 (5%) unable to enter due to medical or social problems (Figure 3.1).

The mean age at follow up (± SD) was 33.7±9.3 years in the 95 index cases and 40.1±10.9 years in the 92 unaffected siblings (p < 0.001).

### 3.3.2 Survival

Date of birth, gestation, sex, maternal and infant names were identified for all 242 perinatal survivors, 34 (81%) neonatal deaths and 58 (31%) fetal deaths.
Eleven perinatal survivors (5%) died in infancy at a median age of 94 days (range 29 to 339 days). Primary causes of death were infection 5 (45%), respiratory failure 2 (18%), congenital abnormalities 2 (18%), necrotising enterocolitis 1 (9%), and anaesthetic complications 1 (9%).

Three perinatal survivors (1%) died in adulthood. Causes and age of death were: trauma (29 years), suicide (30 years), and complications of cerebral palsy (36 years).

Although 139 of the IUT recipients were not identified, survival status was known for all 473 (Table 3.2). As there was no mechanism to reliably identify fetuses who received IUT prior to 1978, it is likely that the IUT recipients not identified by our searching strategy were born prior to this time. Using this assumption, survival status was assessed separately for babies born from 1963 to 1985 and from 1986 to 1992, to allow comparison of survival before and after introduction of the technique of intravascular IUT.

Table 3.2: Survival of IUT recipients born before and after the introduction of intravascular IUT in 1986

<table>
<thead>
<tr>
<th></th>
<th>Total IUT recipients n=473</th>
<th>Birth 1963-85 n=406</th>
<th>Birth 1986-92 n=67</th>
<th>p-value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal deaths</td>
<td>189 (40%)</td>
<td>182 (45%)</td>
<td>7 (10%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neonatal deaths</td>
<td>42 (9%)</td>
<td>41 (10%)</td>
<td>1 (1%)</td>
<td>0.006</td>
</tr>
<tr>
<td>Infant deaths</td>
<td>11 (2%)</td>
<td>11 (3%)</td>
<td>0 (0%)</td>
<td>0.07</td>
</tr>
<tr>
<td>Adult deaths</td>
<td>3 (1%)</td>
<td>3 (1%)</td>
<td>0 (0%)</td>
<td>0.34</td>
</tr>
<tr>
<td>Adult survivors</td>
<td>228 (48%)</td>
<td>169 (42%)</td>
<td>59 (88%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are n (%). * For comparison between birth date cohorts.
Figure 3.2: Location and numbers of participants of the Fetal Anaemia Study who resided in New Zealand
3.3.3 Perinatal Characteristics

3.3.3.1 Fetal deaths, neonatal/infant deaths and post-infancy survivors
Results are presented for 58 of 189 fetal deaths (31%), 45 of 53 neonatal or infant deaths (85%) and all 231 post-infancy survivors (forthwith referred to as “survivors”).

3.3.3.1.1 Maternal characteristics
Mothers whose babies survived were of lower gravidity and parity than those whose babies died. They also had had fewer rhesus affected pregnancies, received fewer IUTs, and had experienced fewer rhesus related perinatal deaths (Table 3.3).

3.3.3.1.2 Fetal and neonatal characteristics
Of the 58 fetuses who died, 51 (88%) were born before 1986. Perhaps unsurprisingly, since they were born at a mean gestation of 27.4±4.0 weeks, they received fewer IUTs and hence less blood, with first IUT given at an earlier gestation (25.6±3.9 weeks) than those who survived (Table 3.4).

Babies who survived were more likely to be born after 1986 than those who died in the first 12 months. Compared with those who died as neonates/infants, survivors were given their first IUT at a similar gestation (28.0±3.1 versus 27.5±4.2 weeks, p=0.63) but received more IUTs, particularly via the intravascular route. Consequently, survivors received a greater volume of blood and amount of haemoglobin in total although a similar volume of blood per IUT than those who died as neonates/infants. Survivors were also born at a greater gestational age and birth weight, and in better condition, as reflected in better Apgar scores and less need for respiratory support. They were more likely to receive phototherapy, although this was not associated with a reduction in the number of exchange transfusions, and were more likely to receive a higher number of top-up transfusions to a later age (Table 3.4).

3.3.3.2 IUT recipient survivors with and without follow up
Ninety five of the 228 IUT recipients who survived to adulthood participated in the Fetal Anaemia Study (42%). There were no significant differences in fetal characteristics between those who participated and those who did not (Table 3.5). Participants had a larger head circumference at birth, both absolute and corrected for gestation using z-scores, than those who did not participate. There were no significant differences in other neonatal characteristics between study participants and non-participants.
3.3.3.3 IUT recipients born pre and post 1986
Participants born from 1986 to 1992 were given their first IUT at an earlier gestation and received more IUTs in total than those born from 1963 to 1985 (Table 3.6). As the total volume of blood transfused was the same in both groups, the mean volume of blood transfused per IUT was less in those born later. Following the introduction of intravascular IUT, 56 (84%) of fetuses received intraperitoneal IUT. Those born later were more likely to have had a fetal blood sample taken and to have ascites or oedema detected prior to an IUT.

Birth weight and birth weight z-scores were similar for those born before or after 1986. Birth length and head circumference were larger in those born later, and a difference between groups persisted when head circumference, but not birth length, was expressed as a z-score. Those born later were more likely to have an Apgar score greater than 7 at five minutes, a lower peak serum bilirubin and to have received phototherapy and were less likely to have received exchange transfusion. Survival to adulthood following IUT was more common in those from born after 1986 (Table 3.6).

3.3.3.4 Affected and unaffected Fetal Anaemia Study participants
In comparison to unaffected siblings, affected participants were born at an earlier gestation, with 96% born preterm, compared to 11% of unaffected siblings (Table 3.7). Consequently affected participants were of lower birth weight than unaffected siblings, but birth weight z-score was greater for affected participants, presumably due at least in part to oedema and ascites. Affected participants were also less likely to have been born by normal vaginal delivery.
Table 3.3: Maternal characteristics of IUT recipients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mothers of Fetal Deaths</th>
<th>Mothers of Neonatal/Infant Deaths</th>
<th>Mothers of Survivors</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=50*</td>
<td>n=43*</td>
<td>n=209*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fetal Deaths vs Survivors</td>
<td>Neonatal/Infant Deaths vs Survivors</td>
</tr>
<tr>
<td>Gravidity</td>
<td>5 (2 – 11)</td>
<td>5 (2 – 11)</td>
<td>4 (1 – 11)</td>
<td>0.04</td>
</tr>
<tr>
<td>Parity</td>
<td>4 (1 – 10)</td>
<td>4 (2 – 9)</td>
<td>4 (1 – 10)</td>
<td>0.03</td>
</tr>
<tr>
<td>Number of RhHD affected pregnancies</td>
<td>3 (1 – 7)</td>
<td>3 (1 – 8)</td>
<td>2 (1 – 8)</td>
<td>0.006</td>
</tr>
<tr>
<td>≥ 1 RhHD related fetal deaths</td>
<td>50 (100%)</td>
<td>23 (53%)</td>
<td>55 (26%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥ 1 RhHD related neonatal or infant death</td>
<td>12 (24%)</td>
<td>43 (100%)</td>
<td>46 (22%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥ 1 perinatal survivor post IUT</td>
<td>27 (54%)</td>
<td>15 (35%)</td>
<td>209 (100%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Number of pregnancies requiring IUT</td>
<td>2 (1 – 4)</td>
<td>1 (1 – 4)</td>
<td>1 (1 – 4)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Data are median (range) or n (%). IUT=intrauterine transfusion, RhHD=rhesus haemolytic disease. * As some mothers had more than one affected fetus, the number of mothers in each group is less than the number of IUT recipients. † p-values from post-hoc analysis.
Table 3.4: Perinatal characteristics of IUT recipients who were fetal deaths, neonatal and infant death, and survivors

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Fetal Deaths</th>
<th>Neonatal/Infant Deaths</th>
<th>Survivors</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of IUT recipients</td>
<td>58</td>
<td>45</td>
<td>231</td>
<td></td>
</tr>
<tr>
<td><strong>Fetal characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age at LMP (years)</td>
<td>28.1±5.6</td>
<td>28.8±4.3</td>
<td>29.5±5.0</td>
<td>231</td>
</tr>
<tr>
<td>Gestation at first IUT (weeks)</td>
<td>25.6±3.9</td>
<td>28.0±3.1</td>
<td>27.5±4.2</td>
<td>230</td>
</tr>
<tr>
<td>Number of IUTs</td>
<td>1 (1 – 5)</td>
<td>2 (1 – 5)</td>
<td>3 (1 – 9)</td>
<td>231</td>
</tr>
<tr>
<td>Received intraperitoneal IUT &gt; 1</td>
<td>56 (97%)</td>
<td>45 (100%)</td>
<td>222 (96%)</td>
<td>231</td>
</tr>
<tr>
<td>intraperitoneal IUT</td>
<td>19 (33%)</td>
<td>34 (76%)</td>
<td>172 (74%)</td>
<td>222</td>
</tr>
<tr>
<td>Received intravascular IUT &gt; 1</td>
<td>5 (9%)</td>
<td>0 (0%)</td>
<td>57 (25%)</td>
<td>231</td>
</tr>
<tr>
<td>intravascular IUT</td>
<td>2 (3%)</td>
<td>0 (0%)</td>
<td>46 (20%)</td>
<td>231</td>
</tr>
<tr>
<td>Volume of blood transfused (ml)</td>
<td>90.9±70.4</td>
<td>196.0±90.2</td>
<td>226.4±87.9</td>
<td>222</td>
</tr>
<tr>
<td>Mean volume of blood per IUT (ml)</td>
<td>57.6±38.4</td>
<td>88.4±20.9</td>
<td>88.2±34.0</td>
<td>222</td>
</tr>
<tr>
<td>Amount of haemoglobin transfused (g)</td>
<td>25.7±19.9</td>
<td>53.8±24.2</td>
<td>62.1±24.9</td>
<td>193</td>
</tr>
<tr>
<td>Fetal blood sample taken</td>
<td>6 (10%)</td>
<td>0 (0%)</td>
<td>58 (25%)</td>
<td>230</td>
</tr>
<tr>
<td>Ascites present at time of IUT</td>
<td>24 (45%)</td>
<td>18 (45%)</td>
<td>108 (53%)</td>
<td>204</td>
</tr>
<tr>
<td>Oedema present at time of IUT</td>
<td>12 (23%)</td>
<td>5 (13%)</td>
<td>42 (20%)</td>
<td>206</td>
</tr>
<tr>
<td><strong>Neonatal characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Born before 1986</td>
<td>51 (88%)</td>
<td>44 (98%)</td>
<td>172 (74%)</td>
<td>231</td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>27.4±4.0</td>
<td>32.1±3.0</td>
<td>34.5±1.6</td>
<td>231</td>
</tr>
<tr>
<td>Male sex</td>
<td>34 (64%)</td>
<td>24 (53%)</td>
<td>140 (61%)</td>
<td>231</td>
</tr>
<tr>
<td>Received antenatal steroids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal vaginal delivery</td>
<td>20 (44%)</td>
<td>45</td>
<td>98 (43%)</td>
<td>228</td>
</tr>
</tbody>
</table>

* p-values indicate statistical significance between groups.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Fetal Deaths</th>
<th>Neonatal/Infant Deaths</th>
<th>Survivors</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>Total number of IUT recipients</td>
<td>58</td>
<td>45</td>
<td>231</td>
<td></td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.0±0.6</td>
<td>2.5±0.5</td>
<td>231</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Birth weight z-score</td>
<td>1.0±1.3</td>
<td>0.8±1.2</td>
<td>231</td>
<td>0.22</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>41.8±4.7</td>
<td>45.9±3.2</td>
<td>205</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Birth length z-score</td>
<td>-0.5±1.4</td>
<td>0.2±1.3</td>
<td>174†</td>
<td>0.03</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>30.4±2.8</td>
<td>32.4±2.0</td>
<td>203</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Head circumference z-score</td>
<td>0.4±1.2</td>
<td>0.9±1.4</td>
<td>203</td>
<td>0.01</td>
</tr>
<tr>
<td>Hydropic at birth</td>
<td>39 (93%)</td>
<td>116 (51%)</td>
<td>229</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5 minute Apgar score &gt;7</td>
<td>16 (41%)</td>
<td>161 (77%)</td>
<td>209</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ventilatory support in first day</td>
<td>28 (64%)</td>
<td>50 (22%)</td>
<td>231</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cord serum bilirubin (mmol.L⁻¹)</td>
<td>106±52</td>
<td>111±61</td>
<td>227</td>
<td>0.68</td>
</tr>
<tr>
<td>Received phototherapy</td>
<td>6 (13%)</td>
<td>124 (54%)</td>
<td>231</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Received exchange transfusion</td>
<td>38 (86%)</td>
<td>192 (83%)</td>
<td>231</td>
<td>0.59</td>
</tr>
<tr>
<td>Number of exchange transfusions</td>
<td>1 (0 – 11)</td>
<td>1 (0 – 8)</td>
<td>231</td>
<td>0.93</td>
</tr>
<tr>
<td>Lowest haemoglobin (g.L⁻¹)</td>
<td>86±32</td>
<td>88±21</td>
<td>222</td>
<td>0.61</td>
</tr>
<tr>
<td>Received top up transfusion</td>
<td>7 (16%)</td>
<td>139 (60%)</td>
<td>230</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Number of top up transfusions</td>
<td>0 (0 – 2)</td>
<td>1 (0 – 14)</td>
<td>230</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age at last top up transfusion (days)</td>
<td>10.3±12.3</td>
<td>39.3±20.6</td>
<td>139</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are mean±SD, median (range) or n (%). IUT=intrauterine transfusion, LMP=last menstrual period. * p-values from post-hoc analysis. † Data for calculation of birth length z-score not available below 33 weeks’ gestation.
Table 3.5: Perinatal characteristics of Fetal Anaemia Study participants and non-participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Participants</th>
<th>Non-participants</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total number of IUT recipients</strong></td>
<td>95</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td><strong>Fetal characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age at LMP (years)</td>
<td>29.7±4.4</td>
<td>29.4±5.3</td>
<td>0.77</td>
</tr>
<tr>
<td>Gestation at first IUT (weeks)</td>
<td>27.5±4.3</td>
<td>27.5±4.1</td>
<td>0.91</td>
</tr>
<tr>
<td>Number of IUTs</td>
<td>3 (1 – 9)</td>
<td>3 (1 – 7)</td>
<td>0.95</td>
</tr>
<tr>
<td>Received intraperitoneal IUT</td>
<td>93 (98%)</td>
<td>126 (95%)</td>
<td>0.23</td>
</tr>
<tr>
<td>&gt; 1 intraperitoneal IUT</td>
<td>72 (76%)</td>
<td>97 (73%)</td>
<td>0.63</td>
</tr>
<tr>
<td>Received intravascular IUT</td>
<td>26 (27%)</td>
<td>31 (23%)</td>
<td>0.49</td>
</tr>
<tr>
<td>&gt; 1 intravascular IUT</td>
<td>20 (21%)</td>
<td>26 (20%)</td>
<td>0.78</td>
</tr>
<tr>
<td>Volume of blood transfused (ml)</td>
<td>223.1±78.8</td>
<td>226.9±93.7</td>
<td>0.89</td>
</tr>
<tr>
<td>Mean volume of blood per IUT (ml)</td>
<td>88.5±32.6</td>
<td>88.3±35.3</td>
<td>0.97</td>
</tr>
<tr>
<td>Amount of blood transfused (g)</td>
<td>60.3±23.9</td>
<td>62.8±25.8</td>
<td>0.52</td>
</tr>
<tr>
<td>Fetal blood sample taken</td>
<td>26 (27%)</td>
<td>32 (24%)</td>
<td>0.57</td>
</tr>
<tr>
<td>Ascites present at time of IUT</td>
<td>39 (41%)</td>
<td>67 (50%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Oedema present at time of IUT</td>
<td>15 (16%)</td>
<td>25 (19%)</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Neonatal characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth pre 1986</td>
<td>69 (73%)</td>
<td>100 (75%)</td>
<td>0.66</td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>34.5±1.6</td>
<td>34.5±1.6</td>
<td>1.00</td>
</tr>
<tr>
<td>Male sex</td>
<td>51 (54%)</td>
<td>87 (65%)</td>
<td>0.07</td>
</tr>
<tr>
<td>Received antenatal steroids</td>
<td>24 (26%)</td>
<td>42 (32%)</td>
<td>0.27</td>
</tr>
<tr>
<td>Mode of delivery (normal vaginal)</td>
<td>40 (43%)</td>
<td>56 (42%)</td>
<td>0.84</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.6±0.4</td>
<td>2.5±0.5</td>
<td>0.16</td>
</tr>
<tr>
<td>Birth weight z-score</td>
<td>0.9±1.1</td>
<td>0.7±1.2</td>
<td>0.10</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>46.1±3.5</td>
<td>45.9±2.9</td>
<td>0.32</td>
</tr>
<tr>
<td>Birth length z-score</td>
<td>0.2±1.4</td>
<td>0.2±1.2</td>
<td>0.84</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>32.9±2.2</td>
<td>32.2±1.9</td>
<td>0.01</td>
</tr>
<tr>
<td>Head circumference z-score</td>
<td>1.2±1.6</td>
<td>0.7±1.2</td>
<td>0.03</td>
</tr>
<tr>
<td>Hydropic at birth</td>
<td>45 (48%)</td>
<td>69 (52%)</td>
<td>0.51</td>
</tr>
<tr>
<td>5 minute Apgar score &gt;7</td>
<td>64 (81%)</td>
<td>95 (75%)</td>
<td>0.30</td>
</tr>
<tr>
<td>Ventilatory support in first day after birth</td>
<td>25 (26%)</td>
<td>25 (19%)</td>
<td>0.18</td>
</tr>
<tr>
<td>Cord serum bilirubin (mmol.l⁻¹)</td>
<td>113.0±75.3</td>
<td>110.7±49.1</td>
<td>0.71</td>
</tr>
<tr>
<td>Highest serum bilirubin (mmol.l⁻¹)</td>
<td>276.6±109.2</td>
<td>279.0±149.6</td>
<td>0.43</td>
</tr>
<tr>
<td>Received phototherapy</td>
<td>54 (57%)</td>
<td>68 (51%)</td>
<td>0.39</td>
</tr>
<tr>
<td>Received exchange transfusion</td>
<td>81 (85%)</td>
<td>108 (81%)</td>
<td>0.42</td>
</tr>
<tr>
<td>Number of exchange transfusions</td>
<td>2 (0 – 7)</td>
<td>1 (0 – 8)</td>
<td>0.48</td>
</tr>
<tr>
<td>Cord haemoglobin (g.l⁻¹)</td>
<td>119.7±26.8</td>
<td>124.3±32.3</td>
<td>0.47</td>
</tr>
<tr>
<td>Lowest haemoglobin (g.l⁻¹)</td>
<td>88.2±20.2</td>
<td>88.4±21.5</td>
<td>0.98</td>
</tr>
<tr>
<td>Received top up transfusion</td>
<td>59 (63%)</td>
<td>77 (58%)</td>
<td>0.46</td>
</tr>
<tr>
<td>Number of top up transfusions</td>
<td>1 (0 – 11)</td>
<td>1 (0 – 14)</td>
<td>0.50</td>
</tr>
<tr>
<td>Age at last top up transfusion (days)</td>
<td>37.9±20.9</td>
<td>40.3±20.8</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Data are n (%), median (range) or mean±SD. IUT=intraperitoneal transfusion, LMP=last menstrual period. * Data for calculation of birth length z-score not available below 33 weeks’ gestation.
Table 3.6: Perinatal characteristics of IUT recipients born before and after the introduction of intravascular transfusion in 1986

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>Total number of IUT recipients</td>
<td>267</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td><strong>Fetal characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age at LMP (years)</td>
<td>29.1±5.1</td>
<td>267</td>
<td>29.73±4.6</td>
</tr>
<tr>
<td>Gestation at first IUT (weeks)</td>
<td>28.1±3.7</td>
<td>266</td>
<td>23.8±3.9</td>
</tr>
<tr>
<td>Number of IUTs</td>
<td>2 (1 – 7)</td>
<td>267</td>
<td>4 (1 – 9)</td>
</tr>
<tr>
<td>Received intraperitoneal IUT &gt;1 intraperitoneal IUT</td>
<td>267 (100%)</td>
<td>267</td>
<td>56 (84%)</td>
</tr>
<tr>
<td>Received intravascular IUT &gt; 1 intravascular IUT</td>
<td>0 (0%)</td>
<td>267</td>
<td>62 (93%)</td>
</tr>
<tr>
<td>Volume of blood transfused (ml)</td>
<td>196.9±100.5</td>
<td>250</td>
<td>207.3±94.0</td>
</tr>
<tr>
<td>Mean volume of blood per IUT (ml)</td>
<td>91.5±33.0</td>
<td>266</td>
<td>50.9±23.5</td>
</tr>
<tr>
<td>Amount of blood transfused (g)</td>
<td>53.7±27.9</td>
<td>261</td>
<td>56.9±24.9</td>
</tr>
<tr>
<td>Fetal blood sample taken</td>
<td>1 (0%)</td>
<td>267</td>
<td>63 (94%)</td>
</tr>
<tr>
<td>Ascites present at time of IUT</td>
<td>111 (42%)</td>
<td>267</td>
<td>39 (58%)</td>
</tr>
<tr>
<td>Oedema present at time of IUT</td>
<td>36 (13%)</td>
<td>267</td>
<td>23 (34%)</td>
</tr>
<tr>
<td><strong>Neonatal characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>32.7±3.6</td>
<td>267</td>
<td>33.7±3.5</td>
</tr>
<tr>
<td>Male sex</td>
<td>160 (60%)</td>
<td>264</td>
<td>38 (58%)</td>
</tr>
<tr>
<td>Received antenatal steroids</td>
<td>70 (33%)</td>
<td>212</td>
<td>15 (26%)</td>
</tr>
<tr>
<td>Normal vaginal delivery</td>
<td>94 (44%)</td>
<td>214</td>
<td>24 (41%)</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.4±0.5</td>
<td>215</td>
<td>2.5±0.4</td>
</tr>
<tr>
<td>Birth weight z-score</td>
<td>0.8±1.2</td>
<td>214</td>
<td>0.7±1.0</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>45.0±3.9</td>
<td>188</td>
<td>46.3±3.1</td>
</tr>
<tr>
<td>Birth length z-score</td>
<td>0.1±1.2</td>
<td>145</td>
<td>0.2±1.6</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>31.9±2.2</td>
<td>181</td>
<td>32.8±2.2</td>
</tr>
<tr>
<td>Head circumference z-score</td>
<td>0.7±1.2</td>
<td>181</td>
<td>1.1±1.7</td>
</tr>
<tr>
<td>Hydropic at birth</td>
<td>130 (62%)</td>
<td>211</td>
<td>25 (42%)</td>
</tr>
<tr>
<td>5 minute Apgar score &gt;7</td>
<td>129 (68%)</td>
<td>189</td>
<td>48 (81%)</td>
</tr>
<tr>
<td>Ventilatory support in first day after birth</td>
<td>61 (28%)</td>
<td>216</td>
<td>17 (28%)</td>
</tr>
<tr>
<td>Cord serum bilirubin (mmol.1⁻¹)</td>
<td>113.7±62.9</td>
<td>210</td>
<td>99.8±43.4</td>
</tr>
<tr>
<td>Highest serum bilirubin (mmol.1⁻¹)</td>
<td>284.0±146.6</td>
<td>207</td>
<td>243.6±82.5</td>
</tr>
<tr>
<td>Received phototherapy</td>
<td>77 (36%)</td>
<td>216</td>
<td>53 (88%)</td>
</tr>
<tr>
<td>Received exchange transfusion</td>
<td>195 (91%)</td>
<td>215</td>
<td>35 (58%)</td>
</tr>
<tr>
<td>Number of exchange transfusions</td>
<td>2 (0 – 11)</td>
<td>215</td>
<td>1 (0 – 4)</td>
</tr>
<tr>
<td>Cord haemoglobin (g.1⁻¹)</td>
<td>121.5±34.7</td>
<td>210</td>
<td>112.4±24.5</td>
</tr>
<tr>
<td>Lowest haemoglobin (g.1⁻¹)</td>
<td>87.6±22.5</td>
<td>200</td>
<td>87.4±24.0</td>
</tr>
<tr>
<td>Received top up transfusion</td>
<td>119 (56%)</td>
<td>214</td>
<td>27 (45%)</td>
</tr>
<tr>
<td>Number of top up transfusions</td>
<td>1 (0 – 14)</td>
<td>214</td>
<td>0 (0 – 3)</td>
</tr>
<tr>
<td>Age at last top up transfusion (days)</td>
<td>39.1±20.2</td>
<td>119</td>
<td>32.6±24.8</td>
</tr>
<tr>
<td>Adult survivor post IUT</td>
<td>169 (63%)</td>
<td>267</td>
<td>59 (88%)</td>
</tr>
</tbody>
</table>

Data are n (%), median (range) or mean±SD. IUT=intrauterine transfusion, LMP=last menstrual period. * Data for calculation of birth length z-score not available below 33 weeks’ gestation.
Table 3.7: Neonatal characteristics of affected and unaffected Fetal Anaemia Study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Affected Participants</th>
<th>Unaffected Participants</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>34.5±1.6</td>
<td>95</td>
<td>39.5±2.1</td>
</tr>
<tr>
<td>Birth &lt; 37 weeks</td>
<td>91 (96)</td>
<td>95</td>
<td>10 (11)</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>51 (54%)</td>
<td>95</td>
<td>41 (45%)</td>
</tr>
<tr>
<td>Normal vaginal delivery</td>
<td>40 (43%)</td>
<td>92</td>
<td>66 (74%)</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.5±0.5</td>
<td>95</td>
<td>3.3±0.6</td>
</tr>
<tr>
<td>Birth weight z-score</td>
<td>0.8±1.2</td>
<td>95</td>
<td>-0.1±1.2</td>
</tr>
</tbody>
</table>

Data are mean±SD or n (%).

3.4 Discussion

This chapter examines the survival and perinatal characteristics of the oldest and largest single centre cohort of IUT recipients in the world. Survival data are presented for 473 IUT recipients born over thirty years from 1963 to 1992. Of the total cohort, 40% died in utero and 9% died in the neonatal period resulting in 242 (51%) perinatal survivors. Of these, 228 (48% of all recipients and 94% of perinatal survivors) were believed to be alive at the time of this study, 18 to 47 years later.

Survival following IUT has been reported by a number of groups (Table 3.8). These studies were initiated over a number of decades, from the 1960s to the 1990s, and improvements in perinatal mortality will in part reflect advancement in perinatal medicine as a whole during this time. However, the advent of intravascular IUT heralded a significant improvement in survival from the mid 1980s, and our cohort comprised both intravascular and intraperitoneal transfusion recipients. Therefore, to allow meaningful comparison of survival findings with other reported IUT cohorts, the Fetal Anaemia Study cohort was divided into those born from 1963 to 1985 and from 1986 to 1992, using the assumption that fetal and neonatal deaths not identified by our searching strategy were likely to have been born before the introduction of the Special Procedures Book in 1978.

Survival rates in our study compare favourably with 4 previous reports of survival of small cohorts of recipients of intraperitoneal IUT (McCrostie 1966; White, Goplerud et al. 1978; Hardyment, Salvador et al. 1979; Harman, Bowman et al. 1990)(Table 3.8). They also compare favourably with those of 11 cohorts of recipients for whom the intravascular route
Table 3.8: Summary of survival statistics from reported cohorts of intraperitoneal and intravascular IUT recipients

<table>
<thead>
<tr>
<th>Study</th>
<th>Time frame</th>
<th>Number of IUT recipients</th>
<th>Country of origin</th>
<th>Predominant IUT technique</th>
<th>Fetal deaths</th>
<th>Neonatal deaths</th>
<th>Perinatal survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal Anaemia Study</td>
<td>1963–85</td>
<td>406</td>
<td>New Zealand</td>
<td>IPT</td>
<td>182 (45%)</td>
<td>41 (10%)</td>
<td>183 (45%)</td>
</tr>
<tr>
<td>McCrostie, 1966</td>
<td>1964–65</td>
<td>10</td>
<td>New Zealand</td>
<td>IPT</td>
<td>4 (40%)</td>
<td>1 (10%)</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>White et al, 1978</td>
<td>1965–76</td>
<td>84</td>
<td>USA</td>
<td>IPT</td>
<td>49 (58%)</td>
<td>5 (6%)</td>
<td>30 (36%)</td>
</tr>
<tr>
<td>Hardyment et al 1979</td>
<td>1966–75</td>
<td>57</td>
<td>Canada</td>
<td>IPT</td>
<td>15 (26%)</td>
<td>15 (26%)</td>
<td>27 (47%)</td>
</tr>
<tr>
<td>Harman et al, 1990</td>
<td>1980–86</td>
<td>44</td>
<td>Canada</td>
<td>IPT</td>
<td>10 (23%)</td>
<td>5 (11%)</td>
<td>29 (66%)</td>
</tr>
<tr>
<td>Fetal Anaemia Study</td>
<td>1986–92</td>
<td>67</td>
<td>New Zealand</td>
<td>IVT</td>
<td>7 (10%)</td>
<td>1 (1%)</td>
<td>59 (88%)</td>
</tr>
<tr>
<td>Grannum et al, 1988</td>
<td>1984–87</td>
<td>26</td>
<td>USA</td>
<td>IVT</td>
<td>4 (15%)</td>
<td>1 (4%)</td>
<td>21 (81%)</td>
</tr>
<tr>
<td>Lemery et al, 1989</td>
<td>1984–89</td>
<td>15</td>
<td>France</td>
<td>IVT</td>
<td>4 (27%)</td>
<td>1 (7%)</td>
<td>10 (67%)</td>
</tr>
<tr>
<td>Poissonnier et al, 1989</td>
<td>1985–88</td>
<td>107</td>
<td>France</td>
<td>IVT</td>
<td>15 (14%)</td>
<td>8 (7%)</td>
<td>84 (79%)</td>
</tr>
<tr>
<td>Weiner et al, 1991</td>
<td>1985–91</td>
<td>48</td>
<td>USA</td>
<td>IVT</td>
<td>2 (4%)</td>
<td>0 (0%)</td>
<td>46 (96%)</td>
</tr>
<tr>
<td>Nicolini et al, 1989</td>
<td>1986–87</td>
<td>31</td>
<td>England</td>
<td>IVT</td>
<td>4 (13%)</td>
<td>1 (3%)</td>
<td>26 (84%)</td>
</tr>
<tr>
<td>Orsini et al, 1988</td>
<td>1986–88</td>
<td>15</td>
<td>Italy</td>
<td>IVT</td>
<td>4 (27%)</td>
<td>1 (7%)</td>
<td>10 (67%)</td>
</tr>
<tr>
<td>Harman et al, 1990</td>
<td>1986–90</td>
<td>44</td>
<td>Canada</td>
<td>IVT</td>
<td>3 (7%)</td>
<td>1 (2%)</td>
<td>40 (91%)</td>
</tr>
<tr>
<td>Grab et al, 1999</td>
<td>1986–91</td>
<td>43</td>
<td>Germany</td>
<td>IVT</td>
<td>5 (12%)</td>
<td>3 (7%)</td>
<td>35 (81%)</td>
</tr>
<tr>
<td>Lindenburg et al, 2011</td>
<td>1988–08</td>
<td>451</td>
<td>Netherlands</td>
<td>IVT</td>
<td>31 (7%)</td>
<td>11 (2%)</td>
<td>409 (91%)</td>
</tr>
<tr>
<td>Farrant et al, 2001</td>
<td>1991–00</td>
<td>41</td>
<td>New Zealand</td>
<td>IVT</td>
<td>4 (10%)</td>
<td>0 (0%)</td>
<td>37 (90%)</td>
</tr>
<tr>
<td>Weisz et al, 2009</td>
<td>1996–04</td>
<td>54</td>
<td>Israel</td>
<td>IVT</td>
<td>6 (11%)</td>
<td>1 (2%)</td>
<td>47 (87%)</td>
</tr>
</tbody>
</table>

IPT=intraperitoneal transfusion, IVT=intravascular transfusion.
was the primary IUT technique (Grannum, Copel et al. 1988; Orsini, Pilu et al. 1988; Lemery, Urbain et al. 1989; Nicolini, Kochenour et al. 1989; Poissonnier, Brossard et al. 1989; Harman, Bowman et al. 1990; Weiner, Williamson et al. 1991; Grab, Paulus et al. 1999; Farrant, Battin et al. 2001; Weisz, Rosenbaum et al. 2009; Lindenburg, Smits-Wintjens et al. 2011). Apart from the report of Lindenburg et al, these cohorts are small, with a median of 44 IUT recipients (Table 3.8).

Ours is the first inclusive report of the maternal, fetal and neonatal characteristics of IUT recipients. Previous cohorts have included limited perinatal information and frequently exclude fetal and/or neonatal deaths or hydropic fetuses (Poissonnier, Brossard et al. 1989; Harman, Bowman et al. 1990; Utter, Socol et al. 1990; Weiner, Williamson et al. 1991; Roberts, Grannum et al. 1993; Janssens, de Haan et al. 1997; De Boer, Zeestraten et al. 2008). However, three reports provide some perinatal data on IUT survivors that can be compared with our findings (Grannum, Copel et al. 1988; McGlone, Simpson et al. 2009; Lindenburg, Smits-Wintjens et al. 2011) (Table 3.9). Gestation at first IUT, number of IUTs required and gestation at delivery were similar for all four groups. However, a greater proportion of infants in the Fetal Anaemia Study cohort were hydropic at birth and received exchange transfusion; features which likely reflect changes in both obstetric and neonatal practice over the 45 years from which these cohorts are drawn.

Table 3.9: Comparison of fetal and neonatal characteristics of IUT survivor cohorts

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number IUT survivors</td>
<td>231</td>
<td>291</td>
<td>116</td>
<td>21</td>
</tr>
<tr>
<td>Gestation at first IUT (weeks)</td>
<td>27.5±4.2</td>
<td>26.0±4.2</td>
<td>26 (20 – 33)</td>
<td>27.2±4.0</td>
</tr>
<tr>
<td>Number IUTs per fetus</td>
<td>3 (1 – 9)</td>
<td>3 (1 – 6)</td>
<td>4 (1 – 9)</td>
<td>3(1 – 6)</td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>34.5±1.6</td>
<td>36 (35–37)*</td>
<td>35</td>
<td>34.4±1.9</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.5±0.5</td>
<td>2.8 (2.5–3.2)*</td>
<td>-</td>
<td>2.3±0.5</td>
</tr>
<tr>
<td>Hydropic at birth</td>
<td>116 (51%)</td>
<td>75 (26%)</td>
<td>-</td>
<td>3 (14%)</td>
</tr>
<tr>
<td>Received exchange transfusion</td>
<td>192 (83%)</td>
<td>168 (58%)</td>
<td>23 (20%)</td>
<td>13 (62%)</td>
</tr>
<tr>
<td>Received phototherapy</td>
<td>124 (54%)</td>
<td>-</td>
<td>116 (100%)</td>
<td>-</td>
</tr>
<tr>
<td>Received top up transfusion</td>
<td>139 (60%)</td>
<td>-</td>
<td>63 (54%)</td>
<td>-</td>
</tr>
</tbody>
</table>

*Median (IQR), otherwise data are mean±SD, median (range) or n (%). IUT=intruterine transfusion.

Our longitudinal data collection allows us to explore the influence of changes in practice with time, in particular the introduction of intravascular IUT. The increased number of IUTs and decreased mean volume of blood per transfusion for IUT recipients born from 1986 to
1992 reflects the trend for more frequent, smaller volume IUTs with the advent of intravascular procedures (Nicolini, Kochenour et al. 1989). The increased incidence of ascites or oedema at the time of IUT in those born from 1986 is likely due to improved antenatal diagnostic ability, rather than a true increase in the incidence of fetal hydrops, especially as infants born later were less likely to be hydropic at birth. There was no difference in birth weight between those born before and after the introduction of intravascular IUT in 1986, in contrast to a previous report of low birth weight in intraperitoneal IUT recipients (Binks, Lind et al. 1973). Although phototherapy was introduced at National Women’s Hospital in 1971, it was not routinely used for the management of hyperbilirubinaemia until 1980. Thus, increased use of phototherapy and decreased requirement for exchange transfusion in infants born later are in keeping with this change in practice and are consistent with more recent reports of the neonatal characteristics of IUT survivor cohorts (McGlone, Simpson et al. 2009; Lindenburg, Smits-Wintjens et al. 2011).

Two comparisons are critical for interpretation of the remainder of the results of this study. Firstly, comparison of IUT survivors who participated in the Fetal Anaemia Study and those who did not indicates no clinically relevant differences in perinatal characteristics. Thus, affected participants of the Fetal Anaemia Study are likely to be reasonably representative of the entire cohort of IUT survivors over this time period, even though we were only able to study approximately half of them.

Secondly, comparison of affected and unaffected participants reveals a number of important differences. As expected, given that infants with severe rhesus disease are commonly born preterm, affected participants were born at an earlier gestation and of lower birth weight than unaffected participants, and were less likely to have a normal vaginal delivery. However, birth weight z-score was higher in affected participants. This is likely to be due to increased maternal parity and the presence of hydrops at birth in affected participants, since maternal factors such as smoking status, ethnicity and body habitus were the same for affected participants and unaffected siblings (Seidman, Ever-Hadani et al. 1988; Roberts, Grannum et al. 1993).

There are several potential limitations to the findings reported in this chapter. Firstly, we have no details for 131 (69%) fetal deaths and 8 (15%) neonatal deaths, so our findings related to fetal and neonatal deaths are not representative of the entire cohort. However, as
there was no reliable way to identify fetuses who received IUT before the introduction of Special Procedures Book in 1978, it is reasonable to assume that all missing IUT recipients were born before this time. This assumption was used to calculate survival statistics before and after 1986. However, it was not possible to gather perinatal data on infants who were not identified. Therefore, it is likely that the differences found between those born before and after 1986 are less marked than they would be if case capture had been complete.

Secondly, for the 334 IUT recipients for whom perinatal data was collected, details were frequently missing from medical records. For example, birth length and head circumference were not documented in approximately 30% of cases and the amount of haemoglobin transfused was not documented for more than half of the fetuses transfused from 1986. Occasionally the information was simply not available; e.g. Apgar Scores were not routinely recorded until the late 1960s. However, for the remaining perinatal characteristics, data were missing for only a small number of IUT recipients and for similar numbers in each of the comparison subgroups, making important bias unlikely.

Finally, it is possible that some of the significant differences reported in this chapter may be explained by chance due to multiple comparisons in the analyses. In total, 157 comparisons were made in this chapter. With statistical significance defined as a p-value of less than or equal to 0.05, approximately 8 comparisons would be expected to be statistically significant by chance alone (type one errors). However, we found 81 statistically significant associations, and the majority of p-values are small, so it is unlikely that this is a major source of error.

3.5 Summary
The Fetal Anaemia Study cohort is both the largest and oldest cohort of IUT recipients in the world. Our findings from recipients treated at a single centre over the last 50 years clearly show greater severity of illness in those who did not survive, and improved survival from the mid 1980s following the introduction of intravascular IUT. Furthermore, given the similarity between IUT survivors who participated and those who did not, our findings with respect to adult outcomes following IUT are likely to be representative of the entire cohort.

The value of this description of the perinatal characteristics of the Fetal Anaemia Study cohort is not purely historical. Given the paucity of data in the literature, this information may act as a baseline with which future cohorts may be compared, thus providing a
mechanism by which changes in practice may be assessed. Furthermore, it is likely that the IUT survivors included in the Fetal Anaemia Study are representative of other adult IUT survivors around the world, given that the technique of IUT and the diagnostic tools used to determine when transfusion was required were established in New Zealand and were widely accepted as the standard of care prior to the development of more sophisticated diagnostic and IUT techniques in the mid 1980s. Thus, it is the perinatal characteristics of this older cohort, rather than those born more recently, that are currently of relevance when considering the potential cardiovascular implications for adult survivors of fetal anaemia and IUT throughout the world.
Chapter 4: Adult Characteristics of the Cohort

4.1 Summary of Chapter Contents
This chapter describes the parental characteristics, socioeconomic status and cardiovascular risk factors of IUT recipients who participated in the Fetal Anaemia Study and their unaffected siblings.

4.2 Introduction
Cardiovascular disease and its risk factors, including hypertension, hyperlipidaemia and diabetes mellitus, are a major burden on health care resources (Capewell, Ford et al. 2010). A large amount of epidemiological evidence now supports the association between an unfavourable intrauterine environment and cardiovascular risk factors and disease in later life (Barker and Bagby 2005). Impaired fetal nutrition is associated with hypertension, impaired glucose tolerance and hyperlipidaemia in adulthood which may be modulated through alterations to renal, pancreatic and hepatic blood flow as the undernourished fetus attempts to preserve nutrient and oxygen supply to the brain (Osmond and Barker 2000). Furthermore, an unfavourable intrauterine environment may also result in preterm birth, which may contribute to the risk of hypertension and impaired glucose tolerance in later life (Dalziel, Parag et al. 2007).

Intrauterine anaemia causes fetal hypoxia and increased blood flow to the brain, heart and adrenal glands in order to maintain oxygen supply to these organs. However blood flow to other regions, including the gastrointestinal tract and kidneys, remains unchanged, hence oxygen supply to these tissues is decreased (Fumia, Edelstone et al. 1984). Therefore, it is plausible that fetal anaemia may act in a similar way as impaired fetal nutrition to programme an individual for an unfavourable cardiovascular risk factor profile in later life. However, the influence of fetal anaemia on cardiovascular outcome in adulthood has not previously been investigated.

An individual’s ability to achieve socially and economically may be influenced by a number of subtle abnormalities, for example, mild impairment of cognitive function, behavioural problems and learning difficulties (van Klink, Koopman et al. 2011). Several studies have reported neurodevelopmental outcome in childhood following IUT, with most concluding that survivors of IUT are at no greater risk of neurological impairment than other high or low risk infant groups (Doyle, Kelly et al. 1993; Janssens, de Haan et al. 1997; Hudon,
Moise et al. 1998; Grab, Paulus et al. 1999; Farrant, Battin et al. 2001; Harper, Swingle et al. 2006; Weisz, Rosenbaum et al. 2009; Lindenburg, Smits-Wintjens et al. 2011). Those findings suggest that the socioeconomic status of IUT survivors should not differ from that of their unaffected siblings, but adult outcomes have not previously been reported.

4.3 Chapter Hypothesis
That exposure to fetal anaemia does not alter socioeconomic status, educational achievement, smoking status, alcohol intake, physical activity, body size, general health, medication use, heart rate variability or prevalence of cardiovascular disease, hypertension, hyperlipidaemia, diabetes or impaired glucose tolerance in adulthood.

4.4 Results
4.4.1 Parental Characteristics and Sibling Relationships
One hundred and sixty seven (89%) participants (78 sibling groups) were full siblings, thus parental characteristics are presented for all participants combined (Table 4.1). For 9 of the 10 sibling groups who were not full siblings, the mother was the common parent. As unaffected siblings were frequently the first born in a family, parents were younger at delivery of unaffected participants (24.0±4.4 years versus 29.9±4.5 years for mothers and 28.0±5.2 versus 33.4±5.5 years for fathers, both p<0.001). Cardiovascular death was more common in fathers than mothers, as was a history of coronary artery or cerebrovascular disease. With the exception of hyperlipidaemia, cardiovascular risk factors were also more common in fathers (Table 4.1). Within sibling groups, parental characteristics were reported consistently, with more than 90% concordance between responses from affected and unaffected participants to questions regarding parental medical history.

4.4.2 Sex Distribution, Age and Ethnicity
Approximately half of affected and unaffected participants were male. Affected participants were a median of 6 years younger than unaffected siblings (Table 4.2). One hundred and fifty six (83%) of the entire group identified themselves as European, 22 (12%) as Maori, 2 (1%) as of Pacific origin and 7 (4%) identified themselves with other ethnic groups.

4.4.3 Socioeconomic Status
Sixty percent of participants were married or living with a partner and a similar proportion of affected and unaffected participants had undertaken post secondary school education
Table 4.1: Parental characteristics of Fetal Anaemia Study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
<th>n=89</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular death*</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>History of coronary artery disease</td>
<td>7 (8%)</td>
<td></td>
</tr>
<tr>
<td>History of cerebrovascular disease</td>
<td>3 (3%)</td>
<td></td>
</tr>
<tr>
<td>History of hypertension</td>
<td>33 (37%)</td>
<td></td>
</tr>
<tr>
<td>History of hyperlipidaemia</td>
<td>31 (35%)</td>
<td></td>
</tr>
<tr>
<td>History of diabetes</td>
<td>6 (7%)</td>
<td></td>
</tr>
<tr>
<td>Smoker during pregnancy†</td>
<td>37 (20%)</td>
<td></td>
</tr>
<tr>
<td>Smoker during participant’s childhood†</td>
<td>52 (28%)</td>
<td></td>
</tr>
<tr>
<td><strong>Paternal characteristics</strong></td>
<td></td>
<td>n=97</td>
</tr>
<tr>
<td>Cardiovascular death†</td>
<td>10 (10%)</td>
<td></td>
</tr>
<tr>
<td>History of coronary artery disease</td>
<td>23 (24%)</td>
<td></td>
</tr>
<tr>
<td>History of cerebrovascular disease</td>
<td>9 (9%)</td>
<td></td>
</tr>
<tr>
<td>History of hypertension</td>
<td>41 (42%)</td>
<td></td>
</tr>
<tr>
<td>History of hyperlipidaemia</td>
<td>39 (40%)</td>
<td></td>
</tr>
<tr>
<td>History of diabetes</td>
<td>13 (13%)</td>
<td></td>
</tr>
<tr>
<td>Smoker during participant’s childhood†</td>
<td>67 (36%)</td>
<td></td>
</tr>
</tbody>
</table>

Data are n (%). * 6 maternal deaths and 22 paternal deaths in total. † n=187 participants.

(\(p=0.24\)). However, of those who did not undertake post secondary school education, affected participants were more likely to have remained at secondary school for more than 3 years (\(p=0.003\)). These findings did not alter following adjustment for age. There were no differences between affected and unaffected participants for occupation type or income, with more than half of participants reporting a household income of over $70,000 per annum. New Zealand deprivation index scores were also similar in affected and unaffected participants (Table 4.2).

### 4.4.4 Lifestyle Variables

There were no differences between affected and unaffected participants for smoking status, with 35 (19%) reporting that they were current smokers. There were also no differences between participant groups for alcohol use, with over half of participants defined as heavy drinkers according to revised Alcohol Advisory Committee guidelines. (www.alac.org.nz/alcohol-you/your-drinking-okay/low-risk-alcohol-drinking-advice). An apparent trend towards more affected participants using illicit drugs in the last year was no longer evident following adjustment for age (\(p=0.22\)). The majority of participants reported exercising to a level at or above that recommended by the European guidelines on cardiovascular disease prevention (Perk, De Backer et al. 2012), with no difference between groups (Table 4.2).
Table 4.2: Socioeconomic status, lifestyle characteristics, past medical history and medication use of Fetal Anaemia Study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Affected n=95</th>
<th>Unaffected n=92</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>51 (54%)</td>
<td>41 (45%)</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Age at time of study (years)</strong></td>
<td>37.2 (17.3–47.8)</td>
<td>43.3 (18.2–60.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Marital status</strong></td>
<td></td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td>Married or living with partner</td>
<td>58 (61%)</td>
<td>55 (60%)</td>
<td></td>
</tr>
<tr>
<td>Separated/divorced/widowed</td>
<td>5 (5%)</td>
<td>11 (12%)</td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>32 (34%)</td>
<td>26 (28%)</td>
<td></td>
</tr>
<tr>
<td><strong>Highest educational achievement</strong></td>
<td></td>
<td></td>
<td>0.005</td>
</tr>
<tr>
<td>≤ 3 years secondary schooling</td>
<td>11 (12%)</td>
<td>20 (22%)</td>
<td></td>
</tr>
<tr>
<td>&gt; 3 years secondary schooling</td>
<td>31 (33%)</td>
<td>13 (14%)</td>
<td></td>
</tr>
<tr>
<td>Post secondary school education</td>
<td>53 (56%)</td>
<td>59 (64%)</td>
<td></td>
</tr>
<tr>
<td><strong>Occupation</strong></td>
<td></td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>Professional</td>
<td>33 (35%)</td>
<td>37 (40%)</td>
<td></td>
</tr>
<tr>
<td>Trade</td>
<td>21 (22%)</td>
<td>14 (15%)</td>
<td></td>
</tr>
<tr>
<td>Administrative or sales work</td>
<td>14 (15%)</td>
<td>21 (23%)</td>
<td></td>
</tr>
<tr>
<td>Manual or unskilled labour</td>
<td>16 (17%)</td>
<td>14 (15%)</td>
<td></td>
</tr>
<tr>
<td>Beneficiary or student</td>
<td>11 (12%)</td>
<td>6 (7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Household income</strong></td>
<td></td>
<td></td>
<td>0.87</td>
</tr>
<tr>
<td>&lt; $40,000</td>
<td>10 (15%)</td>
<td>12 (17%)</td>
<td></td>
</tr>
<tr>
<td>$40,000 to $70,000</td>
<td>21 (31%)</td>
<td>19 (28%)</td>
<td></td>
</tr>
<tr>
<td>&gt; $70,000</td>
<td>37 (54%)</td>
<td>38 (55%)</td>
<td></td>
</tr>
<tr>
<td><strong>New Zealand deprivation index score</strong>†</td>
<td></td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>1–2</td>
<td>26 (27%)</td>
<td>24 (26%)</td>
<td></td>
</tr>
<tr>
<td>3–4</td>
<td>25 (26%)</td>
<td>19 (21%)</td>
<td></td>
</tr>
<tr>
<td>5–6</td>
<td>20 (21%)</td>
<td>22 (24%)</td>
<td></td>
</tr>
<tr>
<td>7–8</td>
<td>19 (20%)</td>
<td>13 (14%)</td>
<td></td>
</tr>
<tr>
<td>9–10</td>
<td>5 (5%)</td>
<td>14 (15%)</td>
<td></td>
</tr>
<tr>
<td><strong>Tobacco use</strong></td>
<td></td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>55 (58%)</td>
<td>51 (55%)</td>
<td></td>
</tr>
<tr>
<td>Former smoker</td>
<td>20 (21%)</td>
<td>26 (28%)</td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>20 (21%)</td>
<td>15 (16%)</td>
<td></td>
</tr>
<tr>
<td><strong>Alcohol use</strong></td>
<td></td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td>Non drinker</td>
<td>12 (13%)</td>
<td>13 (14%)</td>
<td></td>
</tr>
<tr>
<td>Social drinker</td>
<td>25 (26%)</td>
<td>31 (34%)</td>
<td></td>
</tr>
<tr>
<td>Heavy drinker</td>
<td>58 (61%)</td>
<td>48 (52%)</td>
<td></td>
</tr>
<tr>
<td><strong>Illicit drug use</strong></td>
<td></td>
<td></td>
<td>0.06†</td>
</tr>
<tr>
<td>20 (21%)</td>
<td>10 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Physical activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No exercise</td>
<td>11 (12%)</td>
<td>7 (8%)</td>
<td></td>
</tr>
<tr>
<td>Exercises below CVD prevention criteria</td>
<td>21 (22%)</td>
<td>17 (18%)</td>
<td>0.48</td>
</tr>
<tr>
<td>Exercises at or above CVD prevention criteria</td>
<td>63 (66%)</td>
<td>68 (74%)</td>
<td></td>
</tr>
<tr>
<td><strong>Cardiovascular health</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>15 (16%)</td>
<td>20 (22%)</td>
<td>0.30</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>11 (12%)</td>
<td>11 (12%)</td>
<td>0.94</td>
</tr>
<tr>
<td>Characteristic</td>
<td>Affected n=95</td>
<td>Unaffected n=92</td>
<td>p-value</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>---------------</td>
<td>----------------</td>
<td>---------</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2 (2%)</td>
<td>0 (0%)</td>
<td>0.10</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>0 (0%)</td>
<td>2 (2%)</td>
<td>0.09</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>‡</td>
</tr>
<tr>
<td>General health</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma or atopy</td>
<td>61 (64%)</td>
<td>50 (54%)</td>
<td>0.17</td>
</tr>
<tr>
<td>Other medical problems</td>
<td>29 (31%)</td>
<td>27 (29%)</td>
<td>0.86</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>4 (4%)</td>
<td>8 (9%)</td>
<td>0.48</td>
</tr>
<tr>
<td>Endocrine</td>
<td>4 (4%)</td>
<td>5 (5%)</td>
<td></td>
</tr>
<tr>
<td>Psychiatric or neurological</td>
<td>9 (9%)</td>
<td>7 (8%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>13 (14%)</td>
<td>7 (8%)</td>
<td></td>
</tr>
<tr>
<td>Medication use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regular medication (excluding OCP)</td>
<td>23 (24%)</td>
<td>26 (28%)</td>
<td>0.53</td>
</tr>
<tr>
<td>Oral contraceptive use (females only)</td>
<td>13 (30%)</td>
<td>8 (16%)</td>
<td>0.10†</td>
</tr>
<tr>
<td>One or more children</td>
<td>49 (52%)</td>
<td>67 (73%)</td>
<td>0.003***</td>
</tr>
</tbody>
</table>

Data are n (%) or median (range). CVD=cardiovascular disease, OCP=oral contraceptive pill. * Figures given in New Zealand dollars; 27 affected and 23 unaffected declined to answer. † Deprivation score of 1 represents those living in the least deprived areas, and score of 10 represents those living in the most deprived areas of New Zealand (Tobias, Bhattacharya et al. 2008). ‡ After adjustment for age p=0.22. § Based on European guidelines on cardiovascular disease prevention (Perk, De Backer et al. 2012). ¶ Not calculable. || After adjustment for age p=0.77. ** After adjustment for age p=0.17.

### 4.4.5 Past Medical History and Medication Use

There were no differences between affected and unaffected participants in reported history of cardiovascular disease.

A previous diagnosis of hypertension was reported by 15 (16%) affected and 20 (22%) unaffected participants (RR=0.73, 95% CI=0.40 to 1.33, p=0.30, Table 4.2). Four (4%) affected and 7 (8%) unaffected participants were currently taking antihypertensive medication (RR=0.55, 95% CI=0.17 to 1.82, p=0.32).

A previous diagnosis of hyperlipidaemia was reported by 11 (12%) of both affected and unaffected participants (RR=0.97, 95% CI=0.44 to 2.12, p=0.94, Table 4.2). Two (2%) affected and 2 (2%) unaffected participants were currently taking lipid reducing agents (RR=0.97, 95% CI=0.14 to 6.73, p=0.97).

A previous diagnosis of diabetes was reported by 2 (2%) of affected and 0 (0%) unaffected participants (RR=0.98, 95% CI=0.95 to 1.01, p=0.10, Table 4.2).
Other general health problems were also reported with equal frequency in affected and unaffected groups, with over half reporting a history of asthma, eczema or hayfever. Approximately 25% of all participants reported regular medication use. Although oral contraceptive use appeared to be more common in affected participants, this trend was not evident following adjustment for age (p=0.77). Similarly, after adjustment for age, unaffected participants were not more likely to have children than affected participants (p=0.17, Table 4.2).

4.4.6 Clinical Findings

4.4.6.1 Body size, blood pressure, lipid status, and glucose tolerance
There were no differences between affected and unaffected participants for any measure of body size or for measured resting systolic or diastolic blood pressure. Exclusion of participants taking antihypertensive medication did not alter results (Table 4.3). In addition, there were no differences between groups for fasting plasma cholesterol, LDL or triglyceride concentrations or total cholesterol/HDL ratio, but fasting HDL concentration was lower in affected participants (difference between adjusted means -0.12 mmol.l\(^{-1}\), 95% CI=-0.24 to -0.004, p=0.04, Table 4.3). These findings did not alter following exclusion of participants taking lipid reducing agents.

Two (2%) affected participants with a previous diagnosis of diabetes were excluded from the glucose tolerance test. There was no difference between affected and unaffected participants for any measure of glucose or insulin at 0, 30 or 120 minutes following ingestion of a 75 g glucose load (Table 4.3).

There was no relationship between gestational age and systolic or diastolic blood pressure (Figure 4.1), or any measures of lipid status or glucose tolerance (data not shown).

4.4.6.2 Heart rate variability
Heart rate variability (HRV) was assessed in 59 (62%) affected and 59 (64%) unaffected participants comprising 57 (65%) sibling groups, and a trace suitable for analysis was obtained in 55 (58%) affected and 56 (61%) unaffected participants comprising 54 (61%) sibling groups. LF\(_{nu}\) and LF/HF ratio were higher in affected participants (ratio of geometric means 1.21, 95% CI=1.00 to 1.46, p=0.05 and 1.53, 95% CI=1.04 to 2.25, p=0.03 respectively), indicating increased sympathetic tone, and HF\(_{nu}\) was lower in affected participants (ratio of geometric means 0.79, 95% CI=0.64 to 0.97, p=0.03), indicating
decreased parasympathetic tone (Table 4.3). There was no relationship between gestational age and LFnu, HFnu or LF/HF ratio in affected or unaffected participants (Figure 4.2).

4.4.6.3 Haematological parameters
Haemoglobin concentration and haematocrit were higher in affected participants (difference between adjusted means 3.7 g.l⁻¹, 95% CI=0.8 to 6.6, p=0.01 and 0.9%, 95% CI=0.1 to 1.7, p=0.02 respectively, Table 4.3). Other haematological parameters were similar in affected and unaffected participants. There was no relationship between gestational age and haematological parameters in affected or unaffected participants (data not shown).
Table 4.3: Body size, blood pressure, fasting lipids, glucose tolerance, heart rate variability and haematological findings of Fetal Anaemia Study participants

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th>Adjusted†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Affected n</td>
<td>Unaffected</td>
</tr>
<tr>
<td><strong>Body size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>80.6±17.2</td>
<td>77.2±15.7</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.7±0.11</td>
<td>1.72±0.09</td>
</tr>
<tr>
<td>Body mass index (kg.m²)</td>
<td>26.6±5.5</td>
<td>26.0±4.6</td>
</tr>
<tr>
<td>Body surface area (m²)</td>
<td>1.97±0.24</td>
<td>1.92±0.23</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>58.1±1.8</td>
<td>57.8±2.8</td>
</tr>
<tr>
<td><strong>Blood pressure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic (mmHg)</td>
<td>122.7±13.9</td>
<td>125.0±14.4</td>
</tr>
<tr>
<td>Diastolic (mmHg)</td>
<td>73.0±9.9</td>
<td>74.3±10.7</td>
</tr>
<tr>
<td><strong>Fasting lipids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol.L⁻¹)</td>
<td>4.75±0.88</td>
<td>5.01±0.82</td>
</tr>
<tr>
<td>HDL cholesterol (mmol.L⁻¹)</td>
<td>1.42±0.33</td>
<td>1.59±0.44</td>
</tr>
<tr>
<td>LDL cholesterol (mmol.L⁻¹)</td>
<td>2.85±0.80</td>
<td>2.94±0.81</td>
</tr>
<tr>
<td>Triglycerides (mmol.L⁻¹)</td>
<td>1.05±0.52</td>
<td>1.09±0.69</td>
</tr>
<tr>
<td>Total cholesterol/HDL ratio</td>
<td>3.50±1.03</td>
<td>3.40±1.08</td>
</tr>
<tr>
<td><strong>Glucose Tolerance</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose (mmol.L⁻¹)</td>
<td>4.71±0.51</td>
<td>4.88±0.59</td>
</tr>
<tr>
<td>30 minute glucose (mmol.L⁻¹)</td>
<td>7.66±1.46</td>
<td>7.72±1.83</td>
</tr>
<tr>
<td>120 minute glucose (mmol.L⁻¹)</td>
<td>5.13±1.87</td>
<td>5.64±2.09</td>
</tr>
<tr>
<td>Fasting insulin (mU.L⁻¹)</td>
<td>5.2±3.1</td>
<td>5.5±3.6</td>
</tr>
<tr>
<td>30 minute insulin (mU.L⁻¹)</td>
<td>36.2±24.9</td>
<td>31.7±16.7</td>
</tr>
<tr>
<td>120 minute insulin (mU.L⁻¹)</td>
<td>24.2±20.7</td>
<td>28.4±33.9</td>
</tr>
<tr>
<td><strong>Heart rate variability</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDNN (ms)</td>
<td>51.0 (45.6 to 57.1)</td>
<td>41.3 (35.9 to 47.6)</td>
</tr>
<tr>
<td>pNN50</td>
<td>9.4 (6.2 to 14.3)</td>
<td>5.9 (3.5 to 9.8)</td>
</tr>
<tr>
<td>Total power (ms²)</td>
<td>2333 (1783 to 3053)</td>
<td>1492 (1138 to 1957)</td>
</tr>
<tr>
<td>LF (ms⁻¹)</td>
<td>726 (564 to 936)</td>
<td>412 (301 to 562)</td>
</tr>
<tr>
<td>LFₙu</td>
<td>53 (47 to 60)</td>
<td>46 (40 to 52)</td>
</tr>
<tr>
<td>HF (ms⁻¹)</td>
<td>496 (355 to 693)</td>
<td>383 (263 to 559)</td>
</tr>
<tr>
<td>HFₙu</td>
<td>36 (32 to 41)</td>
<td>42 (37 to 49)</td>
</tr>
</tbody>
</table>
Unadjusted data are mean±SD or geometric mean (95%CI). Adjusted data are mean or geometric mean (95%CI) and difference between means or † ratio of geometric means (95%CI). A 95% CI for a ratio of geometric means is non-significant if it includes 1. * Adjusted for age, sex, birth weight z-score and sibship. HDL=high density lipoprotein, LDL=low density lipoprotein, SDNN=standard deviation of NN intervals, pNN50=percentage of NN intervals that differ by >50ms from the preceding interval, LF=low frequency power, LF\textsubscript{nu}=low frequency power expressed in normalised units, HF=high frequency power, HF\textsubscript{nu}=high frequency power expressed in normalised units.

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th></th>
<th>Adjusted *</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Affected</td>
<td>n</td>
<td>Unaffected</td>
<td>n</td>
<td>Affected</td>
<td>n</td>
<td>Unaffected</td>
</tr>
<tr>
<td>LF/HF ratio</td>
<td>1.46 (1.15 to 1.87)</td>
<td>55</td>
<td>1.07 (0.83 to 1.38)</td>
<td>56</td>
<td>1.55 (1.20 to 2.01)</td>
<td>1.02 (0.79 to 1.31)</td>
<td>1.53 (1.04 to 2.25)†</td>
</tr>
<tr>
<td>Haematology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g.l\textsuperscript{-1})</td>
<td>148.1±14.0</td>
<td>95</td>
<td>142.4±12.6</td>
<td>92</td>
<td>147.3 (145.3 to 149.2)</td>
<td>143.6 (141.6 to 145.5)</td>
<td>3.7 (0.8 to 6.6)</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>44.8±3.6</td>
<td>95</td>
<td>43.5±3.1</td>
<td>92</td>
<td>44.6 (44.1 to 45.2)</td>
<td>43.7 (43.2 to 44.3)</td>
<td>0.9 (0.1 to 1.7)</td>
</tr>
<tr>
<td>White cell count (x10\textsuperscript{9}.l\textsuperscript{-1})</td>
<td>6.1±1.8</td>
<td>95</td>
<td>5.8±1.7</td>
<td>92</td>
<td>6.1 (5.8 to 6.5)</td>
<td>5.7 (5.4 to 6.1)</td>
<td>0.4 (-0.2 to 1.0)</td>
</tr>
<tr>
<td>Platelet count (x 10\textsuperscript{9}.l\textsuperscript{-1})</td>
<td>250±59</td>
<td>95</td>
<td>245±55</td>
<td>92</td>
<td>250 (239 to 262)</td>
<td>244 (232 to 256)</td>
<td>6 (-11 to 24)</td>
</tr>
</tbody>
</table>
Figure 4.1: Effect of gestational age at birth on mean (A) systolic blood pressure, and (B) diastolic blood pressure

Figure legends are regression coefficient ± SE, $r^2$ and p-values for each regression line
Figure 4.2: Effect of gestational age on (A) normalised low frequency power, (B) normalised high frequency power, and (C) low frequency to high frequency ratio.

Figure legends are regression coefficient ± SE, $r^2$ and p-values for each regression line.
Heart rate variability data not available for single participant with gestational age of 26 weeks.
LF = low frequency, HF = high frequency.
4.5 Discussion

This chapter provides the first description of the socioeconomic status, general health and prevalence of cardiovascular risk factors in adult survivors of IUT.

Our findings indicate that exposure to intrauterine anaemia and subsequent treatment with IUT is not associated with altered educational achievement, occupation, income or socioeconomic status in adults aged 18 to 47 years. This conclusion is consistent with several reports of favourable neurological outcome in children following fetal anaemia and treatment with IUT (Doyle, Kelly et al. 1993; Janssens, de Haan et al. 1997; Hudon, Moise et al. 1998; Grab, Paulus et al. 1999; Farrant, Battin et al. 2001; Harper, Swingle et al. 2006; Weisz, Rosenbaum et al. 2009; Lindenburg, Smits-Wintjens et al. 2011). However, the majority of these studies included children less than 6 years of age and longitudinal follow up was not performed.

Studies of preterm cohorts have demonstrated the importance of longitudinal follow up, with persistent and often worsening neurological, behavioural and emotional difficulties reported with increasing age (Botting, Powls et al. 1998; Resnick, Gomatam et al. 1998; Saigal, Hoult et al. 2000; Walther, den Ouden et al. 2000; Rickards, Kelly et al. 2001; Hack, Flannery et al. 2002; Pritchard, Clark et al. 2009). These trends would not have been apparent without repeated assessment throughout childhood and adolescence and led to predictions that up to half of children born extremely preterm would not be fully independent in adulthood (Saigal and Streiner 2009). Those preterm cohorts were born in the 1970s and 1980s, thus received similar obstetric and neonatal care to our affected participants. However, they were of much lower birth weight and gestational age than our participants, albeit without the morbidity associated with severe RhHD. Therefore, findings from those preterm cohorts may not be applicable to our IUT recipients. Furthermore, several recent reports of large cohorts of adults born preterm indicate that most fare better than predicted from earlier assessments, becoming independent and productive members of society with no long-term social or emotional sequelae, even though they are likely to have lower educational attainment and income than term born peers (Strauss 2000; Saigal, Stoskopf et al. 2006; Lindstrom, Winbladh et al. 2007; Moster, Lie et al. 2008; Mathiasen, Hansen et al. 2009; Heinonen, Eriksson et al. 2013). Thus it is clear that isolated neurodevelopmental assessment in early childhood is a poor proxy for social and educational functioning in adulthood.
Most IUT recipients in our cohort were born moderately preterm, with a mean gestational age of 34.5±1.6 weeks (range 26.1 to 37.1 weeks). Socioeconomic status and psychological functioning has been reported in a similar group of moderately preterm infants, born at National Women’s Hospital between December 1969 and February 1974 (Dalziel, Lim et al. 2007). That study reported that the socioeconomic status and cognitive functioning at 30 years of age of individuals born preterm was similar to those born at term. Our findings that adult survivors of IUT attain similar socioeconomic status and educational achievement to that of their unaffected siblings are in keeping with that report and indicate that neither moderately preterm birth nor exposure to intrauterine anaemia have a major influence on these outcomes.

We found similar rates of tobacco use and alcohol consumption in affected and unaffected participants, indicating that these factors are unlikely to alter the cardiovascular risk profile of IUT recipients compared to unaffected siblings. Interestingly, there was also no difference between affected and unaffected participants for a history of illicit drug use in the preceding 12 months. Taken together, these findings suggest that IUT recipients in our cohort do not display reduced risk taking behaviour, which has been observed in several cohorts of adults born preterm (< 32 weeks’ gestation) or of very low birth weight (<1,500 g) (Hack, Flannery et al. 2002; Hille, Dorrepaal et al. 2008; Hallin and Stjernqvist 2011). Furthermore, similar levels of exercise in affected and unaffected participants suggest that this is also unlikely to alter cardiovascular risk in IUT recipients. These findings are again in contrast to reports of reduced physical activity in adults born preterm or of low birth weight (Rogers, Fay et al. 2005; Kajantie, Strang-Karlsson et al. 2010; Welsh, Kirkby et al. 2010; Clemm, Roksund et al. 2012; Kaseva, Wehkalampi et al. 2012). While the lower birth weight and gestational age of those preterm cohorts compared to our IUT recipients may explain these differences, it is possible that use of siblings as the comparison group in our study provided better control for environmental influences on risk taking behaviour and physical activity, thus diminishing differences between affected and unaffected groups.

Our finding that blood pressure and glucose tolerance were similar in affected and unaffected participants is of particular note. An association between impaired fetal growth and hypertension in later life is well established, with renal, vascular and neuroendocrine pathways implicated in the pathogenesis of this association (Barker, Osmond et al. 1989a; Alexander and Intapad 2012). However, more recently numerous reports have emerged of increased systolic blood pressure in adolescents and adults born preterm or of low birth
weight (Doyle, Faber et al. 2003; Hack, Schluchter et al. 2005; Johansson, Iliadou et al. 2005; Dalziel, Parag et al. 2007; Hovi, Andersson et al. 2007; Relton, Pearce et al. 2008; Keijzer-Veen, Dulger et al. 2010; Crump, Winkleby et al. 2011; Kerkhof, Breukhoven et al. 2012). Indeed, a recent meta-analysis of the relationship between preterm birth and systolic blood pressure in later life, including 10 studies comprised of 1342 preterm infants with mean gestational age 30.2 weeks and birth weight 1280 g, demonstrated a 2.5 mmHg increase in systolic blood pressure at a mean age of 17.8 years (de Jong, Monuteaux et al. 2012). Similarly, an association between preterm birth and insulin resistance is also well reported (Hofman, Regan et al. 2004; Lawlor, Davey Smith et al. 2006; Regan, Cutfield et al. 2006; Dalziel, Parag et al. 2007; Hovi, Andersson et al. 2007). However, despite significant differences in both gestational age and birth weight between affected and unaffected participants in our study, we found no such differences between groups, and no association between blood pressure and gestational age.

There are several possible explanations for this. Firstly, our cohort may not have been large enough to detect the relatively small differences in systolic blood pressure and glucose tolerance seen in preterm cohorts. For example, the 95% confidence intervals around the adjusted means for both systolic and diastolic blood pressures in our study span 6-8 mmHg, so an increase in systolic blood pressure of 2.5 mmHg, as reported from the meta-analysis of preterm cohorts discussed above, cannot be reliably excluded by our study (de Jong, Monuteaux et al. 2012). A similar problem may exist in our cohort for detection of differences in glucose and insulin concentrations between affected and unaffected participants, given that increases in fasting glucose and insulin of 0.08 mmol.l\(^{-1}\) and 0.8 mIU.l\(^{-1}\) respectively have previously been reported in young adults born preterm (Hovi, Andersson et al. 2007), increases which are considerably smaller than the 95% confidence intervals in our data, which span 0.34 mmol.l\(^{-1}\) for glucose and 2.2 mIU.l\(^{-1}\) for insulin.

Secondly, the gestational age of our affected participants may not have been low enough to influence these outcomes, although changes in blood pressure and increased insulin resistance in adulthood have been documented across a continuous distribution of gestational age to term (Dalziel, Parag et al. 2007; Lawlor, Hubinette et al. 2007; Relton, Pearce et al. 2008).

Thirdly, it is possible that differences between our IUT recipients and the previously described preterm cohorts may have mitigated the effect of decreased gestational age on
systolic blood pressure and insulin resistance. For example, relatively higher birth weight for gestational age in affected participants may have reduced the likelihood of rapid catch up growth, a factor known to be associated with hypertension in later life (Huxley, Shiell et al. 2000; Ekelund, Ong et al. 2007; De Matteo, Stacy et al. 2008). Furthermore, preterm birth in all affected participants in our cohort was due to RhHD, whereas in the previously reported preterm cohorts, early delivery occurred for a multitude of reasons, some of which may also influence blood pressure control or metabolic function in later life (Relton, Pearce et al. 2008).

Finally, it is possible that intrauterine anaemia and its associated cardiovascular changes may have programmed IUT recipients for lower systolic blood pressure than would otherwise have been the case. Although the mechanism for this is unclear, it may be mediated by changes to vascular resistance secondary to remodelling of resistance vessels, similar to the changes postulated to account for the increase in coronary conductance seen in adult sheep exposed to intrauterine anaemia. (Davis, Roullet et al. 2003) Alternatively, it is possible that renal blood flow may be relatively preserved in anaemic fetuses in comparison to growth restricted fetuses, in order to maintain maximal urine output in the face of volume overload. Hence, reduction in nephron number, frequently proposed as a potential mechanism for the relationship between low birth weight and hypertension, may not occur in fetuses exposed to intrauterine anaemia (Hinchliffe, Lynch et al. 1992; Hughson, Farris et al. 2003; Luykx 2005).

We found lower HDL concentrations in affected participants than in unaffected siblings. This finding suggests increased cardiovascular risk in these individuals, since an atherogenic lipid profile is characterised by increased concentrations of total cholesterol, LDL, triglycerides and apolipoprotein B, and decreased concentrations of HDL and apolipoprotein AI (Walldius and Jungner 2004). We found no association between gestational age and lipid profile in our cohort, in keeping with previous reports from other low birth weight and preterm cohorts. Overall, the evidence regarding a relationship between low birth weight and an unfavourable lipid profile in later life supports a weak association at most (Lauren, Jarvelin et al. 2003; Davies, Smith et al. 2004; Huxley, Owen et al. 2004; Lawlor, Owen et al. 2006; Robinson, Batelaan et al. 2006). In addition, a number of studies have found no evidence of an unfavourable lipid profile in adults born preterm, (Finken, Inderson et al. 2006; Dalziel, Parag et al. 2007; Hovi, Andersson et al. 2007; Skilton, Viikari et al. 2011) with one group recently reporting reduced LDL and
apolipoprotein AI levels in subjects aged 18 to 24 years born at a mean gestational age of 32 weeks (Breukhoven, Kerkhof et al. 2012). Exposure to antenatal corticosteroids has also been shown to have no effect on lipid profile at 30 years of age (Dalziel, Walker et al. 2005). Thus it seems unlikely that decreased HDL concentration found in our affected participants is related to preterm birth or low birth weight.

However, there is evidence in both humans and animal models that altered hepatic blood flow in utero may predispose to abnormalities of lipid metabolism in later life. For example, a so-called “liver-sparing” effect, with reduced ductus venosus shunting and increased liver blood flow has been demonstrated in late gestation fetuses of slim mothers and mothers eating an “imprudent diet” (Haugen, Hanson et al. 2005). Although this may allow increased hepatic nutrient conversion and thus may be potentially beneficial in utero, deleterious effects on lipid metabolism may result in the face of a nutrient rich postnatal environment. In addition, animal models of intrauterine hypoxia have demonstrated increased ductus venosus flow in order to preserve oxygen supply to the brain and myocardium (Paulick, Meyers et al. 1990; Jensen, Roman et al. 1991; Tchirikov, Eisermann et al. 1998; Tchirikov, Strohner et al. 2010). This so-called “brain-sparing” effect is supported by the finding of an association between increased LDL concentration in adulthood and reduced abdominal circumference at birth, which may indicate impaired liver growth in utero with consequent reprogramming of liver metabolism (Barker, Martyn et al. 1993). Thus, it is possible that our finding of reduced HDL concentration in affected participants may be related to altered hepatic blood flow in utero in response to the cardiovascular changes induced by severe intrauterine anaemia.

A relationship has also been demonstrated between the rate of weight gain in early life and serum lipids in adulthood, with both rapid and slow growth to 2 years of age associated with an unfavourable lipid profile (Fall, Barker et al. 1992; Kajantie, Barker et al. 2008; Kerkhof, Willemsen et al. 2012). In addition, breast feeding in infancy has been associated with lower total cholesterol concentration in adulthood, with exclusive breast feeding strengthening this relationship (Owen, Whincup et al. 2008). Taken together, these findings suggest that postnatal nutrition may be more important than birth weight or gestational age for the programming of serum lipids in adulthood. Although we do not have data regarding postnatal growth or frequency of breast feeding in affected and unaffected participants in our cohort, it is certainly plausible that their growth and nutrition in the first months of life
was affected by ongoing problems related to RhHD, particularly as most required neonatal intensive care in the first weeks of life.

HRV was altered in our cohort, with affected participants having a higher LF/HF ratio and normalised LF power and lower normalised HF power than their unaffected siblings. As the LF spectral band is governed mainly by sympathetic activity and the HF band by parasympathetic activity (Akselrod, Gordon et al. 1981), these findings are collectively indicative of sympathetic predominance in affected participants. Reduced HRV and increased sympathetic tone have been associated with cardiac failure and increased risk of death following myocardial infarction (Lucini, Mela et al. 2002; Vinik, Maser et al. 2003; Buccelletti, Gilardi et al. 2009). In addition, increased risk of new-onset hypertension has been reported in normotensive subjects with very similar HRV findings to those in our affected participants, suggesting that adult survivors of fetal anaemia may be at greater risk of developing hypertension in the future (Lucini, Mela et al. 2002). Furthermore, numerous studies have documented an association between reduced HRV and increased risk of coronary heart disease and cardiac death in otherwise healthy individuals (Tsuji, Larson et al. 1996; Liao, Cai et al. 1997; Dekker, Crow et al. 2000). The mechanism underlying this association is thought to be instability of the cardiac conducting system, resulting from augmented sympathetic tone and/or reduced parasympathetic tone (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology 1996). Thus, the HRV findings of affected participants in our cohort provide further evidence of increased cardiovascular risk in these individuals.

It is possible that these findings are due to the difference in gestational age between affected and unaffected participants. Reduced HRV with sympathetic predominance has been found in preterm infants up to six months post term equivalent age (Yiallourou, Witcombe et al. 2013). Although one study in children born preterm suggests that HRV parameters normalise by 2 to 3 years of age, the experimental groups in this study were small and sleep state was not taken into account (De Rogalski Landrot, Roche et al. 2007). Furthermore, increased heart rate and urinary catecholamines at rest and with stress have been demonstrated at 9.5 years in children born preterm, suggesting persistence of increased sympathoadrenal activity to middle childhood (Johansson, Norman et al. 2007). HRV parameters in adults born preterm have not been reported, although we have unpublished data in juvenile sheep born preterm demonstrating increased sympathetic activity compared to term controls. However, we found no evidence of an association between HRV
parameters and gestational age in our cohort, thus it seems unlikely that preterm birth accounts for our finding of sympathetic predominance in affected participants.

We also found that haemoglobin concentration and haematocrit were higher in affected participants compared to unaffected siblings. The clinical significance of these findings is uncertain, given that the increases observed are small, with values still well within the normal range. However, these findings imply that exposure to anaemia in utero may influence the programming of haematological parameters in later life, perhaps by permanently increasing the set point for detection of tissue hypoxia by erythropoietin secreting cells in the kidney. In support of this possibility, increased haematocrit, haemoglobin concentration, red blood cell count and erythropoietin concentration have been reported in adult rats following exposure to antenatal glucocorticoids, and the authors of that study speculated that these findings may be due to altered transcription of the erythropoietin gene secondary to intrauterine glucocorticoid exposure (Tang, Seckl et al. 2011). It is possible that fetal anaemia resulted in overproduction of endogenous glucocorticoids, which may have similarly altered erythropoietin and red blood cell production in affected participants.

An alternative explanation for these findings is suggested by a report of increased haematocrit in early adulthood in rats exposed to intrauterine and postnatal iron deficiency, in comparison to iron replete rats. (Hegde, Jensen et al. 2011) Signs of abnormal erythropoesis were not apparent in these animals until the post-weaning phase, coincident with the introduction of an iron adequate diet. Thus, these authors hypothesised that dietary iron supplementation following in utero and postnatal iron deficiency induced an inflammatory reaction, which in turn led to erythrocytosis and increased haematocrit in adulthood. It is possible that a similar mechanism may be responsible for the increase in haemoglobin and haematocrit seen in adult survivors of intrauterine anaemia.

There are several potential limitations to the findings reported in this chapter. Firstly, as data regarding parental history, educational achievement, occupation, income, tobacco and alcohol use, illicit drug use, exercise and past medical history were collected by questionnaire, aspects of this information may have been inaccurately reported. While a concordance rate of over 90% within sibling groups for reporting of parental characteristics is reassuring, it is possible that participants conferred in answering these questions. However, self-reported questionnaires are a practical way to collect this sort data and have
been used extensively in the past by others, with multiple reports of the validity of this technique. (Spitz, Fueger et al. 1988; MacMahon, Norton et al. 1995; Haapanen, Miilunpalo et al. 1997; Ameratunga, Norton et al. 2002; Murabito, Nam et al. 2004; Okura, Urban et al. 2004; Lawlor, Davey Smith et al. 2006; Taylor, Dal Grande et al. 2010).

Secondly, it is not possible to distinguish whether the differences between affected and unaffected participants described in this chapter are due to exposure to intrauterine anaemia or the difference in gestational age between groups. Ideally, adjustment for gestational age would have been made in the multivariate model, but as all affected participants were born preterm and almost all unaffected participants were born at term, gestational age is confounded by affected or unaffected status and cannot be independently adjusted. However, we found no evidence of a relationship between gestational age and any of the outcomes reported, and with the exception of HRV findings, trends for all cardiovascular risk factors described in this chapter are the reverse of those expected from prematurity alone. Thus the difference in gestational age between affected and unaffected participants is unlikely to explain our findings.

Thirdly, the age of participants in this study ranged from 17 to 60 years, with affected participants approximately 6 years younger than unaffected siblings. Younger participants have not had as much time to manifest cardiovascular disease risk factors as older participants. Therefore in analysis of all clinical findings and any other self-reported characteristics for which age could plausibly have influenced outcome, correction was made for age at the time of study participation. Apart from the difference between affected and unaffected participants for number of children, no findings were substantially altered by adjustment for age, making the difference in age an unlikely explanation of our findings.

Finally, it is possible that the significant findings reported in this chapter may be explained by chance due to multiple comparisons in the analyses. In total, 47 comparisons were made in this chapter, of which 2 might be expected to be statistically significant by chance alone. We found 9 statistically significant associations, of which 2 (age at the time of study participation and number of children) were predictably and highly significant. Thus we cannot exclude the possibility that some of our findings were due to a type 1 error, and replication of the study would be required for confirmation.
4.6 Summary

Exposure to fetal anaemia treated by IUT is not associated with altered socioeconomic status, educational achievement, general health, medication use, body size, smoking status, alcohol use, blood pressure or glucose tolerance in adulthood. These conclusions will provide valuable reassurance to IUT recipients, their families and the clinicians caring for them. However, decreased concentration of HDL and increased sympathetic tone suggest that IUT recipients may be at increased risk of cardiovascular disease in later life. Follow up of IUT survivors into their 6th decade of life and beyond is required to ascertain if the increased cardiovascular risks observed in this study translate into increased rates of cardiovascular disease in later life.
Chapter 5: Left Ventricular Function, Size and Stress, Myocardial Viability, and Myocardial Blood Flow

5.1 Summary of Chapter Contents
This chapter reports left ventricular function, radius, wall thickness, wall stress and myocardial blood flow in IUT recipients who participated in the Fetal Anaemia Study and their unaffected siblings.

5.2 Introduction
Alteration of fetal cardiovascular development following exposure to intrauterine anaemia has been well demonstrated in animal studies. Near term fetal sheep whose haematocrit was reduced by half for 7 days have a 50% increase in cardiac output, a six-fold increase in myocardial blood flow (MBF) and a 30% increase in heart to body mass ratio, indicating fetal cardiac hypertrophy (Davis and Hohimer 1991). Furthermore, increased MBF at rest and with maximal vasodilation, and a doubling of coronary conductance but unaltered coronary perfusion reserve have been demonstrated in anaemic fetal sheep, suggesting increased growth of the coronary resistance vessels in the fetus in response to intrauterine anaemia (Davis and Hohimer 1991; Davis, Thornburg et al. 2005). In support of these findings, increased myocardial vascularisation has been reported in anaemic fetal sheep (Martin, Yu et al. 1998). Increased cardiac output and coronary flow have also been demonstrated in human fetuses exposed to intrauterine anaemia (Copel, Grannum et al. 1989; Baschat, Muench et al. 2003).

In sheep, these changes have been shown to persist in adulthood, with a doubling of coronary conductance and coronary perfusion reserve reported in animals who were anaemic before birth (Davis, Roullet et al. 2003). These findings suggest that there may be a functional advantage to the adult animal following fetal anaemia, as an augmented coronary circulation may be better able to compensate for hypoxic stress in adult life. For example, improved indices of left ventricular systolic function during hypoxaemia have been demonstrated in adult sheep following exposure to intrauterine anaemia (Broberg, Giraud et al. 2003). However, increased infarct size following ischaemia-reperfusion injury has also been reported in adult sheep who were anaemic before birth, suggesting decreased tolerance to myocardial ischaemia in these animals (Yang, Hohimer et al. 2008). While these findings confirm that fetal anaemia may influence later cardiovascular function, their conflicting nature means that the implications for adult human survivors of fetal anaemia are uncertain.
There has been no previous report of human cardiovascular outcome in adulthood following exposure to intrauterine anaemia.

In the last two to three decades, cardiovascular magnetic resonance imaging (CMR) has evolved considerably to become the most reliable technique for the assessment of cardiac structure and function (Gandy, Waugh et al. 2008). In addition, myocardial perfusion imaging by CMR has become increasingly sophisticated, with the development of quantitative methods for the assessment of MBF (Jerosch-Herold 2010). Thus, CMR is the tool of choice for the investigation of left ventricular function and MBF in this study of adult survivors of fetal anaemia.

### 5.3 Chapter Hypotheses

That there is no difference between affected and unaffected siblings in:

1. Left ventricular (LV) function, as measured by:
   a. end diastolic, end systolic and stroke volumes
   b. ejection fraction

2. LV mass, global and regional (septal and ventricular free wall) dimensions and wall stress at rest, as measured by:
   a. mass
   b. concentricity
   c. radius at end diastole and end systole
   d. wall thickness at end diastole and end systole
   e. wall thickening
   f. wall stress at end diastole and end systole

3. Myocardial viability, as measured by late gadolinium enhancement imaging.

4. Global and regional (septal and ventricular free wall) MBF, measured:
   a. at rest
   b. during cold pressor stress
   c. at maximal coronary vasodilation during adenosine stress
   d. by myocardial perfusion reserve index (MPRi)
5.4 Results

5.4.1 Left Ventricular Function

Uncorrected LV volumes were larger in affected than in unaffected participants. However, with multivariate linear regression correcting for age, sex, birth weight z-score and sibship, affected participants had smaller end diastolic volume (EDV), end systolic volume (ESV) and stroke volume (SV) than their unaffected siblings; findings which persisted when EDV, ESV and SV were indexed to body surface area to normalise for body size (Table 5.1). Exploratory analysis undertaken to investigate this apparent reversal of differences between groups revealed that age at the time of scan was the most important contributor to adjusted outcomes, with an inverse relationship between indexed LV volumes and age in men. In women, an inverse relationship with age was only evident for indexed EDV and only in unaffected participants (Figure 5.1). There was no relationship between gestational age and indexed LV volumes if all participants were included in the analysis (Figure 5.2), if the single participant with gestational age of 26 weeks was removed (Figure 5.3) or if the effect of gestational age in affected and unaffected participants was analysed according to sex (data not shown).

LV ejection fraction was similar in affected and unaffected participants. There was no association between LV function in affected participants and number of IUTs received, presence of hydrops at birth, cord bilirubin or haemoglobin concentrations, highest postnatal bilirubin concentration, lowest postnatal haemoglobin concentration or birth before or after the introduction of intravascular IUT (data not shown).

5.4.2 Left Ventricular Mass, Dimensions and Stress

LV mass was similar in affected and unaffected participants. However, when indexed to body surface area, there was a trend towards lower LV mass in affected participants (difference between adjusted means -2.5 g.m\(^{-2}\), 95% CI = -5.2 to 0.2, p=0.06, Table 5.1). An inverse relationship between age and indexed LV mass was evident in affected men (regression coefficient±SE: -0.3±0.1, p=0.02) and a similar trend was apparent in unaffected men (-0.2±0.1, p=0.06, figure 5.1). There was no relationship between indexed LV mass and gestational age (Figures 5.2 and 5.3).
### Table 5.1: Resting left ventricular volumes, size, and stress of Fetal Anaemia Study participants

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th></th>
<th></th>
<th>Adjusted*</th>
<th></th>
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<td></td>
<td>Affected</td>
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<td>Affected</td>
<td>Unaffected</td>
<td>Difference</td>
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<tr>
<td>Number of participants</td>
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<td>91</td>
<td>95</td>
<td>91</td>
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<tr>
<td>Volumes and mass</td>
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<td></td>
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<tr>
<td>EDV (ml)</td>
<td>161.7±31.7</td>
<td>157.8±37.5</td>
<td>154.6 (149.6 to 159.6)</td>
<td>166.2 (161.0 to 171.3)</td>
<td>-11.6 (-19.2 to -3.9)</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>EDV/BSA (ml.m⁻²)</td>
<td>82.3±12.7</td>
<td>82.1±15.0</td>
<td>79.3 (76.9 to 81.7)</td>
<td>85.4 (83.0 to 87.9)</td>
<td>-6.1 (-9.7 to -2.4)</td>
<td>0.001</td>
<td></td>
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<tr>
<td>ESV (ml)</td>
<td>61.6±16.4</td>
<td>60.0±19.8</td>
<td>58.1 (55.3 to 60.9)</td>
<td>64.1 (61.2 to 67.0)</td>
<td>-6.0 (-10.3 to -1.7)</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>ESV/BSA (ml.m⁻²)</td>
<td>31.3±7.3</td>
<td>31.1±8.7</td>
<td>29.8 (28.3 to 31.2)</td>
<td>32.9 (31.4 to 34.3)</td>
<td>-3.1 (-5.3 to -1.0)</td>
<td>0.005</td>
<td></td>
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<tr>
<td>SV (ml)</td>
<td>100.0±19.0</td>
<td>97.8±20.5</td>
<td>96.3 (93.2 to 99.5)</td>
<td>102.1 (98.8 to 105.3)</td>
<td>-5.7 (-10.6 to -0.9)</td>
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<tr>
<td>SV/BSA (ml.m⁻²)</td>
<td>50.9±7.5</td>
<td>50.9±7.9</td>
<td>49.5 (48.0 to 50.9)</td>
<td>52.5 (51.0 to 54.0)</td>
<td>-3.0 (-5.3 to -0.8)</td>
<td>0.007</td>
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<td>Ejection fraction (%)</td>
<td>62.2±4.8</td>
<td>62.7±5.4</td>
<td>62.7 (61.6 to 63.7)</td>
<td>62.2 (61.1 to 63.2)</td>
<td>0.5 (-1.1 to 2.0)</td>
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<tr>
<td>LV mass (g)</td>
<td>131.2±31.7</td>
<td>126.2±34.6</td>
<td>126.9 (122.6 to 131.3)</td>
<td>131.8 (127.3 to 136.2)</td>
<td>-4.8 (-11.4 to 1.8)</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>LV mass/BSA (g.m⁻²)</td>
<td>66.2±11.2</td>
<td>65.2±12.8</td>
<td>64.7 (62.9 to 66.4)</td>
<td>67.2 (65.4 to 69.0)</td>
<td>-2.5 (-5.2 to 0.2)</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Wall thickness (end diastolic)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Global (mm)</td>
<td>7.37±0.93</td>
<td>7.25±0.99</td>
<td>7.34 (7.20 to 7.48)</td>
<td>7.31 (7.17 to 7.45)</td>
<td>0.03 (-0.18 to 0.25)</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Septal (mm)</td>
<td>7.72±0.99</td>
<td>7.66±1.10</td>
<td>7.71 (7.55 to 7.87)</td>
<td>7.70 (7.54 to 7.87)</td>
<td>-0.01 (-0.23 to 0.25)</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Free wall (mm)</td>
<td>7.74±1.05</td>
<td>7.57±1.07</td>
<td>7.71 (7.55 to 7.86)</td>
<td>7.64 (7.48 to 7.80)</td>
<td>0.07 (-0.17 to 0.30)</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Concentricity</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV mass/EDV (g.ml⁻¹)</td>
<td>0.81±0.11</td>
<td>0.80±0.12</td>
<td>0.82 (0.80 to 0.84)</td>
<td>0.79 (0.77 to 0.81)</td>
<td>0.03 (-0.003 to 0.06)</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>LV mass/(EDV)⁰.⁶⁷ (g.ml⁻⁰.⁶⁷)</td>
<td>4.32±0.65</td>
<td>4.23±0.70</td>
<td>4.31 (4.21 to 4.41)</td>
<td>4.26 (4.15 to 4.36)</td>
<td>0.05 (-0.10 to 0.21)</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>End diastolic wall thickness to volume ratio†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global (mm².ml⁻¹)</td>
<td>0.091±0.018</td>
<td>0.091±0.019</td>
<td>0.094 (0.091 to 0.098)</td>
<td>0.088 (0.084 to 0.091)</td>
<td>0.007 (0.001 to 0.012)</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Septal (mm².ml⁻¹)</td>
<td>0.096±0.018</td>
<td>0.096±0.020</td>
<td>0.099 (0.095 to 0.103)</td>
<td>0.092 (0.089 to 0.096)</td>
<td>0.007 (0.001 to 0.012)</td>
<td>0.02</td>
<td></td>
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<tr>
<td>Free wall (mm².ml⁻¹)</td>
<td>0.096±0.019</td>
<td>0.095±0.020</td>
<td>0.099 (0.095 to 0.103)</td>
<td>0.092 (0.088 to 0.095)</td>
<td>0.008 (0.002 to 0.013)</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Wall thickening¶</td>
<td></td>
<td></td>
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<tr>
<td>Global (%)</td>
<td>56.7±8.1</td>
<td>57.3±8.8</td>
<td>57.4 (55.7 to 59.0)</td>
<td>56.5 (54.8 to 58.2)</td>
<td>0.9 (-1.7 to 3.4)</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Septal (%)</td>
<td>39.8±8.2</td>
<td>39.3±7.9</td>
<td>39.3 (37.7 to 40.9)</td>
<td>39.7 (38.1 to 41.4)</td>
<td>-0.5 (-2.9 to 2.0)</td>
<td>0.72</td>
<td></td>
</tr>
</tbody>
</table>

† Significance of the difference between affected and unaffected groups.

‡ Adjusted for gestational age.

§ Adjusted for sex.

¶ Adjusted for maternal weight.

||
### Radius (end diastolic)

<table>
<thead>
<tr>
<th></th>
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<th>Adjusted*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Affected</td>
<td>Unaffected</td>
</tr>
<tr>
<td>Free wall (%)</td>
<td>61.2±9.5</td>
<td>63.1±11.0</td>
</tr>
<tr>
<td>Global (mm)</td>
<td>25.3±1.7</td>
<td>25.1±2.1</td>
</tr>
<tr>
<td>Septal (mm)</td>
<td>27.6±2.0</td>
<td>27.4±2.6</td>
</tr>
<tr>
<td>Free wall (mm)</td>
<td>29.6±2.2</td>
<td>29.3±2.6</td>
</tr>
</tbody>
</table>

### Radius (end systolic)

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</thead>
<tbody>
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<td></td>
<td>Affected</td>
<td>Unaffected</td>
</tr>
<tr>
<td>Global (mm)</td>
<td>19.8±1.6</td>
<td>19.6±2.0</td>
</tr>
<tr>
<td>Septal (mm)</td>
<td>23.5±2.1</td>
<td>23.2±2.6</td>
</tr>
<tr>
<td>Free wall (mm)</td>
<td>22.4±2.1</td>
<td>22.2±2.4</td>
</tr>
</tbody>
</table>

### Wall stress** (end systolic)

<table>
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</thead>
<tbody>
<tr>
<td></td>
<td>Affected</td>
<td>Unaffected</td>
</tr>
<tr>
<td>Global (x 10³ N.m⁻²)</td>
<td>11.0±1.8</td>
<td>11.2±1.6</td>
</tr>
<tr>
<td>Septal (x 10³ N.m⁻²)</td>
<td>14.6±2.2</td>
<td>14.8±2.2</td>
</tr>
<tr>
<td>Free wall (x 10³ N.m⁻²)</td>
<td>11.6±2.1</td>
<td>11.8±1.8</td>
</tr>
</tbody>
</table>

Unadjusted data are mean±SD. Adjusted data are mean (95% CI) and difference between means (95% CI). EDV=end diastolic volume, BSA=body surface area, ESV=end systolic volume, SV=stroke volume. * Adjusted for age, sex, birth weight z-score and sibship. † End diastolic wall thickness to volume ratio=end diastolic wall thickness/(EDV/BSA). ‡ Average from all slices. †† Average from inferoseptal and anteroseptal regions from all slices. ‡‡ Average from anterior, inferior, anterolateral and inferolateral regions of all slices. || Wall thickening = (end systolic wall thickness – end diastolic wall thickness)/end diastolic wall thickness x 100. ** Wall stress = 0.133 x systolic blood pressure x (LV radius/2 x LV wall thickness x (1 + LV wall thickness/2 x LV radius) (Zhong, Su et al. 2009)
End diastolic wall thickness was similar in affected and unaffected participants when assessed globally, in the septum or in the ventricular free wall. Concentricity, the ratio of mass to volume, and concentricity$^{0.67}$ were also similar in affected and unaffected participants (Table 5.1). However, end diastolic wall thickness to volume ratio was higher in affected than in unaffected participants when assessed globally, in the septum and in the free wall. There was a positive relationship between end diastolic wall thickness to volume ratio and age in affected and unaffected men and women in all three locations, (Figure 5.4) but no relationship with gestational age (Figures 5.5 and 5.6). There were no differences between affected and unaffected participants for wall thickening assessed globally, in the septum or in the free wall (Table 5.1).

LV radius at end diastole and end systole was lower in affected than in unaffected participants, assessed either globally or at the free wall (Table 5.1), with an inverse relationship between these parameters and age apparent in affected and unaffected men, but not women (Figure 5.7, end systolic data only shown). Wall stress was also lower in affected participants when assessed globally and in the free wall at end systole. There was an inverse relationship between age and free wall stress in both affected and unaffected men but a similar relationship with global wall stress was apparent only in unaffected men (Figure 5.7). In addition, an inverse relationship between gestational age and end systolic global and free wall stress in affected participants was no longer apparent if the participant with a gestational age of 26 weeks was excluded (Figures 5.8 and 5.9).

There was no association between LV size and wall stress in affected participants and number of IUTs received, presence of hydrops at birth, cord bilirubin or haemoglobin concentrations, highest postnatal bilirubin concentration, lowest postnatal haemoglobin concentration or birth before or after the introduction of intravascular IUT (data not shown).

### 5.4.3 Myocardial Viability

Assessment of myocardial viability by late gadolinium enhancement (LGE) imaging was undertaken on 175 (94%) of the 187 study participants, with reportable images obtained from 167 (89%). Findings for LGE imaging were similar in both groups, with equivocal findings in 1 unaffected and 4 affected participants and a positive test result in 1 affected participant (p=0.43, Table 5.2).
Five of the six participants with equivocal or positive LGE findings were men, all were of European ethnicity and none had a history of coronary artery disease. Of those with equivocal findings, three were ex-smokers and one had mild hypertension. The single participant with a positive result had no risk factors for cardiovascular disease.

Table 5.2: Late gadolinium enhancement imaging findings of Fetal Anaemia Study participants

<table>
<thead>
<tr>
<th>LGE Finding</th>
<th>Affected (n=89)</th>
<th>Unaffected (n=86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>80 (90)</td>
<td>81 (94)</td>
</tr>
<tr>
<td>Equivocal</td>
<td>4 (4)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Positive</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Not reportable</td>
<td>4 (4)</td>
<td>4 (5)</td>
</tr>
</tbody>
</table>

Data are n (%). LGE=late gadolinium enhancement. There are no significant differences between groups, p=0.43.

### 5.4.4 Myocardial Blood Flow

Compared to unaffected siblings, MBF at rest and with cold pressor stress was lower in affected participants when measured globally and in the ventricular free wall (Table 5.3). There were no differences between affected and unaffected participants for MBF with adenosine-induced hyperaemia. An inverse relationship was apparent between age at time of scan and MBF measured in the free wall at rest and with cold pressor in affected men only (Figure 5.10). There was no relationship between gestational age and MBF (Figures 5.11 and 5.12). Myocardial perfusion reserve index (MPRi) was higher in affected participants when assessed globally and in the free wall, but not in the septum. Mean arterial pressure and heart rate prior to MBF assessment at rest and during adenosine-induced hyperaemia were similar in both groups (Table 5.3).

Participants born after the introduction of intravascular IUT in 1986 had lower global and septal MBF at rest, and higher global and free wall MPRi than participants born prior to 1986 (Table 5.4). There was no association between MBF and potential indicators of the severity of the fetal anaemia, including number of IUTs received, presence of hydrops at birth, cord bilirubin or haemoglobin concentration, highest postnatal bilirubin or lowest postnatal haemoglobin concentration (data not shown).
Figure 5.1: Effect of age on indexed (A) end diastolic volume, (B) end systolic volume, (C) stroke volume, and (D) left ventricular mass

Figure legends are regression coefficient ± SE, r² and p-values for each regression line
EDV=end diastolic volume, ESV=end systolic volume, SV=stroke volume, LV=left ventricular, BSA=body surface area
Figure 5.2: Effect of gestational age at birth on indexed (A) end diastolic volume, (B) end systolic volume, (C) stroke volume and (D) left ventricular mass

Figure legends are regression coefficient ± SE, $r^2$ and p-values for each regression line.

EDV=end diastolic volume, ESV=end systolic volume, SV=stroke volume, LV=left ventricular, BSA=body surface area.
Figure 5.3: Effect of gestational age at birth on indexed (A) end diastolic volume, (B) end systolic volume, (C) stroke volume, and (D) left ventricular mass (with outlier excluded).

Figure legends are regression coefficient ± SE, $r^2$ and p-values for each regression line.

EDV=end diastolic volume, ESV=end systolic volume, SV=stroke volume, LV=left ventricular, BSA=body surface area.

*Single participant with gestational age 26 weeks excluded.
Figure 5.4: Effect of age on end diastolic wall thickness to volume ratio* measured (A) globally, (B) in the septum, and (C) in the ventricular free wall.

Figure legends are regression coefficient ± SE, \( r^2 \) and \( p \)-values for each regression line.

* End diastolic wall thickness to volume ratio=end diastolic wall thickness/(EDV/BSA)
Figure 5.5: Effect of gestational age at birth on end diastolic wall thickness to volume ratio* measured (A) globally, (B) in the septum, and (C) in the ventricular free wall.

Figure legends are regression coefficient ± SE, $r^2$ and $p$-values for each regression line.

*End diastolic wall thickness to volume ratio=end diastolic wall thickness/(EDV/BSA)
Figure 5.6: Effect of gestational age at birth on end diastolic wall thickness to volume ratio* measured (A) globally, (B) in the septum, and (C) in the ventricular free wall (with outlier excluded†)

Figure legends are regression coefficient ± SE, $r^2$ and p-values for each regression line

* End diastolic wall thickness to volume ratio=end diastolic wall thickness/(EDV/BSA)
† Single participant with gestational age 26 weeks excluded
Figure 5.7: Effect of age on end systolic (A) global radius, (B) ventricular free wall radius, (C) global wall stress and (D) ventricular free wall stress

Figure legends are regression coefficient ± SE, $r^2$ and p-values for each regression line
ES=end systolic, FW=free wall, BSA=body surface area
Figure 5.8: Effect of gestational age at birth on end systolic (A) global radius, (B) ventricular free wall radius, (C) global wall stress, and (D) ventricular free wall stress.

Figure legends are regression coefficient ± SE, r² and p-values for each regression line.
ES=end systolic, FW=free wall, BSA=body surface area.
Figure 5.9: Effect of gestational age at birth on end systolic (A) global radius, (B) ventricular free wall radius, (C) global wall stress, and (D) ventricular free wall stress (with outlier excluded)

Figure legends are regression coefficient ± SE, $r^2$ and p-values for each regression line
ES=end systolic, FW=free wall, BSA=body surface area
*Single participant with gestational age 26 weeks excluded
Table 5.3: Myocardial blood flow (MBF) at rest, with cold pressor and with adenosine-induced hyperaemia (with associated blood pressure and heart rate data where available), and myocardial perfusion reserve index (MPRi) of Fetal Anaemia Study participants

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th></th>
<th></th>
<th>Adjusted*</th>
<th></th>
<th></th>
<th>p-value</th>
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<tbody>
<tr>
<td></td>
<td>Affected</td>
<td>Unaffected</td>
<td></td>
<td>Affected</td>
<td>Unaffected</td>
<td></td>
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<tr>
<td></td>
<td>n</td>
<td></td>
<td></td>
<td>n</td>
<td></td>
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<tr>
<td>At rest</td>
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</tr>
<tr>
<td>Global MBF‡ (ml.min⁻¹.g⁻¹)</td>
<td>0.69 (0.66 to 0.72)</td>
<td>94</td>
<td>0.76 (0.73 to 0.79)</td>
<td>90</td>
<td>0.68 (0.66 to 0.71)</td>
<td>0.77 (0.74 to 0.80)</td>
<td>0.89 (0.84 to 0.95)†</td>
</tr>
<tr>
<td>Septal MBF§ (ml.min⁻¹.g⁻¹)</td>
<td>0.87 (0.83 to 0.90)</td>
<td>94</td>
<td>0.88 (0.84 to 0.92)</td>
<td>90</td>
<td>0.85 (0.82 to 0.89)</td>
<td>0.89 (0.85 to 0.94)</td>
<td>0.96 (0.89 to 1.03)†</td>
</tr>
<tr>
<td>Free wall MBF¶ (ml.min⁻¹.g⁻¹)</td>
<td>0.69 (0.66 to 0.72)</td>
<td>94</td>
<td>0.76 (0.73 to 0.79)</td>
<td>90</td>
<td>0.68 (0.66 to 0.71)</td>
<td>0.77 (0.74 to 0.80)</td>
<td>0.89 (0.84 to 0.95)†</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>90 (87 to 93)</td>
<td>87</td>
<td>91 (88 to 94)</td>
<td>86</td>
<td>91 (88 to 94)</td>
<td>90 (87 to 93)</td>
<td>1.42 (-2.92 to 5.75)</td>
</tr>
<tr>
<td>Heart rate (beats.min⁻¹)</td>
<td>66 (64 to 68)</td>
<td>90</td>
<td>66 (64 to 68)</td>
<td>89</td>
<td>66 (64 to 69)</td>
<td>65 (63 to 68)</td>
<td>0.92 (-2.55 to 4.39)</td>
</tr>
<tr>
<td>With cold pressor stress</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global MBF‡ (ml.min⁻¹.g⁻¹)</td>
<td>1.01 (0.97 to 1.05)</td>
<td>89</td>
<td>1.06 (1.01 to 1.11)</td>
<td>89</td>
<td>1.00 (0.96 to 1.04)</td>
<td>1.06 (1.02 to 1.11)</td>
<td>0.93 (0.88 to 1.00)†</td>
</tr>
<tr>
<td>Septal MBF§ (ml.min⁻¹.g⁻¹)</td>
<td>1.17 (1.12 to 1.21)</td>
<td>89</td>
<td>1.20 (1.14 to 1.27)</td>
<td>89</td>
<td>1.16 (1.10 to 1.21)</td>
<td>1.21 (1.15 to 1.27)</td>
<td>0.96 (0.89 to 1.03)†</td>
</tr>
<tr>
<td>Free wall MBF¶ (ml.min⁻¹.g⁻¹)</td>
<td>1.01 (0.96 to 1.05)</td>
<td>89</td>
<td>1.08 (1.03 to 1.14)</td>
<td>89</td>
<td>1.00 (0.95 to 1.05)</td>
<td>1.08 (1.03 to 1.14)</td>
<td>0.92 (0.86 to 0.99)†</td>
</tr>
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<td>With hyperaemia</td>
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<tr>
<td>Global MBF‡ (ml.min⁻¹.g⁻¹)</td>
<td>2.31 (2.22 to 2.41)</td>
<td>83</td>
<td>2.37 (2.27 to 2.49)</td>
<td>72</td>
<td>2.31 (2.21 to 2.40)</td>
<td>2.38 (2.28 to 2.49)</td>
<td>0.97 (0.91 to 1.03)†</td>
</tr>
<tr>
<td>Septal MBF§ (ml.min⁻¹.g⁻¹)</td>
<td>2.43 (2.33 to 2.54)</td>
<td>83</td>
<td>2.47 (2.36 to 2.59)</td>
<td>72</td>
<td>2.44 (2.34 to 2.55)</td>
<td>2.46 (2.35 to 2.58)</td>
<td>0.99 (0.92 to 1.06)†</td>
</tr>
<tr>
<td>Free wall MBF¶ (ml.min⁻¹.g⁻¹)</td>
<td>2.35 (2.26 to 2.45)</td>
<td>83</td>
<td>2.32 (2.21 to 2.44)</td>
<td>72</td>
<td>2.35 (2.25 to 2.45)</td>
<td>2.32 (2.22 to 2.43)</td>
<td>1.01 (0.95 to 1.08)†</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>87 (84 to 90)</td>
<td>84</td>
<td>88 (85 to 91)</td>
<td>83</td>
<td>88 (85 to 91)</td>
<td>87 (84 to 90)</td>
<td>0.59 (-3.86 to 5.04)</td>
</tr>
<tr>
<td>Heart rate (beats.min⁻¹)</td>
<td>89 (85 to 93)</td>
<td>87</td>
<td>87 (83 to 91)</td>
<td>84</td>
<td>88 (84 to 92)</td>
<td>88 (84 to 92)</td>
<td>-0.43 (-6.37 to 5.50)</td>
</tr>
<tr>
<td>MPRi†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global</td>
<td>3.37 (3.20 to 3.56)</td>
<td>82</td>
<td>3.12 (2.92 to 3.33)</td>
<td>71</td>
<td>3.41 (3.21 to 3.62)</td>
<td>3.08 (2.88 to 3.28)</td>
<td>1.11 (1.01 to 1.22)†</td>
</tr>
<tr>
<td>Septal</td>
<td>2.81 (2.65 to 2.98)</td>
<td>82</td>
<td>2.77 (2.56 to 2.99)</td>
<td>71</td>
<td>2.86 (2.67 to 3.06)</td>
<td>2.71 (2.52 to 2.92)</td>
<td>1.05 (0.95 to 1.18)†</td>
</tr>
<tr>
<td>Free wall</td>
<td>3.46 (3.28 to 3.64)</td>
<td>82</td>
<td>3.04 (2.85 to 3.24)</td>
<td>71</td>
<td>3.50 (3.30 to 3.71)</td>
<td>3.00 (2.82 to 3.20)</td>
<td>1.16 (1.06 to 1.28)†</td>
</tr>
</tbody>
</table>

Data are mean or geometric mean (95%CI), differences between means (95%CI) or † ratio of geometric means (95% CI). A 95% CI for a ratio of geometric means is non-significant if it includes 1. MAP=mean arterial pressure, MBF=myocardial blood flow, MPRi=myocardial perfusion reserve index. * Adjusted for age, sex, birth weight z-score and sibship. ‡ Average of global MBF from all slices. § Average of MBF from inferoseptal and anteroseptal regions from all slices. ¶ Average of MBF from anterior, inferior, anterolateral and inferolateral regions from all slices. || MPRi= stress/resting MBF.
Figure 5.10: Effect of age on myocardial blood flow measured (A) globally at rest, (B) in the ventricular free wall at rest, and (C) in the ventricular free wall during cold pressor stress.
Figure 5.11: Effect of gestational age at birth on myocardial blood flow measured (A) globally at rest, (B) in the ventricular free wall at rest, and (C) in the ventricular free wall during cold pressor stress.

Figure legends are regression coefficient ± SE, $r^2$ and p-values for each regression line.
Figure 5.12: Effect of gestational age at birth on myocardial blood flow measured (A) globally at rest, (B) in the ventricular free wall at rest, and (C) in the ventricular free wall during cold pressor stress (with outlier excluded).

Figure legends are regression coefficient ± SE, $r^2$ and p-values for each regression line.
*Single participant with gestational age 26 weeks excluded.
Table 5.4: Myocardial blood flow (MBF) in affected participants born before and after the introduction of intravascular transfusion in 1986

|                      | Unadjusted |  | Adjusted* |  |          |  |          |  | Difference |  | p-value |
|----------------------|------------|  |-----------|  |-----------|  |-----------|  |------------|  |---------|
| At rest              |            |  |           |  |           |  |           |  |           |  |         |
| Global MBF\(‡\) (ml.min\(^{-1}\) \(g\^{-1}\)) | 0.69 (0.66 to 0.73) | 68 | 0.69 (0.65 to 0.74) | 26 | 0.73 (0.69 to 0.77) | 0.61 (0.54 to 0.68) | 0.84 (0.72 to 0.98)\(†\) | 0.02 |
| Septal MBF\(§\) (ml.min\(^{-1}\) \(g\^{-1}\)) | 0.88 (0.84 to 0.93) | 68 | 0.83 (0.77 to 0.89) | 26 | 0.91 (0.86 to 0.96) | 0.77 (0.68 to 0.87) | 0.85 (0.72 to 1.00)\(†\) | 0.05 |
| Free wall MBF\(¶\) (ml.min\(^{-1}\) \(g\^{-1}\)) | 0.68 (0.65 to 0.72) | 68 | 0.71 (0.66 to 0.76) | 26 | 0.72 (0.68 to 0.76) | 0.63 (0.56 to 0.71) | 0.88 (0.75 to 1.02)\(†\) | 0.09 |
| MAP (mmHg)           | 90 (87 to 93) | 68 | 89 (83 to 94) | 18 | 88 (85 to 92) | 93 (84 to 103) | -5 (-16 to 6) | 0.34 |
| Heart rate (beats.min\(^{-1}\)) | 65 (62 to 67) |  | 70 (65 to 75) | 21 | 65 (62 to 69) | 67 (58 to 75) | -1 (-11 to 9) | 0.82 |
| **With cold pressor stress** |  |  |  |  |  |  |  |  |
| Global MBF\(‡\) (ml.min\(^{-1}\) \(g\^{-1}\)) | 1.01 (0.96 to 1.06) | 65 | 1.01 (0.94 to 1.08) | 24 | 1.01 (0.96 to 1.07) | 1.00 (0.89 to 1.12) | 0.99 (0.84 to 1.15)\(†\) | 0.85 |
| Septal MBF\(§\) (ml.min\(^{-1}\) \(g\^{-1}\)) | 1.17 (1.12 to 1.23) | 65 | 1.15 (1.06 to 1.24) | 24 | 1.19 (1.12 to 1.25) | 1.13 (1.01 to 1.27) | 0.95 (0.82 to 1.11)\(†\) | 0.53 |
| Free wall MBF\(¶\) (ml.min\(^{-1}\) \(g\^{-1}\)) | 0.98 (0.94 to 1.00) | 65 | 1.07 (0.96 to 1.20) | 24 | 0.99 (0.93 to 1.05) | 1.08 (0.95 to 1.23) | 1.10 (0.93 to 1.30)\(†\) | 0.28 |
| **With hyperaemia** |  |  |  |  |  |  |  |  |
| Global MBF\(‡\) (ml.min\(^{-1}\) \(g\^{-1}\)) | 2.27 (2.18 to 2.37) | 62 | 2.42 (2.19 to 2.68) | 21 | 2.26 (2.14 to 2.39) | 2.51 (2.22 to 2.84) | 1.11 (0.95 to 1.30)\(†\) | 0.19 |
| Septal MBF\(§\) (ml.min\(^{-1}\) \(g\^{-1}\)) | 2.46 (2.35 to 2.57) | 62 | 2.36 (2.12 to 2.63) | 21 | 2.44 (2.30 to 2.58) | 2.46 (2.15 to 2.81) | 1.01 (0.85 to 1.20)\(†\) | 0.90 |
| Free wall MBF\(¶\) (ml.min\(^{-1}\) \(g\^{-1}\)) | 2.30 (2.21 to 2.40) | 62 | 2.52 (2.29 to 2.78) | 21 | 2.30 (2.18 to 2.43) | 2.54 (2.25 to 2.87) | 1.10 (0.94 to 1.29)\(†\) | 0.22 |
| MAP (mmHg)           | 87 (84 to 90) | 65 | 88 (81 to 95) | 19 | 86 (83 to 90) | 92 (82 to 101) | -5 (-17 to 6) | 0.36 |
| Heart rate (beats.min\(^{-1}\)) | 87 (83 to 92) | 66 | 95 (87 to 102) | 21 | 90 (85 to 95) | 82 (70 to 95) | 8 (-7 to 23) | 0.31 |
| **MPRi\(§\)** |  |  |  |  |  |  |  |  |
| Global\(§\) | 3.33 (3.13 to 3.54) | 61 | 3.50 (3.10 to 3.95) | 21 | 3.14 (2.91 to 3.38) | 4.19 (3.53 to 4.96) | 1.33 (1.07 to 1.66)\(†\) | 0.01 |
| Septal\(§\) | 2.81 (2.61 to 3.01) | 61 | 2.83 (2.57 to 3.11) | 21 | 2.69 (2.48 to 2.92) | 3.19 (2.66 to 3.83) | 1.19 (0.94 to 1.50)\(†\) | 0.16 |
| Free wall\(§\) | 3.42 (3.22 to 3.63) | 61 | 3.58 (3.22 to 3.99) | 21 | 3.25 (3.02 to 3.49) | 4.13 (3.51 to 4.86) | 1.27 (1.03 to 1.57)\(†\) | 0.03 |

Data are mean or geometric mean (95%CI), differences between means (95%CI) or \(†\) ratio of geometric means (95% CI). A 95% CI for a ratio of geometric means is non-significant if it includes 1. MAP=mean arterial pressure, MBF=myocardial blood flow, MPRi=myocardial perfusion reserve index. * Adjusted for age, sex, and birth weight z-score. ‡ Average of global MBF from all slices. § Average of MBF from inferoseptal and anteroseptal regions from all slices. † Average of MBF from anterior, inferior, anterolateral and inferolateral regions from all slices. || MPRi= stress/resting MBF.
5.5  Discussion

This chapter provides the first report of LV function, size and stress, myocardial viability, and MBF in adult survivors of fetal anaemia managed with IUT.

5.5.1  Left Ventricular Function, Size and Stress

In the last decade, CMR has become the gold standard for the assessment of cardiac size, structure and function (Hudsmith, Petersen et al. 2005). In recent years there have been several reports of normal values for LV volumes and mass determined by CMR in healthy subjects devoid of cardiovascular risk factors or disease (Lorenz, Walker et al. 1999; Marcus, DeWaal et al. 1999; Sandstede, Lipke et al. 2000; Alfakih, Plein et al. 2003; Drazner, Dries et al. 2005; Hudsmith, Petersen et al. 2005; Maceira, Prasad et al. 2006; Natori, Lai et al. 2006; Cain, Ahl et al. 2009; Prakken, Velthuis et al. 2010). However, notable variation is evident on comparison of these published reference ranges. Several factors explain these variations. Firstly, there is interstudy variability in the CMR protocols used to acquire images, with only four recent studies providing reference ranges for images assessed using steady state free precession pulse sequences, the imaging technique utilised in our study (Alfakih, Plein et al. 2003; Hudsmith, Petersen et al. 2005; Maceira, Prasad et al. 2006; Prakken, Velthuis et al. 2010). Several authors have demonstrated that, in comparison to older gradient echo sequences, acquisition of images using steady state free precession results in larger LV volumes and smaller LV mass measurements, with improved image quality and reduced signal-to-noise ratio (Plein, Bloomer et al. 2001; Thiele, Nagel et al. 2001; Alfakih, Plein et al. 2003; Ozgun, Hoffmeier et al. 2005). Thus, comparison of CMR measurements of LV volumes and mass obtained using different imaging techniques is difficult.

Secondly, differing approaches are common for three aspects of image analysis of particular importance for determining LV volumes and mass; specifically inclusion or exclusion of papillary muscles in the LV mass, determination of end diastolic and end systolic frames and inclusion or exclusion of outflow tract below the aortic valve in the LV volumes. In our study, papillary muscles were excluded from the LV mass, in keeping with the most widely accepted current approach (Scharhag, Schneider et al. 2002; Prakken, Velthuis et al. 2010), and LV volumes were determined using a semi-automated cardiac contouring method integral to the software used to analyse our images. By comparison, of the four studies reporting references ranges for images acquired using steady state free precession, three included the papillary muscles in the LV mass (Alfakih, Plein et al. 2003; Hudsmith,
Thirdly, it is well accepted that age and sex are important determinants of LV volumes and mass, both of which are consistently higher in men than women (Marcus, DeWaal et al. 1999; Sandstede, Lipke et al. 2000). Furthermore, LV volumes decrease with age in men, with some authors reporting a similar, albeit less significant, relationship in women. Most also report that LV mass decreases with age in men, but not women (Hudsmith, Petersen et al. 2005; Maceira, Prasad et al. 2006; Cain, Ahl et al. 2009). Thus, comparison to age- and sex-specific reference ranges allows more accurate definition of the upper and lower limits of normal for a given individual.

These expected relationships are also evident in our data. Furthermore, when comparison is made to the four studies in which measurements of LV volumes and mass were taken from images acquired using a steady state free precession CMR protocol, our findings largely appear to fall within normal limits. However, none of these studies utilised exactly the same image analysis methodology as our study (Table 5.5), and not all these reported references ranges are applicable to our data. In fact, surprisingly, Prakken et al, who used image analysis methodology similar to ours, reported much larger LV volumes, and much smaller LV mass values than found in our study (Prakken, Velthuis et al. 2010). These differences may be due, at least in part, to the fact that they based their normal values on a group of volunteers who engaged in very little exercise, meaning that this was unlikely to have been a representative sample of the normal population (Prakken, Velthuis et al. 2010). In contrast, comparison to the other three most relevant studies showed that all but two of our participants with findings for indexed EDV or LV mass outside these published ranges had values well within three standard deviations of the reported means (Table 5.5). Assuming these data are normally distributed, we would expect 4.7% of our study participants (approximately 9 individuals) to have values between two and three standard deviations from the mean. Thus, with the exception of the two individuals discussed below, it is likely that participants with LV volumes and mass measurements outside reported reference ranges represent the extremes of normal.
Table 5.5: Comparison of Fetal Anaemia Study findings for indexed end diastolic volume and left ventricular mass to published values acquired using similar scan protocols

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<td>CIM v 7.0 (University of Auckland)</td>
<td>Argus software (Siemens)</td>
<td>Manual cardiac contour tracing and selection of end systolic + end diastolic frames</td>
<td>MASS software (Medis)</td>
<td>CMRTTools software (CV Imaging Solutions)</td>
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<td>Semi–automated cardiac contour tracing and selection of end systolic + end diastolic frames</td>
<td>Manual cardiac contour tracing and selection of end systolic + end diastolic frames</td>
<td>Blood below aortic valve included in LV volumes</td>
<td>Semi–automated cardiac contour tracing and selection of end systolic + end diastolic frames</td>
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<td>Papillary muscles excluded from LV mass</td>
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<td>Subjects n (%male) age range</td>
<td>IUT recipients (affected) + unaffected sibling(s) 186 (49) 17–60y</td>
<td>Healthy volunteers 108 (58) 21–68y</td>
<td>Healthy volunteers 60 (50) 20–65y</td>
<td>Healthy volunteers 120 (50) 20–80y</td>
<td>Sedentary volunteers 114 (49) 18–39y</td>
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<td>Subjects n (%male) age range</td>
<td>Raw and indexed volumes + mass (BSA)</td>
<td>Raw and indexed volumes + mass (BSA), all–age, &lt;35y and ≥35y</td>
<td>Raw and indexed volumes + mass (BSA, height), all–age, &lt;40y and ≥40y</td>
<td>Raw and indexed volumes + mass (BSA)</td>
<td>Indexed volumes + mass (BSA), all–age</td>
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<td>EDV/BSA* (ml.m^2)</td>
<td>All–age: 2 F (U) &lt;95%CI; 6 M (1A) + 1 F (A) &gt;95%CI&lt;35y: 1 M (A) &lt;95%CI; 3 M (1A) + 1 F (U) &gt;95%CI ≥35y: 5 M (1A) + 1 F (A) &gt;95%CI</td>
<td>All–age: 1 M (A) + 1 F (U) &lt;95%CI; 3 M (1A) + 2 F (1A) &gt;95%CI &lt;40y: 2 M (1A) &gt;95%CI ≥40y: 1 F (U) &lt;95%CI 2 F (A) &gt;95%CI</td>
<td>All–age: 2 F (1A) &lt;95%CI 6 M (1A) &gt;95%CI 30–39y: 1 F (A) &lt;95%CI 4 M (3A) + 1 F (U) &gt;95%CI 40–49y: 3 M (2A) &lt;95%CI 4 M (2A) + 1 F (A) &gt;95%CI 50–59y: 3 F (U) &lt;95%CI 1 F (U) &gt;95%CI 60–69y: none &lt; or &gt;95%CI</td>
<td>10 M (8A) + 23 F (8A) &lt;95%CI 1 M (U) &gt;95%CI</td>
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<td>Number of FAS subjects with EDV/BSA outside 95% CI</td>
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<td>Number of FAS subjects with LV mass/BSA outside 95% CI</td>
<td>All–age: 24 M (12A) + 1 F (U) &gt;95%CI</td>
<td>All–age: 17 M (7A) + 11 F (3A) &gt;95%CI</td>
<td>20–29y: 3 F (U) &gt;95%CI</td>
<td>30–39y: none &lt; or &gt;95%CI</td>
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<td>&lt;35y: 4 M (2A) &gt;95%CI</td>
<td>24 M (12A) + 2 F (U) &gt;95%CI</td>
<td>30–39y: none &lt; or &gt;95%CI</td>
<td>40–49y: M (U) + 1 F (A) &gt;95%CI</td>
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<td>≥35y: 16 M (9A) + 6 F (2A) &gt;95%CI</td>
<td>≥40y: 1 F (U) &lt;95%CI</td>
<td>30–39y: none &lt; or &gt;95%CI</td>
<td>50–59y: 3 F (U) &lt;95%CI</td>
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<td>3 M (2A) &gt;95%CI</td>
<td>30–39y: none &lt; or &gt;95%CI</td>
<td>1 F (U) &gt;95%CI</td>
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<td>≥40y: 11 M (5A) + 14 F (5A) &gt;95%CI</td>
<td>30–39y: none &lt; or &gt;95%CI</td>
<td>60–69y: none &lt; or &gt;95%CI</td>
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EDV=end diastolic volume, BSA=body surface area, LV=left ventricular, IUT=intrauterine transfusion, FAS=Fetal Anaemia Study, M=male, F=female, A=affected and U=unaffected FAS participants, 95%CI=95% confidence interval. * Data are range (FAS) or normal range (95% confidence interval) for comparative studies.
One unaffected man and one affected woman had values for indexed LV volumes which were substantially more than three standard deviations beyond the means for all three comparison studies, suggesting that these individuals may have pathologically enlarged left ventricles. The unaffected man was a competitive triathlete who reported engaging in 30 hours of moderate or vigorous exercise per week. His LV volumes were in keeping with exercise-induced cardiac remodelling, the so-called “athlete’s heart”, a well described physiological adaptation to prolonged physical training (Scharf, Brem et al. 2010; Pelliccia, Maron et al. 2012). However, the affected woman with large LV volumes reported engaging in no physical activity whatsoever. Although she reported no history of cardiovascular disease, she was found to have mild systolic hypertension and was a smoker, with a 28 pack-year history. In addition, this woman was born at 26 weeks’ gestation; the only participant in our cohort born extremely preterm. Indeed, analysis of the relationship between LV volume data and gestational age revealed that her results frequently skewed findings for the entire cohort. Although caution must be taken not to over-interpret these results, her data raise the possibility that extremely preterm birth may have different implications for cardiac structure in adult life than birth at less preterm gestations.

We investigated a number of other aspects of LV geometry, and found that these all fell within the published normal ranges, including LV end diastolic wall thickness whether measured globally, in the septum or in the free ventricular wall (Dweck, Joshi et al. 2012; Kawel, Turkbey et al. 2012), LV concentricity (Dweck, Joshi et al. 2012; Lee, Dweck et al. 2013), concentricity$^{0.67}$, diastolic wall thickness to volume ratio (Petersen, Selvanayagam et al. 2005), LV wall thickening assessed globally, in the septum and in the free ventricular wall (Prasad, Ramesh et al. 2010; Dawson, Maceira et al. 2011), LV radius measured at end diastole and end systole (Puntmann, Gebker et al. 2013), and end systolic wall stress whether assessed globally, at the septum or in the free wall (Zhong, Su et al. 2009). LV concentricity, the ratio of LV mass to EDV, is often used as a CMR equivalent of echocardiogram-derived relative wall thickness. Concentricity$^{0.67}$ (LV mass/EDV$^{0.67}$) is thought to be more highly correlated with both systolic blood pressure and LV wall thickness than concentricity (Khoury, Peshock et al. 2010). Diastolic wall thickness to volume ratio is also used as a measure of wall thickness relative to heart size. Taken together, these findings suggest that LV function, mass and geometry were within normal limits in all our study participants, except for one participant born extremely preterm in whom LV volumes were larger than expected.
However, our results suggest that exposure to intrauterine anaemia may alter LV function. Specifically, compared to unaffected siblings, affected participants had lower raw and indexed EDV, ESV and SV, higher end diastolic wall thickness to volume ratio, lower end diastolic and end systolic LV radius when measured globally and at the free wall, and lower global and free ventricular wall stress. A trend towards lower indexed LV mass was also apparent. To our knowledge, only one other investigation of cardiac function following intrauterine anaemia has been published. This reported lower left atrial area and LV mass and a trend to lower LV end diastolic dimension (p=0.053) in 25 children exposed to intrauterine anaemia due to rhesus disease compared to age and sex matched controls, as assessed with transthoracic echocardiogram at a median age of 10.1 years (Dickinson, Sharpe et al. 2010). These results are in keeping with ours, although our findings provide statistically stronger evidence of lower LV volumes in affected participants, which several advantages in study design may have allowed us to detect. Firstly, our study was much larger, thus had greater power to detect differences between groups. Secondly, our study was conducted in adults, in whom maturation of the cardiovascular system would be expected to be complete. Finally, use of CMR may have allowed for more accurate assessment of LV function and size in our study.

It is possible that confounding differences between affected and unaffected participants influenced our findings. Both groups were similar for several important cardiovascular risk factors, specifically rates of smoking, hypertension, diabetes and hypercholesterolaemia, body habitus, and socioeconomic status. Thus, these factors were unlikely to have influenced outcomes. Furthermore, by using unaffected siblings as our comparison group, we hoped to minimise the impact of social, familial and genetic diversity on our findings. However, this strategy resulted in two unavoidable, and potentially important, differences between groups.

Firstly, as rhesus disease is uncommon in a first born child, most unaffected participants were older than their affected siblings. Since LV volumes decrease with increasing age, particularly in men (Sandstede, Lipke et al. 2000), we adjusted for age in our linear regression model. Furthermore, LV volumes were lower in affected participants than in their older unaffected siblings, the opposite outcome to that expected based on age alone, so it is unlikely that the differences in age account for our findings.
Secondly, due to the difficulties they experienced in utero, affected participants were born at an earlier gestation, and consequently of lower birth weight, than their unaffected siblings. Several studies have demonstrated subclinical cardiac dysfunction in children born preterm, of low birth weight and small for gestational age (Mikkola, Leipala et al. 2007; Crispi, Bijnens et al. 2010; Altin, Kараarslan et al. 2012; Kwinta, Jagla et al. 2013). In addition, in a recent CMR investigation of LV function in 102 adults aged 23 to 28 years born at a mean gestation of 30.3 weeks, Lewandowski et al reported an approximate 10% reduction in indexed EDV, ESV and SV compared to age and sex matched term-born controls; findings that are similar to ours. These authors also reported a 30% greater LV mass index in those born preterm, which is the opposite of the trend to lower LV mass in our affected participants (Lewandowski, Augustine et al. 2013). However, it is often difficult to determine the extent to which preterm delivery, size at birth, or any one of a number of other adverse perinatal factors associated with preterm delivery, may contribute to the differences identified in these studies. In an attempt to account for these factors, Lewandowski et al included 5 key perinatal variables in their regression analysis (gestational age, birth weight z-score, postnatal weight gain in the first 2 weeks, days of ventilation and maternal preeclampsia), and concluded that the altered cardiac structure identified in their cohort was determined solely by gestational age, with the severity of changes graded according to the degree of prematurity.

By comparison, in our cohort, we found that birth weight z-score was an independent predictor of variables pertaining to LV function and size, and thus was included in our linear regression model. However, gestational age was not an independent predictor of LV outcomes, with the exception of end systolic wall stress, and even for this measure, the relationship was no longer apparent when the sole participant born extremely preterm was excluded. Furthermore, gestational age and affected/unaffected status were highly correlated ($r^2=0.68$) and on multiple logistic regression, only gestational age was significantly associated with affected/unaffected status ($p<0.0001$). Thus, it was neither possible, nor necessary, to adjust for gestational age in analysis of our data.

Our finding that gestational age was not independently associated with LV outcomes in our cohort is at odds with the report of Lewandowski et al. One key difference between that study and ours is that Lewandowski’s preterm group was born at an earlier gestation (mean±SD: 30.3±2.5 weeks, with 69% born less than 31 weeks versus 34.3±1.5 weeks, with only 2 participants (1%) born less than 31 weeks in our study). Thus, it is possible that
gestational age is only an important predictor of LV function for those born very preterm. This in turn suggests that the lower LV volumes we found in affected participants are more likely to be related to fetal anaemia rather than prematurity. If this were the case, it is possible to speculate that decreased LV volumes in Lewandowski’s preterm group may be due, at least in part, to postnatal anaemia prior to term equivalent age, which occurs almost universally in infants born preterm (Widness 2008). Furthermore, as findings for LV mass in our affected participants were opposite to those reported in Lewandowski’s preterm group, it is possible that exposure to fetal anaemia and preterm birth influence myocardial development in different ways.

Clues to potential pathways by which fetal anaemia leads to alterations in LV function may be found in animal studies. In fetal sheep, chronic anaemia results in increased stroke volume and heart rate, with a consequent doubling of cardiac output, together with a 30% increase in heart to body weight ratio (Davis and Hohimer 1991). Increased ventricular size is accompanied by myocardial hypertrophy, an adaptive response that ensures the ratio between ventricular radius and wall thickness is kept constant, thus normalising wall stress (Thornburg, Jonker et al. 2011). Furthermore, in order to maintain myocardial oxygen supply, coronary blood flow increases nearly six fold, in comparison to, at most, a two fold increase in flow to other regional circulations (Davis and Hohimer 1991). Thus, the fetal response to anaemia is characterised by increased cardiac size and mass, and expansion of the coronary circulation.

Under normal intrauterine conditions, heart growth in fetal sheep is achieved through cardiomyocyte proliferation and enlargement (Smolich, Walker et al. 1989). In addition, terminal differentiation, characterised by the transition of cardiomyocytes from mononucleation to binucleation, becomes increasingly common in the last third of gestation, with at least 70% of cells binucleated by term (Thornburg, Jonker et al. 2011). Importantly, this process of cellular maturation results in a progressive decrease in the proliferative capacity of the myocardium, so that after birth cardiac growth is achieved almost entirely by cellular enlargement (Bergmann, Bhardwaj et al. 2009). However, adverse intrauterine conditions may disrupt this process leading to a reduction in the number of cardiomyocytes endowed at birth, in turn resulting in greater work per cell and cellular hypertrophy in order to achieve adequate contractile function (Thornburg, Jonker et al. 2011). Indeed, in sheep structural remodelling of the myocardium has been shown to occur in response to fetal anaemia (Jonker, Giraud et al. 2010; Jonker, Scholz et al. 2011), as well as fetal
hypertension (Barbera, Giraud et al. 2000; Jonker, Faber et al. 2007) and hypotension (O'Tierney, Anderson et al. 2010), intrauterine hypoxia and undernutrition (Bubb, Cock et al. 2007; Louey, Jonker et al. 2007), and preterm birth (Bensley, Stacy et al. 2010).

The effect of fetal anaemia on ovine myocardial structure has been investigated by Jonker et al, who reported that in comparison to nonanaemic twins, fetal sheep in whom haematocrit was reduced by approximately 50% in late gestation (from 129 to 138 days, term gestation 145 days) had a 39% increase in heart weight due to a balanced combination of increased cardiomyocyte proliferation, enlargement and terminal differentiation (Jonker, Giraud et al. 2010). However, there was no evidence of increased terminal differentiation or cardiomyocyte enlargement in fetal sheep exposed to a similar degree of anaemia earlier in gestation (from days 109 to 119), suggesting that the 45% increase in heart weight in these animals was mostly due to increased cardiomyocyte proliferation (Jonker, Scholz et al. 2011). Intrauterine transfusion at gestational day 119 decreased cardiomyocyte proliferation and terminal differentiation, and substantially slowed heart growth in anaemic fetuses (Jonker, Scholz et al. 2011). Thus, the effect of fetal anaemia on myocardial development is dependent upon the timing of exposure to anaemia as well as receipt of intrauterine transfusion, with the balance of these factors determining cardiomyocyte endowment at birth. We are not aware of any reports of cardiomyocyte number in adult sheep exposed to fetal anaemia +/- intrauterine transfusion, and thus whether fetal anaemia alters long-term myocardial structure remains uncertain.

Our findings indicate that LV volumes and radius, and possibly LV mass, are reduced in affected participants, and the data from fetal sheep suggest that this may reflect a reduction in cardiomyocyte number. Furthermore, our finding of reduced end systolic wall stress, determined by the relationship between LV radius and wall thickness, may reflect a response to fetal anaemia in the face of decreased cardiomyocyte number. Thus, we propose that fetal anaemia and intrauterine transfusion may lead to altered cardiomyocyte maturation and decreased cardiomyocyte number at birth with resultant long-term structural changes to the myocardium, which may be detected by CMR assessment of LV function and size in adulthood.

5.5.2 Myocardial Viability

LGE is an extremely useful tool for detection of myocardial scarring, with a reported sensitivity of 99% for acute infarction and 94% for chronic infarction in a recent large
multicentre trial (Kim, Albert et al. 2008). However, LGE is not specific for coronary artery disease, as it can also be present in a variety of nonischaemic conditions, such as acute and chronic myocarditis, sarcoidosis, amyloidosis and cardiomyopathy (Ordonez and Higgins 2011). In our study, LGE was negative in 96% of participants in whom this investigation was undertaken. However, equivocal results were found in 5 participants and a positive result in one participant, although no differences were found between affected and unaffected groups. All those with equivocal results were reported to have small and/or patchy midwall defects in the septum, consistent with the distribution of lesions usually found in those with LGE unrelated to myocardial infarction (Hunold, Schlosser et al. 2005). Thus, it is possible that these individuals had previously suffered a nonischaemic myocardial insult, for example due to viral myocarditis, although as none reported a history in keeping with this, any such illnesses went undiagnosed. By comparison, the one participant with a positive result was reported to have LGE localised to the subendocardium of the LV, a distribution in keeping with previous myocardial infarction (Hunold, Schlosser et al. 2005). However, this individual had no risk factors for, or history of, cardiovascular disease and further cardiac investigation, undertaken by his primary health care provider, as a result of this incidental finding, revealed no evidence of coronary artery disease.

Given the lack of history to support either nonischaemic myocardial damage or myocardial infarction in these individuals, the possibility that these were false positive results must be considered. As LGE is usually undertaken only in those with a clear clinical indication for CMR imaging, there is little evidence regarding the false positive rate in healthy subjects with no history of cardiovascular disease. However, the diagnostic value of LGE images can be critically affected by selection of an inappropriate inversion time, which determines the relative signal intensity of infarcted and viable myocardium, permitting identification of myocardial scar (Gupta, Lee et al. 2004). Several factors affect the inversion time, such as body habitus, cardiac function, and length of time since contrast administration. Thus, it is possible that the standard approach to selection of inversion time used in our scan protocol was not appropriate for all participants.

5.5.3 Myocardial Blood Flow

Given the clear advantages of CMR over other forms of noninvasive perfusion imaging, there has been increasing interest in quantitative MBF assessment by CMR in recent years. Many reports of normal values for MBF at rest and with hyperaemic stress induced with adenosine or dipyridamole are now available (Koskenvuo, Sakuma et al. 2001; Muhling,
Dickson et al. 2001; Muehling, Jerosch-Herold et al. 2004; Elkington, Gatehouse et al. 2005; Hsu, Rhoads et al. 2006; Parkka, Niemi et al. 2006; Ritter, Brackertz et al. 2006; Rosen, Lima et al. 2006; Wang, Jerosch-Herold et al. 2006a; Wang, Jerosch-Herold et al. 2006b; Fritz-Hansen, Hove et al. 2008; Jerosch-Herold, Vazquez et al. 2008; Pack and DiBella 2010; Radjenovic, Biglands et al. 2010; Larghat, Biglands et al. 2012; Larghat, Maredia et al. 2013) and two recent reports document MBF with cold pressor stress in healthy volunteers (Moro, Flavian et al. 2011; Ritter, Kowalski et al. 2012). However, as for LV function data, a number of different methods exist for both the acquisition of perfusion data by CMR and the quantitative analysis of images obtained, and consequently there is considerable variation in reported normal values. However, comparison to studies reporting complete perfusion data (rest, stress and MPRi, or rest and cold pressor stress) from at least 10 people suggests that our MBF results are in keeping with most other reported values (Table 5.6).

There are several potential explanations for the variation in reported values for MBF assessed by CMR. Firstly, results may be influenced by differences in CMR imaging protocol, for example, magnet strength, acquisition method, gadolinium formulation, dose and administration protocol, and whether resting perfusion is assessed before or after perfusion with hyperaemic stress. However, Pack et al report no differences in MBF assessment if subjects were imaged with a 1.5T or 3T scanner, if images were obtained using a gradient echo pulse sequence or a fast low angle shot pulse sequence, or if different formulations of gadolinium were used (Pack and DiBella 2010). Furthermore, all 11 comparative studies reporting normal MBF values were undertaken on a 1.5T scanner. Thus, these factors are unlikely to be responsible for the variation in reported MBF values. However, the two studies which report the lowest resting MBF values (Ritter, Brackertz et al. 2006; Ritter, Kowalski et al. 2012) used steady state free precession image acquisition techniques, a dual bolus gadolinium protocol and assessed hyperaemic stress perfusion prior to rest perfusion (Table 5.6). Thus, it is possible that these differences account, at least in part, for lower resting MBF values in these studies.
Table 5.6: Comparison of Fetal Anaemia Study findings to published values for myocardial perfusion reserve index and global myocardial blood flow at rest, with hyperaemic stress and with cold pressor stress

<table>
<thead>
<tr>
<th>Author</th>
<th>Acquisition method</th>
<th>Rest images acquired</th>
<th>Gadolinium dose (mmol.kg⁻¹)</th>
<th>Analysis method</th>
<th>Subjects n (%male), age (mean±SD or range)</th>
<th>Rest MBF (ml.min⁻¹.g⁻¹)</th>
<th>Stress MBF (ml.min⁻¹.g⁻¹)</th>
<th>MPRi</th>
<th>Cold pressor MBF (ml.min⁻¹.g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal Anaemia Study</td>
<td>SR-FLASH 1st</td>
<td>0.04</td>
<td>Fermi</td>
<td>184† (48) IUT recipients + siblings, 37±11y</td>
<td>0.73±0.14</td>
<td>2.23±0.46</td>
<td>3.36±0.90</td>
<td>1.06±0.23</td>
<td></td>
</tr>
<tr>
<td>Muehling et al, 2004</td>
<td>SR-FLASH 1st</td>
<td>0.03</td>
<td>Fermi</td>
<td>17 healthy volunteers, 34±9y</td>
<td>1.10±0.40</td>
<td>4.20±1.10</td>
<td>4.10±1.40</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Wang et al, 2006</td>
<td>SR-FLASH 1st</td>
<td>0.04</td>
<td>Model-independent</td>
<td>222 (56) MESA participants, 45-84y</td>
<td>1.01±0.23</td>
<td>3.02±0.84</td>
<td>3.05±0.84</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Hsu et al, 2006</td>
<td>SR-GRE/EPI 1st</td>
<td>0.005/0.1*</td>
<td>Fermi</td>
<td>10 (80) healthy volunteers, 33±4y</td>
<td>1.02±0.22</td>
<td>3.39±0.59</td>
<td>3.43±0.71</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ritter et al, 2006</td>
<td>SR-SSFP 2nd</td>
<td>~0.005/0.03*</td>
<td>Fermi</td>
<td>12 (75) healthy volunteers, 27±7y</td>
<td>0.52±0.11</td>
<td>1.78±0.53</td>
<td>3.59±1.26</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Parkka et al, 2006</td>
<td>SR-FLASH 1st</td>
<td>0.05</td>
<td>Two-compartment</td>
<td>18 (100) healthy volunteers, 40±14y</td>
<td>0.71±0.24</td>
<td>1.72±0.67</td>
<td>2.51±0.95</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pack et al, 2010</td>
<td>SR-FLASH 1st</td>
<td>0.02</td>
<td>Two-compartment</td>
<td>20† (90) healthy volunteers, 50±13y</td>
<td>0.82±0.22</td>
<td>2.42±0.42</td>
<td>3.06±0.86</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pack et al, 2010</td>
<td>SR-FLASH 1st</td>
<td>0.02</td>
<td>Fermi</td>
<td>20† (90) healthy volunteers, 50±13y</td>
<td>0.93±0.24</td>
<td>2.84±0.63</td>
<td>3.09±0.95</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pack et al, 2010</td>
<td>SR-FLASH 1st</td>
<td>0.02</td>
<td>Model-independent</td>
<td>20† (90) healthy volunteers, 50±13y</td>
<td>0.88±0.27</td>
<td>2.32±0.46</td>
<td>2.90±1.10</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pack et al, 2010</td>
<td>SR-FLASH 1st</td>
<td>0.02</td>
<td>Patlak</td>
<td>20† (90) healthy volunteers, 50±13y</td>
<td>0.83±0.27</td>
<td>2.17±0.44</td>
<td>2.90±1.27</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Moro et al, 2011</td>
<td>SR-FLASH 1st</td>
<td>Not reported</td>
<td>Not reported</td>
<td>24 (50) healthy volunteers, 22±4y</td>
<td>0.71±0.23</td>
<td>-</td>
<td>-</td>
<td>1.19±0.55</td>
<td></td>
</tr>
<tr>
<td>Ritter et al, 2012</td>
<td>SR-SSFP 2nd</td>
<td>~0.005/0.03*</td>
<td>Patlak</td>
<td>10 (7) healthy volunteers, 24±2y</td>
<td>0.61±0.22</td>
<td>-</td>
<td>-</td>
<td>1.15±0.34</td>
<td></td>
</tr>
</tbody>
</table>

Secondly, it is possible that differences between the subjects studied influenced MBF results. Apart from the study of Wang et al, which was undertaken on participants of the Multiethnic Study of Atherosclerosis, all the studies with which our findings are compared involved normal healthy volunteers, with no cardiovascular risk factors or history of cardiovascular disease. However, our participants and those of the Multiethnic Study of Atherosclerosis were not required to be free of cardiovascular risk factors, which were reported with similar frequency in both studies. For example, approximately 20% of participants in our study and in the Multiethnic Study of Atherosclerosis reported a history of hypertension, and 10% were found to be hyperlipidaemic (Wang, Jerosch-Herold et al. 2006a). However, despite these similarities, MBF values reported from Multiethnic Study of Atherosclerosis subjects were 35 to 40% greater than that of our participants. Thus, it is unlikely that lower MBF values in our study are due to differences between study populations.

Finally, it is possible that differences in analysis method, particularly the assumptions made in mathematical modelling, may account for the differences in MBF findings. Four different methods for quantitative perfusion analysis (Fermi function modelling, two-compartment modelling, model-independent analysis and Patlak one-compartment plot analysis) have been described and validated by comparison with either PET or Doppler ultrasound measurements of myocardial perfusion (Koskenvuo, Sakuma et al. 2001; Parkka, Niemi et al. 2006; Fritz-Hansen, Hove et al. 2008; Kurita, Sakuma et al. 2009). With no standardised quantitative perfusion analysis software commercially available, these models must be custom-written. However, there are many potential sources of variability within these models, for example the choice of constants to estimate contrast agent influx and efflux and whether factors are included for correction of contrast saturation and coil sensitivity or to account for the time delay between blood and tissue enhancement (Jerosch-Herold 2010; Pack and DiBella 2010). Thus, as no two analysis models will be identical even if the same image acquisition method is utilised, comparison of MBF data from different research groups is fraught with difficulty. However, several studies have shown good interstudy reproducibility for CMR perfusion analysis, with similar results obtained when investigations are repeated in the same patient by the same research group using an identical approach to image analysis (Muhling, Dickson et al. 2001; Elkington, Gatehouse et al. 2005; Jerosch-Herold, Vazquez et al. 2008; Larghat, Maredia et al. 2013). These findings suggest that if a standardised approach to CMR perfusion image analysis could be devised, comparison of results between studies from different groups may be possible and the
clinical application of CMR for perfusion analysis would be greatly improved (Pack and DiBella 2010).

Despite all these potential sources of variability, comparison of MBF findings within our dataset should be valid, as the same analysts assessed images from all our study participants using the same analysis protocol. Overall, global resting, cold pressor and adenosine MBF results were between 2 and 3 standard deviations from the mean for 6, 5 and 3 participants respectively, in keeping with the number expected to fall in this range, assuming the data are normally distributed. However, values more than three standard deviations above the mean were observed for resting MBF in two unaffected participants, for cold pressor stress in one unaffected participant, and for adenosine stress in one affected and one unaffected participant. In addition, one affected and two unaffected participants had findings for MPRi more than 3 standard deviations above the mean, although their rest and stress MBF values were within normal limits.

The individuals from whom these extreme values were obtained were similar to the remaining study participants with regard to frequency of exercise, body size, presence of cardiovascular risk factors, age, LV function and LV size. Thus, the most likely explanation for these outlying values is suboptimal image quality resulting in increased data noise. Image quality can be impaired by poor spatial and temporal resolution, motion artifact from patient movement, breathing or heart pulsation, and a number of technical factors, which can cause signal loss, image blurring or mis-triggering (Stadler, Schima et al. 2007; Meloni, Al-Saadi et al. 2011; Klinke, Muzzarelli et al. 2013). Indeed, decreased signal-to-noise ratio has been observed with higher heart rate, and low spatial and temporal resolution has been associated with increased dark rim artefact in CMR perfusion imaging (Meloni, Al-Saadi et al. 2011). However, these images were deemed to be of acceptable quality for assessment of MBF, and the signal to noise ratio of the scans with outlying MBF values was similar to those of several other participants in whom MBF was normal. Thus, there was no obvious reason for these more extreme values, and no reason for them to be excluded.

Compared to unaffected siblings, affected participants had lower global and free wall MBF at rest and with cold pressor stress, and higher MPRi when assessed globally and in the ventricular free wall. To our knowledge, ours is the first investigation of MBF in adulthood following exposure to fetal anaemia. As discussed for LV function data, it is possible that the two unavoidable differences between affected and unaffected groups, namely age at
time of study participation and gestational age at birth, influenced these outcomes. A decreased MBF response to hyperaemic stress has been reported with increasing age (Wang, Jerosch-Herold et al. 2006a; Wang, Jerosch-Herold et al. 2006b). Thus, we adjusted for age in our linear regression model, although in our cohort an association between age and MBF was found only for affected men. Furthermore, as for LV volumes, MBF was lower in affected participants than in their older unaffected siblings, the opposite finding to that expected based on age alone, so it is unlikely that the difference in age between affected and unaffected groups account for our findings. We are not aware of any investigations of MBF in adulthood following preterm birth. However, as we found no association between gestational age and MBF in our cohort it is unlikely that the gestational age difference between groups is responsible for our MBF findings.

Thus, it is possible that our findings of lower MBF at rest and with cold pressor stress, and increased MPRi in affected participants may be due to exposure to fetal anaemia. However, these findings are very different to those previously reported from sheep exposed to fetal anaemia (Davis, Roulet et al. 2003). In sheep, fetal anaemia results in a nearly two-fold increase in adenosine-induced hyperaemic MBF in adulthood. As resting MBF was similar to that of nonanaemic animals, increased MPRi reported in previously anaemic sheep was due to higher hyperaemic MBF. These findings are the opposite of our observations in affected participants of lower MBF at rest but similar hyperaemic MBF to unaffected siblings, with increased MPRi in affected participants resulting from lower resting MBF.

At rest, MBF is determined primarily by coronary arteriolar resistance (Kaul 2001). Thus, lower resting MBF in affected participants implies that the coronary arterioles may be relatively vasoconstricted in these individuals. Davis et al also hypothesised that the coronary circulation of previously anaemic sheep may be relatively vasoconstricted at rest, given that resting MBF in these animals was similar to that of nonanaemic controls despite higher MBF with hyperaemic stress. Our finding of lower MBF with cold pressor stress also supports this suggestion. The cold pressor test is designed to assess endothelium-dependent vasomotor function. Immersion of an extremity in ice-cold water activates the sympathetic nervous system, with release of adrenaline, noradrenaline and nitric oxide resulting in arteriolar vasodilation, increased heart rate, blood pressure, and myocardial oxygen demand, and ultimately, increased MBF (Moro, Flavian et al. 2011). Reduced response to cold pressor stress has been associated with an increased incidence of cardiovascular events and is thought to indicate impaired endothelial vasoreactivity (Schindler, Nitzsche et al. 2005).
Thus, taken together, our findings of lower MBF at rest and with cold pressor stress suggest that endothelial function may be impaired following exposure to fetal anaemia.

Endothelium-dependent vasodilation is modulated by a number of factors, the most important of which is nitric oxide. As noted above, under normal physiologic conditions, sympathetic stimulation of the vascular endothelium results in arteriolar vasodilation (Vanhoutte 2001). However, chronic sympathetic hyperactivity has been shown to result in paradoxical vasoconstriction, primarily due to reduced nitric oxide bioavailability, leading to the suggestion that sympathetic hyperactivity may be a major contributor to endothelial dysfunction (Davel, Wenceslau et al. 2011). Thus, lower MBF at rest and with cold pressor stress is not only indicative of endothelial dysfunction in affected participants, but may be directly related to our finding of increased sympathetic tone in these individuals, as indicated by our findings of increased LFnu and LF/HF ratio, and decreased HFnu in affected participants. However, higher global and free wall MPRi in affected participants suggests that appropriate arteriolar vasodilation occurs in response to cardiovascular stress.

Our finding that affected participants born from 1986 to 1992 have lower MBF at rest and higher MPRi than those born from 1963 to 1985 is surprising, and potentially very important. Considerable advances were made in fetal and neonatal medicine in the 1980s and 90s, resulting in increased survival of more severely unwell infants, and those born at earlier gestations. Furthermore, widespread availability of antenatal ultrasound scanning from the early 1980s made intravascular IUT possible, allowing more rapid and complete correction of fetal anaemia, as well as closer monitoring of fetal condition. Following the introduction of intravascular IUT at National Women’s Hospital in 1986, survival of infants who had received IUT improved from 60% for those born in the 4 years prior to 1986 to 90% for those born in the subsequent 4 years (Pattison, Roberts et al. 1992). This improved survival suggests that severely affected infants were more likely to survive in the later period, in turn raising the possibility that lower resting MBF in affected participants born from 1986 to 1992 may be related to greater perinatal compromise in these individuals. Alternatively, the different technique used may in some way have contributed to the greater long-term effects on MBF in the later period. However, as ultrasound scanning and direct measurement of fetal haemoglobin concentration allowed earlier diagnosis and more rapid and accurate treatment of fetal anaemia, it is hard to envisage how the techniques themselves may have been responsible for these differences in MBF in those born later.
The fact that affected participants had lower resting and cold pressor MBF in the free ventricular wall but not the septum is also an unexpected finding. The remainder of our findings provide no clues to help explain this regional difference in MBF in affected participants. However, as right and left ventricular pressures are equivalent in the fetus (Johnson, Maxwell et al. 2000), we hypothesise that the septum may be relatively protected from shear wall stress, which is thought to be an important mediator of vascular endothelial cell growth (Qiu and Tarbell 2000). In contrast, the ventricular free wall of the anaemic fetus may be exposed to considerable shear wall stress as a result of cardiac dilation and failure, rendering the vasculature of the free wall more susceptible to the effects of fetal anaemia. Examination of the coronary vasculature of the septum and ventricular free wall from anaemic fetal sheep may help determine the veracity of this speculation.

Unlike MBF at rest, MBF at maximal vasodilation is determined primarily by myocardial capillary resistance, which is in turn inversely related to capillary density (Kaul 2001). Thus, Davis et al speculate that their finding of higher MBF with adenosine in previously anaemic sheep may be due to alterations to the architecture of the coronary circulation, resulting in a “coronary supertree” (Davis, Roullet et al. 2003). Indeed, evidence of increased vascular growth has been found in near-term fetal sheep exposed to chronic intrauterine anaemia, with increased expression of vascular endothelial growth factor and hypoxia-inducible factor 1, decreased intercapillary distance, increased minimal capillary diameter and increased capillary density demonstrated in the right ventricle (Martin, Yu et al. 1998). In addition, young male rats exposed to anaemia postnatally also have increased myocardial capillary density (Olivetti, Lagrasta et al. 1989).

As hyperaemic MBF was similar in affected and unaffected participants in our study, we found no evidence to suggest that the coronary microvasculature is altered in humans following exposure to fetal anaemia. It is possible that we did not have enough power to detect a difference between groups for hyperaemic MBF, especially as measurements of MBF during adenosine infusion are much more variable than at rest (Jerosch-Herold, Vazquez et al. 2008). Variability in MBF measurement by CMR also increases with increased blood flow, which results in decreased contrast-enhancement during first pass and thus increased measurement errors (Klocke, Simonetti et al. 2001). In keeping with these findings, variability in MBF in our study was greatest for measurements made during adenosine-induced hyperaemia and smallest for those made at rest. However, our study was adequately powered to have an 80% chance of detecting a minimum difference between
affected and unaffected participants of 6% for resting and 6% for hyperaemic MBF respectively. In fact, we detected a 12% difference between groups for MBF at rest, and a 3% difference with adenosine. Thus it is unlikely that lack of statistical power prevented the detection of a difference between affected and unaffected participants for hyperaemic MBF.

There are several possible explanations for the differences between our MBF findings, and those in sheep. Firstly, Davis’s experimental sheep were in late adolescence or early adulthood, whereas most participants in our study were in mid adulthood. Thus it is possible that capillary density decreases with increasing age in individuals exposed to fetal anaemia, thereby obliterating the higher hyperaemic MBF seen in younger animals. We are not aware of any investigations of myocardial vascular morphology with increasing age in animals exposed to fetal anaemia, so this possibility remains speculative. However, in healthy sheep who were not anaemic in utero, capillary density does not change from 8 weeks of age to adulthood (Smolich, Walker et al. 1989).

Secondly, it is possible that fetal anaemia induced under controlled experimental conditions in sheep has different effects from fetal anaemia secondary to rhesus disease in humans. As previously discussed, the response of the fetal myocardium to intrauterine anaemia is dependent upon the timing of the anaemic insult and intrauterine transfusion (Jonker, Giraud et al. 2010; Jonker, Scholz et al. 2011). In human fetuses affected by rhesus disease, the timing and severity of anaemia is highly variable, as is the timing and nature of treatment received. Thus, it is plausible that the effect of these insults on myocardial development will also be variable, and may result in different cardiovascular outcomes to those found in sheep. This variability may also explain the differences that we found between the intraperitoneal and intravascular transfusion cohorts.

Thirdly, it is possible that intrinsic differences between humans and sheep affect the response of the myocardium to fetal anaemia.

5.5.4 Implications
The long-term clinical implications of these findings for adults exposed to fetal anaemia remain uncertain. No affected participant in our cohort had a history of cardiovascular disease and all were less than 50 years old, thus younger than the age at which cardiovascular disease typically manifests. However, our findings of reduced LV function,
size and likely lower LV mass, together with lower MBF at rest and with cold pressor stress in affected participants may indicate increased risk of cardiovascular disease.

Once again, investigations in animals may help decipher the implications of our findings. Adult sheep exposed to fetal anaemia demonstrated increased indices of systolic function during hypoxia compared to control animals, and this may be physiologically advantageous (Broberg, Giraud et al. 2003). However, these animals were in early adulthood, and the authors suggested that this apparently beneficial outcome might prove to be disadvantageous with increasing age. In keeping with this suggestion, adult sheep exposed to fetal anaemia for 3 weeks in late gestation had larger infarct area when expressed as a percentage of the LV area-at-risk following ischaemia-reperfusion injury (Yang, Hohimer et al. 2008). In addition, endothelial dysfunction and increased infarct size following ischaemia-reperfusion injury has been demonstrated in adult rats exposed to intrauterine hypoxia (Li, Xiao et al. 2003; Williams, Hemmings et al. 2005). These animals were also found to have increased cardiomyocyte size, a response that may compensate for reduced cardiomyocyte number, but could also be responsible for increased ischaemic injury in these animals.

Taken together, these studies indicate that exposure to intrauterine anaemia or hypoxia alters small vessel contractile response to hypoxic stress and increases susceptibility to myocardial ischaemic injury in adulthood. Our observations of lower resting and cold pressor MBF in affected participants, which suggest impairment of endothelial function, indicate that humans exposed to fetal anaemia may also have increased susceptibility to myocardial injury following ischaemic insult. Moreover, lower MBF suggestive of greater endothelial dysfunction in those affected participants born later implies that these younger members of our affected group may be most susceptible to myocardial ischaemic injury. Thus, improved survival in those born later may come at the cost of increased cardiovascular risk in later life.

There are several potential limitations to the findings reported in this chapter. Firstly, the severity of fetal anaemia experienced by our affected participants, and the treatment they received both in and ex utero was highly variable, which may have increased the variance of the data sufficient to prevent detection of differences between affected and unaffected groups (type 2 errors). However, with the exception of LV mass, p-values for differences
between affected and unaffected groups were small and it seems unlikely that we failed to detect any clinically important differences for other outcomes reported in this chapter.

Secondly, as measures of fetal haemoglobin concentration were not available for most of our affected participants, we were not able to directly relate our findings in adulthood to the severity of fetal anaemia to which these individuals were exposed. However, we found no evidence of an association between the outcomes of interest and surrogate markers of the severity of fetal anaemia; number of IUTs received, presence of hydrops at birth, cord bilirubin or haemoglobin concentration, highest postnatal bilirubin concentration or lowest postnatal haemoglobin concentration.

Thirdly, given the co-dependence of affected status and gestational age, we were unable to adjust for gestational age in our statistical analysis. However, no association between gestational age and the outcomes of interest were identified, so this difference between affected and unaffected participants is unlikely to explain our findings.

Finally, it is possible that the significant findings reported in this chapter may be explained by chance due to multiple comparisons in the analyses. In total, 62 comparisons were made in this chapter, of which 3 might be expected to be statistically significant (p<0.05) by chance alone. However, we found 25 statistically significant associations, and the majority of p-values were small, so it is unlikely that this is a major source of error.

### 5.6 Summary

Exposure to fetal anaemia treated by IUT is associated with reduced LV function and size, increased LV relative wall thickness, decreased LV wall stress, and lower MBF at rest and with cold pressor stress. These findings suggest that fetal anaemia influences development of the ventricular myocardium, and may result in abnormal cardiomyocyte maturation, decreased cardiomyocyte endowment at birth and altered vasomotor responsiveness of the coronary circulation. Although the clinical significance of these findings is uncertain, it is possible that exposure to fetal anaemia and IUT confers increased cardiovascular risk in adulthood. Follow up of adult survivors of fetal anaemia over the next two to three decades will be required to ascertain if these findings are associated with increased rates of cardiovascular disease in later life.
Chapter 6: Preterm Anaemia Pilot Study: Description of the Cohort

6.1 Summary of Chapter Contents
This chapter describes the survival, growth, haematological and biochemical findings in the first 12 months after birth of sheep in the Preterm Anaemia Study.

6.2 Introduction
In sheep, fetal anaemia alters coronary conductance and increases susceptibility to ischaemia-reperfusion injury in adulthood (Broberg, Giraud et al. 2003; Davis, Roullet et al. 2003; Yang, Hohimer et al. 2008). Our findings from the Fetal Anaemia Study indicate that individuals exposed to intrauterine anaemia secondary to rhesus disease may also be at increased risk of cardiovascular disease in later life. As infants born preterm frequently become anaemic in the first few weeks after birth, at a similar gestational age to that of fetuses suffering from rhesus disease (Strauss 2010), it is possible that anaemia of prematurity may also alter cardiovascular outcome in adulthood. However, the influence of preterm anaemia on long-term health outcomes has not previously been investigated.

In order to carry out such an investigation, a process was required for the induction of anaemia in animals born preterm. The sheep is a suitable species for the investigation of preterm birth for two important reasons. Firstly, sheep are not litter bearing and lambs are relatively large and mature at birth, thus they are more likely to survive preterm birth than other small laboratory animals. Secondly, as sheep reach physiological maturity by 12 months of age, investigation of the influence of early life events on long-term health outcomes is possible within a relatively short time frame (De Matteo, Blasch et al. 2010). Although our group were already undertaking studies in preterm lambs (Alsweiler, Harding et al. 2013), experimental induction of anaemia had not previously been undertaken. Therefore, we set out to establish a reliable and safe method of induction of anaemia in sheep born preterm.

To our knowledge, there are no reports of normal changes in haematological parameters after birth in preterm lambs and only one report of these trends from birth to 2 months of age in lambs born at term (Bassett, Borrett et al. 1995). In addition, no reports of normal changes in biochemical parameters after birth in term or preterm lambs could be found. Data regarding postnatal growth in lambs born preterm were also sparse, with only one
report identified of growth from birth to 9 weeks of age (De Matteo, Blasch et al. 2010). Therefore, we also set out to investigate growth, haematological and biochemical outcomes in the first 12 months after birth in preterm and term born lambs, with particular attention to the influence of preterm anaemia on these parameters.

6.3 Chapter Aims

1. To determine the optimal method of experimentally inducing anaemia prior to term equivalent age (TEA) in preterm lambs without significantly compromising survival.
2. To describe the normal changes in preterm and term lambs in the first 12 months after birth in:
   i. Haematological parameters, specifically haemoglobin, haematocrit, white cell count, and platelet count;
   ii. Biochemical parameters, specifically serum sodium, potassium, glucose, lactate, thyroxine, and markers of iron stores.
   iii. Growth, specifically weight, crown rump length, hindlimb length, hock toe length, chest and abdominal circumference, and biparietal diameter.
3. To compare haematological and biochemical parameters and growth in preterm anaemic lambs in the first 12 months after birth with those of preterm and term control lambs.

6.4 Results

6.4.1 Animal Numbers and Survival

Fifty two lambs from 26 twin bearing ewes were included in this study. Seventeen (65%) ewes were randomly allocated to preterm delivery, and 34 lambs were delivered at gestational age 138 days. Of these, 3 (9%) were excluded as they died prior to six hours of age from complications of preterm birth and 31 (91%) were randomly allocated to the preterm control, PT50% or PT30% groups (Figure 6.1). Nine (35%) ewes were allocated to delivery at term, and 18 lambs were delivered at or beyond gestational age 147 days. Of these, 6 (33%) were excluded (4 (22%) due to poor maternal milk supply, and 2 (11%) born beyond 147 days gestation). The remaining 12 (67%) lambs formed the term control group.

Survival was similar in all groups (Figure 6.2). Three (30%) PT30% lambs died prior to TEA, apparently from complications related to severe anaemia. These lambs all had signs of worsening cardiorespiratory failure and haematocrit values less than 10% on the day of
death, and autopsies showed no evidence of any other pathological processes. Haematocrit decreased to less than 10% in only one surviving PT30% lamb. Three further lambs (one PT50% and two TC) were euthanised after TEA for pneumonia, suspected poisoning and persistent failure to thrive.

Figure 6.1: Assignment of lambs to experimental groups in Preterm Anaemia Study

PT50%=preterm 50% anaemic group, PT30%= preterm 30% anaemic group, PC=preterm control group, TC=term control group.

Figure 6.2: Survival in each experimental group to 365 days of age

Log-rank test for difference in survival between groups, p=0.23.
6.4.2 Venesection Volumes

Target haematocrit was reached at a median gestational age of 144 days in PT50% lambs and 145 days in PT30% lambs. Mean volume of blood taken each day from those lambs venesected was similar in PT50% and PT30% lambs (Table 6.1).

Table 6.1: Venesection volumes in preterm anaemic 50% and 30% experimental groups

<table>
<thead>
<tr>
<th>Days Post Term Equivalent Age</th>
<th>Preterm Anaemic 50%</th>
<th>Preterm Anaemic 30%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N (%) venesected</td>
<td>Mean volume (ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-9 days</td>
<td>11/11 (100)</td>
<td>44.6±6.0</td>
</tr>
<tr>
<td>-8 days</td>
<td>11/11 (100)</td>
<td>44.6±6.0</td>
</tr>
<tr>
<td>-7 days</td>
<td>11/11 (100)</td>
<td>52.9±24.3</td>
</tr>
<tr>
<td>-6 days</td>
<td>11/11 (100)</td>
<td>70.0±29.9</td>
</tr>
<tr>
<td>-5 days</td>
<td>11/11 (100)</td>
<td>79.0±23.5</td>
</tr>
<tr>
<td>-4 days</td>
<td>6/11 (55)</td>
<td>81.2±33.4</td>
</tr>
<tr>
<td>-3 days</td>
<td>8/11 (73)</td>
<td>98.6±31.2</td>
</tr>
<tr>
<td>-2 days</td>
<td>2/11 (18)</td>
<td>67.5±10.6</td>
</tr>
<tr>
<td>-1 days</td>
<td>1/11 (9)</td>
<td>30.0</td>
</tr>
</tbody>
</table>

Data are mean±SD. * Mean volume of blood taken from those lambs that were venesected. P-values > 0.05 for all comparisons. Target haematocrits for PT50% and PT30% lambs were calculated from each lamb’s haematocrit on the day of birth. Lambs were venesected daily from birth to 146 days gestation or until target haematocrit was reached. Venesection was recommenced if haematocrit subsequently exceeded target prior to TEA.

6.4.3 Haematological and Biochemical Findings

Haematological and biochemical parameters at birth were similar in PC, PT50% and PT30% groups, except for blood glucose concentration, which was higher in PT30% lambs than in preterm controls (Table 6.2). Haematocrit decreased 52% in PT50% lambs and 70% in PT30% lambs by TEA, as intended. In PC lambs haematocrit decreased 23% to TEA without venesection (Figure 6.3A).

At TEA haemoglobin concentration and haematocrit were lowest in PT30% lambs and highest in TC lambs (Table 6.2). From TEA, haematocrit increased in PT50% and PT30% lambs to day 5 post term, remained static to day 14, then increased again to day 28; decreased in PC lambs a further 8% to 14 days post TEA then increased again; and decreased in TC lambs by 27%, to reach nadir at 12 weeks post TEA. Haematocrit was similar in all groups by 28 days post TEA. Thereafter, out to 12 months of age, PT30% lambs tended to have higher haematocrits than lambs in other groups, although this was statistically significant only at 5, 7 and 8 months post TEA (Figure 6.3B).
Haemoglobin concentration, total iron concentration and total iron binding capacity followed the same patterns as haematocrit, with similar values in all four groups achieved by 12 weeks post TEA (Figures 6.3C, 6.4A and 6.4B). However, these variables were not measured between TEA and 12 weeks post term, so it is possible that recovery occurred earlier, as it did for haematocrit. At 12 months post TEA, haemoglobin concentration was higher in PT50% and PT30% lambs than in preterm controls (Table 6.2 and Figure 6.3C).

Electrolyte concentrations were similar at all ages in all experimental groups except for sodium concentration, which was higher at TEA in TC lambs than in all preterm lamb groups (Table 6.2). Lactate concentration at TEA was highest in PT30% and TC lambs and lowest in PC lambs (Table 6.2 and Figure 6.4C).

6.4.4 Growth
Size at birth was similar in all preterm lamb groups (Table 6.3). However, at TEA PT50% and PC lambs were larger than PT30% lambs and all preterm lambs were larger than TC lambs (Table 6.3 and Figures 6.5A, 6.6A, 6.7A and 6.7B). PT30% and TC lambs grew more quickly than PT50% and PC lambs in the first few weeks post TEA, so that crown rump length and weight were similar in all lambs by 4 and 6 months post TEA respectively (Figures 6.5B and 6.6B). At 12 months post TEA, PC lambs were heavier than PT30% lambs, but growth parameters were otherwise similar in all groups (Table 6.3 and Figures 6.5A, 6.6A, 6.7A and 6.7B).
Table 6.2: Haematological and biochemical data at birth, term equivalent age and 12 months post term equivalent age in each experimental group

<table>
<thead>
<tr>
<th>Days Post Term Equivalent Age</th>
<th>Preterm Anaemic 50% (PT50%)</th>
<th>Preterm Anaemic 30% (PT30%)</th>
<th>Preterm Control (PC)</th>
<th>Term Control (TC)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-9 days (n)</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g.l⁻¹)</td>
<td>146.2±13.6</td>
<td>131.9±21.9</td>
<td>142.4±9.5</td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>47.3±5.9</td>
<td>43.3±7.2</td>
<td>45.9±5.6</td>
<td></td>
<td>0.35</td>
</tr>
<tr>
<td>WCC (x10⁹.l⁻¹)</td>
<td>3.5±1.0</td>
<td>3.2±1.0</td>
<td>3.6±1.2</td>
<td></td>
<td>0.64</td>
</tr>
<tr>
<td>Sodium (mmol.l⁻¹)</td>
<td>152.4±4.3</td>
<td>148.5±4.1</td>
<td>152.4±4.8</td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td>Potassium (mmol.l⁻¹)</td>
<td>4.5±0.4</td>
<td>4.6±0.6</td>
<td>4.4±0.6</td>
<td></td>
<td>0.69</td>
</tr>
<tr>
<td>Glucose (mmol.l⁻¹)</td>
<td>5.6±1.5</td>
<td>6.4±0.9</td>
<td>4.7±0.8</td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>Lactate (mmol.l⁻¹)</td>
<td>3.7±1.0</td>
<td>3.6±1.4</td>
<td>4.6±1.1</td>
<td></td>
<td>0.12</td>
</tr>
<tr>
<td>Thyroxine (µg.dl⁻¹)</td>
<td>18.2±1.8</td>
<td>16.9±2.9</td>
<td>17.3±2.4</td>
<td></td>
<td>0.47</td>
</tr>
<tr>
<td>0 days (n)</td>
<td>11</td>
<td>7</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g.l⁻¹)</td>
<td>66.3±12.5</td>
<td>29.7±8.7</td>
<td>103.7±10.9</td>
<td>140.4±10.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>21.9±3.5</td>
<td>12.4±2.5</td>
<td>33.0±2.3</td>
<td>45.5±3.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WCC (x10⁹.l⁻¹)</td>
<td>4.8±1.2</td>
<td>4.4±1.7</td>
<td>4.8±1.5</td>
<td>6.2±2.3</td>
<td>0.16</td>
</tr>
<tr>
<td>Sodium (mmol.l⁻¹)</td>
<td>146.8±1.9</td>
<td>148.5±7.1</td>
<td>146.0±11.7</td>
<td>150.8±3.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Potassium (mmol.l⁻¹)</td>
<td>4.4±0.2</td>
<td>4.1±0.5</td>
<td>4.5±0.4</td>
<td>4.3±0.5</td>
<td>0.12</td>
</tr>
<tr>
<td>Glucose (mmol.l⁻¹)</td>
<td>7.7±0.6</td>
<td>7.6±0.5</td>
<td>7.5±0.7</td>
<td>7.1±4.9</td>
<td>0.12</td>
</tr>
<tr>
<td>Lactate (mmol.l⁻¹)</td>
<td>1.7±0.3</td>
<td>4.9±3.1</td>
<td>1.3±0.1</td>
<td>4.2±0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Thyroxine (µg.dl⁻¹)</td>
<td>10.4±0.8</td>
<td>9.7±3.6</td>
<td>7.8±3.2</td>
<td>11.4±2.6</td>
<td>0.05</td>
</tr>
<tr>
<td>Total iron (µmol.l⁻¹)</td>
<td>8.9±4.5</td>
<td>5.0±2.2</td>
<td>18.9±5.3</td>
<td>39.8±16.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Iron saturation (%)</td>
<td>7.8±4.4</td>
<td>4.0±2.4</td>
<td>17.1±6.0</td>
<td>62.3±22.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TIBC (µmol.l⁻¹)</td>
<td>122.6±16.9</td>
<td>136.6±18.0</td>
<td>113.9±11.2</td>
<td>63.0±8.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>UIBC (µmol.l⁻¹)</td>
<td>113.7±19.2</td>
<td>131.6±19.9</td>
<td>95.1±15.0</td>
<td>23.2±13.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>365 days (n)</td>
<td>10</td>
<td>7</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Hb (g.l⁻¹)</td>
<td>115.9±6.8</td>
<td>115.1±5.6</td>
<td>105.5±8.0</td>
<td>112.3±7.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>35.1±2.2</td>
<td>35.6±2.6</td>
<td>33.3±2.6</td>
<td>34.3±2.3</td>
<td>0.19</td>
</tr>
<tr>
<td>WCC (x10⁹.l⁻¹)</td>
<td>9.1±2.6</td>
<td>9.1±2.2</td>
<td>9.0±3.1</td>
<td>8.8±1.7</td>
<td>0.99</td>
</tr>
<tr>
<td>Sodium (mmol.l⁻¹)</td>
<td>144.5±2.0</td>
<td>139.7±12.4</td>
<td>140.2±12.3</td>
<td>139.1±10.7</td>
<td>0.80</td>
</tr>
<tr>
<td>Potassium (mmol.l⁻¹)</td>
<td>4.3±0.6</td>
<td>4.0±0.6</td>
<td>4.5±0.7</td>
<td>4.3±0.6</td>
<td>0.45</td>
</tr>
<tr>
<td>Glucose (mmol.l⁻¹)</td>
<td>4.1±0.2</td>
<td>4.0±0.4</td>
<td>4.3±0.8</td>
<td>4.0±0.4</td>
<td>0.84</td>
</tr>
<tr>
<td>Lactate (mmol.l⁻¹)</td>
<td>1.2±0.3</td>
<td>1.5±0.5</td>
<td>1.5±0.9</td>
<td>1.3±0.5</td>
<td>0.78</td>
</tr>
<tr>
<td>Thyroxine (µg.dl⁻¹)</td>
<td>5.4±0.6</td>
<td>5.5±0.9</td>
<td>5.7±0.6</td>
<td>5.0±0.9</td>
<td>0.20</td>
</tr>
<tr>
<td>Total iron (µmol.l⁻¹)</td>
<td>22.6±3.7</td>
<td>26.1±5.5</td>
<td>25.7±3.6</td>
<td>22.2±3.4</td>
<td>0.17</td>
</tr>
<tr>
<td>Iron saturation (%)</td>
<td>38.5±6.3</td>
<td>43.4±7.1</td>
<td>37.8±8.1</td>
<td>37.7±5.2</td>
<td>0.30</td>
</tr>
<tr>
<td>TIBC (µmol.l⁻¹)</td>
<td>58.9±5.1</td>
<td>60.1±7.7</td>
<td>68.9±8.0</td>
<td>59.5±9.2</td>
<td>0.02</td>
</tr>
<tr>
<td>UIBC (µmol.l⁻¹)</td>
<td>36.1±5.1</td>
<td>34.0±6.1</td>
<td>43.1±9.6</td>
<td>37.4±7.7</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Data are n (%) or mean±SD. Hb=haemoglobin, Hct=haematocrit, WCC=white cell count, TIBC=total iron binding capacity, UIBC=unsaturated iron binding capacity. Symbols denote p≤0.05 for post-hoc comparison with: * preterm control group, † term control group, ‡ preterm anaemic 50% group, § preterm anaemic 30% group.
Table 6.3: Anthropometric data at birth, term equivalent age and 12 months post term equivalent age in each experimental group

<table>
<thead>
<tr>
<th>Days Post Term Equivalent Age</th>
<th>Preterm Anaemic 50% (PT50%)</th>
<th>Preterm Anaemic 30% (PT30%)</th>
<th>Preterm Control (PC)</th>
<th>Term Control (TC)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-9 days (n)</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td></td>
<td>0.31</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>4.4±0.5</td>
<td>4.2±0.6</td>
<td>4.1±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRL (cm)</td>
<td>45.4±3.9</td>
<td>45.4±1.9</td>
<td>46.1±3.2</td>
<td></td>
<td>0.49</td>
</tr>
<tr>
<td>Hind limb (cm)</td>
<td>36.9±1.3</td>
<td>35.9±2.0</td>
<td>35.9±1.2</td>
<td></td>
<td>0.22</td>
</tr>
<tr>
<td>Hock toe (cm)</td>
<td>19.7±0.8</td>
<td>19.0±1.1</td>
<td>19.1±0.9</td>
<td></td>
<td>0.21</td>
</tr>
<tr>
<td>Chest circ (cm)</td>
<td>35.4±1.3</td>
<td>34.7±2.0</td>
<td>34.8±1.1</td>
<td></td>
<td>0.51</td>
</tr>
<tr>
<td>Abdominal circ (cm)</td>
<td>35.6±1.4</td>
<td>36.2±1.8</td>
<td>34.3±2.2</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>BPD (cm)</td>
<td>6.1±0.2</td>
<td>6.0±0.2</td>
<td>6.1±0.1</td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td>0 days (n)</td>
<td>11</td>
<td>7</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>7.0±0.8†§</td>
<td>5.7±0.9†§</td>
<td>6.9±0.4†§</td>
<td>4.8±0.7†§</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GV weight -9-0 days (g.kg⁻¹.day⁻¹)</td>
<td>50.3±5.4*§</td>
<td>35.1±15.6*‡</td>
<td>57.0±3.5*§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRL (cm)</td>
<td>54.0±2.6†</td>
<td>51.8±2.2†</td>
<td>55.4±3.4†</td>
<td>47.9±3.4†§</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GV CRL -9-0 days (mm.cm⁻¹.day⁻¹)</td>
<td>0.19±0.09</td>
<td>0.15±0.05</td>
<td>0.20±0.10</td>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td>Hind limb (cm)</td>
<td>40.2±1.8†</td>
<td>38.1±2.6</td>
<td>40.2±1.8*</td>
<td>37.5±1.6†§</td>
<td>0.003</td>
</tr>
<tr>
<td>Hock toe (cm)</td>
<td>21.9±0.9†§</td>
<td>20.6±1.1†</td>
<td>21.8±0.7†</td>
<td>20.6±0.8†‡</td>
<td>0.001</td>
</tr>
<tr>
<td>Chest circ (cm)</td>
<td>42.5±1.9†§</td>
<td>39.9±1.9†</td>
<td>41.7±1.8†</td>
<td>38.0±1.7‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Abdominal circ (cm)</td>
<td>46.0±2.2†§</td>
<td>42.7±2.5†</td>
<td>45.6±1.4†</td>
<td>38.7±2.0‡§</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BPD (cm)</td>
<td>6.6±0.2†</td>
<td>6.4±0.2†</td>
<td>6.6±0.2†</td>
<td>6.3±0.2‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>365 days (n)</td>
<td>10</td>
<td>7</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>58.9±6.3</td>
<td>55.1±8.5§</td>
<td>63.2±3.6§</td>
<td>57.0±5.7</td>
<td>0.05</td>
</tr>
<tr>
<td>GV weight 294-365 days (g.kg⁻¹.day⁻¹)</td>
<td>0.8±1.2</td>
<td>1.5±0.7</td>
<td>0.7±0.6</td>
<td>0.8±0.9</td>
<td>0.16</td>
</tr>
<tr>
<td>CRL (cm)</td>
<td>106.7±2.8</td>
<td>103.9±3.5</td>
<td>107.0±2.6</td>
<td>104.1±4.4</td>
<td>0.11</td>
</tr>
<tr>
<td>GV CRL 294-365 days (mm.cm⁻¹.day⁻¹)</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.12</td>
</tr>
<tr>
<td>Hind limb (cm)</td>
<td>63.7±2.2</td>
<td>61.9±3.1</td>
<td>63.5±2.3</td>
<td>62.4±1.5</td>
<td>0.32</td>
</tr>
<tr>
<td>Hock toe (cm)</td>
<td>30.8±1.4</td>
<td>30.1±1.4</td>
<td>30.5±1.2</td>
<td>30.3±1.4</td>
<td>0.57</td>
</tr>
<tr>
<td>Chest circ (cm)</td>
<td>91.6±4.7</td>
<td>88.5±3.9</td>
<td>93.7±2.7</td>
<td>91.9±3.2</td>
<td>0.06</td>
</tr>
<tr>
<td>Abdominal circ (cm)</td>
<td>105.4±6.1</td>
<td>105.1±5.3</td>
<td>110.3±4.2</td>
<td>106.3±4.4</td>
<td>0.12</td>
</tr>
<tr>
<td>BPD (cm)</td>
<td>9.8±0.5</td>
<td>9.5±0.6</td>
<td>9.7±0.5</td>
<td>9.7±0.7</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Data are n (%) or mean±SD. GV=growth velocity, CRL=crown rump length, circ=circumference, BPD=biparietal diameter. Symbols denote p<0.05 for post-hoc comparison with: * preterm control group, † term control group, ‡ preterm anaemic 50% group, § preterm anaemic 30% group.
Figure 6.3: Haematocrit from birth to 146 days gestation in preterm groups (A), and from term equivalent age to 12 months in each experimental group (B); haemoglobin from birth to 12 months post term equivalent age in each experimental group (C).

Symbols denote p-value ≤ 0.05 on post-hoc analysis as follows:
Preterm control vs * Preterm anaemic 50%, † Preterm anaemic 30% and ‡ Term control;
Term control vs § Preterm anaemic 50% and ¶ Preterm anaemic 30%;
Preterm anaemic 50% vs II Preterm anaemic 30%
Figure 6.4: Total iron concentration (A), total iron binding capacity (B) and lactate concentration (C) from birth to 12 months post term equivalent age in each experimental group.

Symbols denote p-value ≤ 0.05 on post-hoc analysis as follows:
Preterm control vs * Preterm anaemic 50%, † Preterm anaemic 30% and ‡ Term control;
Term control vs § Preterm anaemic 50% and ¶ Preterm anaemic 30%;
Preterm anaemic 50% vs || Preterm anaemic 30%
Figure 6.5: Weight (A) and growth velocity for weight (B) from birth to 12 months post term equivalent age in each experimental group

Symbols denote p-value ≤ 0.05 on post-hoc analysis as follows:
Preterm control vs * Preterm anaemic 50%, † Preterm anaemic 30% and ‡ Term control;
Term control vs § Preterm anaemic 50% and ¶ Preterm anaemic 30%;
Preterm anaemic 50% vs # Preterm anaemic 30%
Figure 6.6: Crown rump length (A) and growth velocity for crown rump length (B) from birth to 12 months post term equivalent age in each experimental group.

Symbols denote p-value ≤ 0.05 on post-hoc analysis as follows:
Preterm control vs * Preterm anaemic 50%, † Preterm anaemic 30% and ‡ Term control;
Term control vs § Preterm anaemic 50% and ¶ Preterm anaemic 30%
Figure 6.7: Abdominal circumference (A) and biparietal diameter (B) from birth to 12 months post term equivalent age in each experimental group.

Symbols denote p-value ≤ 0.05 on post-hoc analysis as follows:
- Preterm control vs † Preterm anaemic 30% and ‡ Term control;
- Term control vs § Preterm anaemic 50% and ¶ Preterm anaemic 30%;
- Preterm anaemic 50% vs || Preterm anaemic 30%.
6.5 Discussion

This chapter provides the first description of survival, haematological and biochemical findings and growth of anaemic preterm lambs in comparison to preterm and term controls. Changes in haematological parameters in the immediate postnatal period have been documented in term born lambs, mice, rats, rabbits and pigs (Holz, Perry et al. 1961; Halvorsen and Halvorsen 1973; Loh 1975; Bassett, Borrett et al. 1995; Moritz, Owens et al. 1996; Dubuque, Dvorak et al. 2002; Halvorsen and Bechensteen 2002). In addition, the effect of induced anaemia on metabolic and cardiovascular function in the neonatal period has been investigated in term born lambs (Van Ameringen, Fouron et al. 1981; Holzman, Tabata et al. 1986; Bernstein, Teitel et al. 1988; Widness, Lowe et al. 2000), and erythropoiesis, iron homeostasis and growth pre- and post weaning have been investigated in iron-deficient rats (Dubuque, Dvorak et al. 2002; Hegde, Jensen et al. 2011). A mutant mouse model of neonatal anaemia also exists, but this has been used largely to investigate the structure and function of red blood cell membrane proteins (Siatacka, Sahr et al. 2010). Thus, to our knowledge, this is the first attempt to induce, and determine the effects of, anaemia in an experimental animal prior to term.

Our findings indicate that anaemia can be successfully induced in preterm lambs prior to TEA without compromising survival at 12 months. Of the 34 lambs delivered preterm, 27 (79%) survived to 12 months of age. This compares favourably with a survival rate of 60% reported recently in preterm lambs, although those animals were delivered slightly earlier than ours (0.9 versus 0.94 of term) (De Matteo, Blasch et al. 2010). However, three lambs in the most anaemic experimental group in our study died in the preterm period from complications of severe anaemia and our findings suggest that a haematocrit of 10% may be the lower limit for survival in preterm lambs. Anaemia of the severity induced in PT30% lambs is unusual in preterm babies, with the average decline in haematocrit prior to term more analogous to that experienced by PT50% lambs, none of whom had a target haematocrit below 15% (Strauss 2010). Therefore, in future studies we plan to use only one preterm anaemic experimental group with a target haematocrit 50% below baseline, and will ensure that animals are not venesected below a haematocrit of 15%, thus allowing induction of anaemia to a level in keeping with that experienced by preterm infants and likely reducing anaemia-related mortality.

This experiment was planned to ensure that enough blood was taken each day to allow target haematocrit to be achieved prior to TEA, whilst minimising the complications of
acute haemorrhage, such as tissue hypoxia or cardiovascular compromise. The volume of blood taken each day from those lambs venesected was similar in both groups, although PT30% lambs required an additional day of venesection to achieve target haematocrit. Using this method, we achieved a reduction in haematocrit and haemoglobin concentration prior to TEA very close to the planned target in both PT30% and PT50% lambs. Therefore, in future studies we plan to use the same approach to determine venesection volumes.

Knowledge of the spontaneous reduction in haematocrit in preterm lambs prior to TEA allowed appropriate haematocrit targets to be determined for preterm anaemic groups. Inclusion of a PT50% group was planned from the outset given that this is the typical reduction in haematocrit seen in preterm infants. In addition, the experiment was initially planned to include a preterm anaemic group with a target haematocrit of 70% of baseline (PT70%), in order to investigate the influence of mild preterm anaemia on the outcomes of interest. However, given that the spontaneous decline in haematocrit in preterm control lambs was close to 30%, it was apparent that a PT70% group would be redundant. Instead, a PT30% group was included, thus allowing investigation of the influence of anaemia of greater severity prior to term, in addition to determination of the lower limit of haematocrit for survival.

In humans born at term, physiologic anaemia occurs as expansion of plasma volume in the first few weeks after birth exceeds erythropoietic capacity, thus diluting red cell volume. Haematocrit declines as much as 30% from baseline by 6 to 12 weeks of age, at which stage relative tissue hypoxia stimulates increased production of erythropoietin, the primary regulator of erythropoiesis (Aher, Malwatkar et al. 2008). Similar patterns were seen in our term control sheep, in whom haematocrit declined by 27% to reach nadir at 12 weeks of age. By contrast, in infants born preterm, a number of additional factors contribute to postnatal anaemia, including iatrogenic phlebotomy loss, decreased red cell survival, and decreased erythropoietin secretion, in addition to inadequate stores and reduced postnatal supply of iron, folate and vitamin B12. Thus, haematocrit decline occurs earlier in preterm than in term born infants and is of greater magnitude, with reductions in haematocrit of up to 50% typically seen, especially in smaller and sicker babies in whom these mechanisms of increased red cell loss and impaired production are magnified (Widness 2008). However, in our preterm control lambs haematocrit declined by 23% prior to term equivalent age, reaching a nadir 39% less than baseline at 14 days post term. Thus, although the spontaneous reduction in haematocrit in preterm control lambs was larger and occurred
earlier than in term lambs, in keeping with patterns seen in preterm infants, the magnitude of haematocrit decline in preterm control lambs was less than that seen in infants born preterm. This is likely to be related to the lack of the previously noted factors that contribute to postnatal anaemia in preterm infants. In particular, unlike most preterm infants, phlebotomy losses in preterm control lambs were only 0.5 ml per day at most prior to TEA.

The changes in haematocrit observed in preterm anaemic and preterm control groups from TEA to 28 days post TEA provide insight into the mechanisms controlling erythropoiesis in the preterm lamb. In both PT50% and PT30% lambs haematocrit increased rapidly from TEA to 5 days post TEA, but in preterm control lambs haematocrit decreased over this time. As iron was administered to preterm anaemic lambs only, it is possible that iron deficiency may have prevented erythropoiesis in preterm control lambs at this time. Previous studies in anaemic iron deficient term born lambs have demonstrated that iron supplementation is required for erythropoiesis to occur (Guiang, Georgieff et al. 1997). However, as haematocrit recovered rapidly in preterm control lambs from 14 to 28 days post TEA in the absence of iron supplementation, it seems unlikely that iron deficiency impeded erythropoiesis in these animals in the first few days post term. Rather, the difference in haematocrit patterns found in preterm lambs in the immediate post term period is likely to be due to differences in erythropoietin concentration between preterm lamb groups. Erythropoietin is produced in response to tissue hypoxia, which is in turn related to haematocrit (Aher, Malwatkar et al. 2008). Since haematocrit was considerably lower in preterm anaemic lambs than preterm controls at TEA, increased erythropoietin concentration would be expected in these animals. Although not measured in our lambs, increased erythropoietin concentration has been demonstrated in late gestation fetal sheep subjected to moderately severe intrauterine haemorrhage of a similar magnitude to that experienced by our preterm anaemic lambs at the same gestational age. However, those authors reported no increase in erythropoietin secretion in preterm lambs subjected to mild anaemia of a similar magnitude to that experienced by our preterm control lambs (Moritz, Cooper et al. 1992). Thus, it appears that anaemia must be of at least moderate severity to stimulate erythropoiesis in preterm lambs, suggesting that the set point for the detection of tissue hypoxia by erythropoietin secreting cells in these animals is low at this age. In preterm infants the main site of erythropoietin synthesis is the liver, which has a lower set point for detection of tissue hypoxia than the kidney, the site of erythropoietin production post TEA. Thus, preterm infants also require a lower haematocrit to stimulate erythropoiesis than infants born at term (Widness 2008; Strauss 2010).
From 5 to 14 days post TEA, haematocrit changed very little in preterm anaemic lambs, whereas haematocrit continued to decrease in preterm control lambs over this time, reaching nadir at 14 days post term. Thereafter haematocrit increased steadily in all preterm lambs, and by 28 days post TEA there were no significant differences in haematocrit between groups. These findings indicate that the initial haematological recovery seen in preterm anaemic lambs was not sustained. This may be because haematocrit increased sufficiently in these animals in the first 5 days post TEA so that tissue oxygenation increased above the set point for stimulation of erythropoietin secretion. Alternatively, the rate of growth related plasma volume expansion may have exceeded the rate of erythropoiesis in preterm anaemia lambs, resulting in static haematological parameters at this age. However, the steady increase in haematocrit from 14 to 28 days post term in all preterm groups suggests that there may have been an increase in the tissue oxygenation set point for erythropoietin secretion in preterm lambs at this time. Although we are aware of no other studies of haematological recovery in experimental animals following preterm anaemia, haematological recovery in preterm infants is slower than the time frame demonstrated in our preterm lambs, despite iron supplementation, thus the switch from hepatic to renal erythropoietin production appears to occur later in humans born preterm (Long, Yi et al. 2012; Mills and Davies 2012). It is postulated that this delay is due primarily to suppression of erythropoietin secretion secondary to repeated red blood cell transfusions throughout the neonatal course of the preterm infant (Freise, Widness et al. 2010).

Changes in haemoglobin concentration in preterm anaemic lambs followed similar patterns to those observed for haematocrit, with one notable exception: at 12 months post TEA, haemoglobin concentration was higher in PT30% and PT50% lambs than in preterm controls. This finding is of particular interest given that we also found higher haemoglobin concentration and haematocrit in adult survivors of fetal anaemia than in their unaffected siblings. The similarity of these findings in sheep and humans suggests they are less likely to be type 1 statistical errors. In humans we have found that intrauterine anaemia is also associated with decreased left ventricular volumes and myocardial blood flow at rest and with cold pressor stress. Thus it is possible that increased haemoglobin concentration may represent an adaptive response to improve oxygen carrying capacity in the face of reduced cardiac output. Although we did not investigate cardiovascular outcome in our preterm anaemic sheep, the similarity of haematological findings in our human and sheep studies suggests that exposure to anaemia prior to TEA, whether in or ex utero, may have similar long-term consequences.
Lactate concentration in PT30% lambs was substantially higher than in PT50% or preterm control lambs at TEA, with values similar to those reported from term born lambs in whom profound anaemia was induced by serial phlebotomy from four to six days of age (Widness, Lowe et al. 2000). Increases in lactate concentration of this magnitude have also been documented in dogs when oxygen delivery is decreased below the critical point at which oxygen consumption outstrips supply, resulting in anaerobic metabolism (Cilley, Scharenberg et al. 1991). Thus, it is likely that the substantial increase in lactate concentration we observed in PT30% lambs at TEA is a marker of tissue hypoxia, suggesting that the severity of anaemia to which these animals were subjected was sufficient to reduce oxygen delivery below the so-called “anaerobic threshold” at that time. By contrast, lactate concentrations in PT50% lambs at TEA were significantly less than that of PT30% lambs, with values similar to those reported from a number of studies of pretransfusion lactate concentrations in anaemic preterm infants (Ross, Christensen et al. 1989; Christensen, Hunter et al. 1992; Izraeli, Ben-Sira et al. 1993; Bard, Fouron et al. 1998). As the mean lactate concentrations of anaemic preterm infants reported in those studies were less than the upper limit of normal for lactate, it is unlikely that the magnitude of anaemia to which these infants were exposed prior to transfusion resulted in tissue hypoxia (Widness, Lowe et al. 2000). Thus, it is also unlikely that tissue oxygenation was impaired in PT50% lambs at TEA. Lactate concentration was not measured again until 3 months of age, at which stage it had decreased substantially in PT30% lambs, with no differences found between any of the four lambs groups. Therefore, although it is not possible to discern how rapidly lactate normalised, there was no evidence of ongoing impairment of tissue oxygenation in PT30% lambs beyond this time.

Although preterm lambs were around 600 grams lighter at birth than term lambs, by TEA all growth parameters of preterm control and PT50% lambs were greater than those of term control lambs. These findings are similar to those reported in preterm sheep by De Matteo and colleagues (De Matteo, Blasch et al. 2010). These authors hypothesised that feeding preterm sheep expressed milk or formula at regular intervals for the first few days after birth until adequate independent feeding was established resulted in initial over feeding and thus provided these animals with an ex utero growth advantage. In De Matteo’s study, preterm lambs were fed 4 to 6 hourly, with intakes of 80 ml.kg\(^{-1}\) from 0 to 24 hours, 100 ml.kg\(^{-1}\) from 24 to 48 hours and 120 ml.kg\(^{-1}\) from 48 to 72 hours. As a similar approach to feeding was taken with our preterm lambs, this may also account for the differences in size found between preterm control, PT50% and term control lambs at TEA. However, this feeding
regime did not extend a growth advantage to PT30% lambs, with only two growth parameters significantly greater than those of term control lambs at TEA. This suggests that the metabolic and cardiovascular adaptations required to compensate for severe anaemia and probable tissue hypoxia in PT30% lambs may have increased energy expenditure and compromised growth in these animals prior to TEA.

The growth patterns of PT50% and PT30% lambs suggest that the severity of anaemia to which lambs were exposed prior to term was more important than preterm birth for the determination of body size in the 12 months after birth. For example, at TEA, PT30% lambs had lower weight, growth velocity for weight and abdominal circumference than PT50% and preterm control groups, and PT30% lambs tended to remain smaller thereafter, being still lighter than preterm controls at 12 months. By comparison, PT50% lambs showed no evidence of growth impairment, and followed similar growth trajectories to preterm and term controls throughout the study period. These findings suggest that severe, but not moderate, anaemia prior to term, has long-term effects on growth, even if the anaemia resolves soon after TEA.

Two potential mechanisms may mediate the effect of severe anaemia on long-term growth observed in our study. Firstly, it is possible that tissue hypoxia in PT30% lambs prior to term may have predisposed to long-term growth impairment. The role of intrauterine hypoxia in impairment of fetal growth is well established in animals and humans (Chang, Rutledge et al. 1984; de Grauw, Myers et al. 1986; Owens, Falconer et al. 1987; Giussani, Phillips et al. 2001; Moore, Shriver et al. 2004; Barry, Rozance et al. 2008). There is also evidence that long-term growth outcomes may be impaired by exposure to severe intrauterine or postnatal hypoxia. For example, severe intrauterine hypoxia in mice is associated with reduced body weight and brain size in adulthood (Meberg 1981). In addition, exposure of newborn mice to constant postnatal hypoxia for four weeks leads to reduced body and liver weight, the latter due mainly to a reduction in hepatocyte number (Farahani, Kanaan et al. 2008). Interestingly, mice exposed to intermittent, rather than constant, hypoxia also had reduced body weight but demonstrated rapid catch up growth which ameliorated growth failure, suggesting that growth outcomes were graded according to the duration of hypoxia exposure. Similarly, varying degrees of postnatal hypoxia in rats leads to growth impairment only in those animals exposed to severely hypoxic conditions (Mortola, Xu et al. 1990). Furthermore, in humans, reduced height has been documented in children and adolescents of similar socioeconomic status residing at high altitude compared
to low altitude controls, with stature shortest in those with the longest period of residence at high altitude (Stinson 1982; Greksa, Spielvogel et al. 1985).

It has been postulated that intrauterine or postnatal hypoxia may impair long-term growth by suppression of protein and DNA synthesis, resulting in changes in cell number, size and function, thus inducing long-term alterations in cellular proliferation and apoptotic remodelling (Fowden, Giussani et al. 2006). Protein synthesis may be impaired due to increased catecholamine release, known to occur as a result of hypoxia (Milley 1997). Alternatively, impairment of growth following intrauterine hypoxia may occur secondary to increased adrenocorticotropic hormone and cortisol production (Allen 2001). Finally, hypoxia may induce activation of a proinflammatory cascade resulting in an attenuated ventilatory response to subsequent hypoxic episodes, which may in turn propagate a vicious cycle of persistent hypoxia (Martin, Wang et al. 2011). Thus, it is plausible that the degree of anaemia experienced by PT30% lambs, but not PT50% lambs, may have resulted in tissue hypoxia of sufficient severity to induce long-term growth impairment.

Secondly, it is possible that iron deficiency may be responsible for impaired growth outcomes following severe preterm anaemia. In rats, inadequate maternal iron intake resulting in fetal iron deficiency and anaemia is associated with reduced birth weight in pups (Crowe, Dandekar et al. 1995; Gambling, Charania et al. 2002). Similarly, in humans, maternal iron deficiency anaemia is associated with reduced birth weight, although it is not clear to what extent preterm birth, also associated with maternal iron deficiency, contributes to this outcome (Brabin, Ginny et al. 1990; Scholl, Hediger et al. 1992; Allen 2001). Several biological mechanisms have been proposed to account for these observations. For example, increased catecholamine production in response to iron deficiency may result in impaired protein synthesis, in a similar manner to that described following intrauterine hypoxia (Dallman 1986). In addition, iron deficiency may increase the susceptibility of red blood cells to oxidative damage, in turn causing further haematological compromise (Allen 2001). However, evidence of long-term growth impairment following intrauterine or early postnatal iron deficiency is sparse, with only one study in rats demonstrating an association between perinatal iron deficiency and reduced weight in adulthood (Hegde, Jensen et al. 2011). Furthermore, no animal studies have investigated the influence of iron deficiency following preterm birth, and there is no evidence to suggest that the use of iron supplementation results in improved long-term growth outcomes in preterm infants (Mills and Davies 2012). Thus, while iron deficiency may account, at least in part, for the
discrepancy in growth parameters between PT30%, PT50% and PC lambs at TEA, there is little evidence to suggest that iron deficiency influenced long-term growth outcomes.

In anaemic preterm infants, improvements in short-term weight gain have been documented following transfusion (Stockman and Clark 1984), but at 12 months of age growth parameters in preterm infants treated with liberal transfusion policies are similar to those treated with restrictive transfusion policies (Whyte and Kirpalani 2011). Thus it seems the degree of anaemia usually experienced by preterm infants is not severe enough to induce long-term growth impairment. This is consistent with our finding that long-term growth impairment did not occur in PT50% lambs, in whom anaemia severity was similar to that usually experienced by preterm infants.

There are some potential limitations to the findings reported in this chapter. Firstly, our lambs were born at a later gestation than are many preterm infants, as earlier delivery would have resulted in unacceptable animal losses, primarily from respiratory insufficiency. Secondly, as this was a pilot study with the primary aim of determining appropriate experimental technique for a larger study, the number of animals included in each experimental group was limited. This may have reduced our ability to detect small differences between groups, particularly with regard to growth and haematological parameters at 12 months. Including more animals in this study was not practical due to restrictions of time, staffing and space. However, further work investigating similar outcomes in preterm anaemic sheep is currently being undertaken by our research group. These findings will be reported together with the findings of this pilot study, which will result in a doubling of the number of animals in each group.

Finally, it is possible that some of the significant findings reported in this chapter may be explained by chance due to multiple comparisons in analyses. In total, 196 comparisons were made in this chapter. With statistical significance defined as a p-value of less than or equal to 0.05, approximately 10 comparisons would be expected to be statistically significant by chance alone (type 1 errors). However, we found 81 statistically significant associations, and the majority of p-values are small, so it is unlikely that this is a major source of error.
6.6 Summary

This is the first report of induction of anaemia in experimental animals prior to TEA, and its long-term consequences. Our findings indicate that anaemia can be successfully induced in preterm lambs delivered at a gestational age equivalent to late preterm birth in humans, although survival of lambs was compromised below a haematocrit of approximately 10%. At 12 months of age only the most severely anaemic lambs demonstrated evidence of growth impairment.

The management of anaemia in preterm infants remains an area of controversy, with evidence to support both restrictive and liberal transfusion thresholds. Use of the experimental approach described in this chapter will allow specific investigation of both short and long-term outcomes in animals exposed to preterm anaemia, in particular growth and cardiovascular status. Further experiments utilising the described techniques for induction of anaemia following preterm birth are underway to investigate these outcomes more thoroughly, and these findings may have implications for the determination of appropriate transfusion thresholds for the preterm infant.
Chapter 7: Preterm Anaemia Pilot Study: Cardiovascular Outcome Assessments

7.1 Summary of Chapter Contents
This chapter describes the process of optimising the method for assessment of cardiovascular outcome in adult sheep, including measurement of coronary blood flow. The final protocol for these experiments is described in Chapter 2, section 2.2.3 Cardiovascular Assessments.

7.2 Introduction
The role of fetal anaemia in the programming of cardiovascular status in later life is supported by evidence from animal studies, with increased coronary conductance in adulthood and altered responses to hypoxic stress reported in adult sheep after exposure to intrauterine anaemia suggestive of extensive remodelling of the coronary tree in these animals (Broberg, Giraud et al. 2003; Davis, Roullet et al. 2003; Yang, Hohimer et al. 2008). Our findings from the Fetal Anaemia Study suggest that cardiovascular changes may also occur in humans exposed to anaemia prior to birth. However, the incidence of rhesus disease, the main cause of human fetal anaemia, has decreased dramatically in the last three to four decades following the introduction of anti-D prophylaxis (Pattison, Roberts et al. 1992). Therefore, although fetal anaemia still occurs as a consequence of either immune or nonimmune haematological processes, substantially fewer fetuses now develop anaemia severe enough to require treatment with IUT.

By contrast, the incidence of preterm birth has increased markedly in recent decades, with over 12% of all infants in the United States born preterm in 2008 (Hamilton, Martin et al. 2010). Furthermore, advances in neonatal care have led to an increasing number of survivors of preterm birth. Preterm birth exposes the morphologically immature heart to the demands of life *ex utero*, including the haemodynamic shift from fetal to postnatal circulation. In sheep, myocardial remodelling and changes in cardiomyocyte maturation have been demonstrated following preterm birth (Bensley, Stacy et al. 2010). Preterm birth in humans is associated with hypertension, obesity and impaired glucose tolerance in adulthood; associations that may represent significant risk for cardiovascular morbidity and mortality (Dalziel, Parag et al. 2007; de Jong, Monuteaux et al. 2012). Therefore, the time between birth and term equivalent age may be a critical period for the cardiovascular development of infants born preterm. Understanding the mechanisms underlying
cardiovascular development in these infants may help to develop strategies to minimise later cardiovascular disease.

Anaemia in the preterm period may be one such mechanism. Most preterm infants become anaemic in the first few weeks after birth, at a similar gestational age to that of fetuses who are anaemic in utero due to rhesus disease (Strauss 2010). There is considerable controversy regarding transfusion thresholds for preterm infants, with restrictive transfusion policies resulting in “permissive” anaemia frequently adopted (Bishara and Ohls 2009; Whyte and Kirpalani 2011). Therefore, investigation of the influence of anaemia in the preterm period on cardiovascular outcome in adulthood could help inform decisions regarding transfusion thresholds in preterm infants to optimise long-term health.

Having successfully generated a cohort of preterm lambs in whom anaemia was induced prior to term equivalent age, as described in the previous chapter, we next set out to pilot a protocol for the investigation of cardiovascular outcomes in adult sheep, particularly measurement of coronary arterial blood flow at rest and during hyperaemic stress induced by adenosine infusion. These investigations have been undertaken previously in sheep exposed to fetal anaemia by our collaborators in the United States, using ultrasonic flow probes applied directly to coronary vessels (Davis, Roullet et al. 2003). However, such experiments require specialised equipment, appropriate facilities and a considerable degree of surgical expertise. Furthermore, there are a number of approaches that can be taken to the complex procedures involved. Thus, our aim was to develop, practise and optimise the experimental approach required to obtain robust, reliable and reproducible data for the assessment of cardiovascular outcome in adult sheep following preterm anaemia.

### 7.3 Chapter Aims

1. To develop a practical and reliable method for assessment of cardiovascular outcomes in adult sheep, including specifically:
   1. Surgical approach;
   2. Arterial and venous line requirements;
   3. Assessment of left ventricular dimensions and function using echocardiography;
   4. Maintenance of stable intraoperative blood pressure;
   5. Manipulation of blood pressure during measurement of coronary flow;
   6. Measurement of coronary flow at rest and during adenosine infusion;
vii. Quantification of myocardial tissue mass supplied by coronary artery from which flow measurements were taken.

2. To calculate coronary conductance and myocardial perfusion reserve at rest and during adenosine infusion in these animals.

7.4 Development of Experimental Method

7.4.1 Animal Numbers, Experiment Duration and Personnel Involved

These experiments were undertaken on 5 sheep from November 2011 to April 2012. (Refer Chapter 2 section 2.2.3.1 Animal selection and care prior to experiments).

My role included research and development of the methodology and coordination of equipment, personnel and animal transportation for each experiment. During the experiments, I was responsible for measurements of coronary flow and pressure, and formulation and delivery of the adenosine infusion. Following each experiment, I calculated coronary conductance and myocardial perfusion reserve, reported these findings to the rest of the group, and recommended protocol modifications. Assistance for other aspects of the experiments was obtained from our colleagues at the Department of Physiology, University of Auckland. Assoc Prof Ian LeGrice and Dr Nigel Lever performed the surgical procedures, with advice as required from Prof Bruce Smaill. Induction and maintenance of anaesthesia was performed by Mrs Linley Nisbet and intraoperative monitoring was undertaken by Dr Greg Sands. (For anaesthetic and monitoring equipment details, refer Chapter 2 section 2.2.3.2 Surgical procedure). Our collaborator in the United States, Prof Kent Thornburg, whose laboratory had performed similar experiments in sheep, was present to assist and advise on methodology for experiments 4 and 5.

7.4.2 Surgical Approach

Midline sternotomy was used to access the thoracic cavity in the first two experiments as this was the approach with which the surgeons were most familiar. However, this proved to be time consuming due to the thickness of the sternum and bleeding from the sternal wound. In addition, significant hypotension resulted when the thoracic cavity was opened with the animal lying supine, and it was difficult to dissect out the circumflex artery for placement of a flow probe using this approach. Thus, for experiments 3, 4 and 5, the surgical approach was changed to left thoracotomy through the third intercostal space, with the animal lying in the right lateral position. This improved intraoperative blood pressure stability and allowed the circumflex artery to be accessed for measurement of coronary flow. Although this
approach was less familiar to the surgical team, it proved to be faster and resulted in less blood loss than midline sternotomy.

7.4.3 Vascular Access, Monitoring and Drug Delivery

7.4.3.1 Pressure monitoring
Continuous monitoring of aortic pressure was achieved in all experiments by placement of a pigtail catheter in the right internal carotid artery. In experiments 1 and 2, LV pressure was monitored intermittently by advancing this catheter into the left ventricle. However, for experiments 3, 4 and 5 a separate catheter was inserted into the left carotid artery to allow continuous LV pressure monitoring. A catheter was also placed in the right internal jugular vein in all experiments to allow continuous monitoring of right atrial pressure. Correct positioning of these catheters was confirmed by transduction of pressure during catheter placement.

7.4.3.2 Cardiac output monitoring
In experiments 1 and 2, a Swan-Ganz catheter was placed in the proximal pulmonary artery via the right internal jugular vein to allow assessment of cardiac output by bolus thermodilution. However, placement of this catheter was difficult due to the need to traverse the right atrium and ventricle, and bolus thermodilution proved too slow for use during coronary flow assessment. As no equipment was available to allow assessment of cardiac output by a more practical technique, this was omitted from subsequent experiments.

7.4.3.3 Venous access and drug delivery
Venous access was obtained in experiment 1 via a catheter placed in the left internal jugular vein. In order to decrease the number of vascular access devices in the animal’s neck, in experiments 2 and 3 this catheter was instead placed in the right femoral vein. However, this location was not easily accessible following the change in surgical approach for experiments 3 to 5. Therefore, venous access was obtained through the left internal jugular vein for the last 2 experiments.

The venous access catheter was used for administration of intraoperative fluids and drugs. For experiments 1 to 4, the adenosine infusion was also administered through this catheter. However, given the short half-life of adenosine, it is important that this drug is delivered as close to the heart as possible in order to obtain maximal pharmacological effect. Therefore, in experiment 5, adenosine was administered via a catheter placed directly into the left atrium.
7.4.4 Left Ventricular Assessment
In experiments 2 to 4, LV end diastolic diameter (EDD), end systolic diameter (ESD), ejection fraction (EF) and fractional shortening (FS) were assessed by placement of an ultrasound probe directly onto the LV wall once the chest cavity was open, before assessment of coronary flow. At the outset of these experiments, only Dr Lever had the expertise required to perform this assessment, thus Dr Sands was also trained to do this. We initially planned to perform these measurements both at rest and with stress. However, the time required to obtain these data was too long to allow measurement during adenosine infusion. Therefore, in all experiments LV assessment was undertaken only at rest.

7.4.5 Blood Pressure Control
7.4.5.1 Intraoperative stability
In experiments 1 and 2, mean aortic pressure decreased by as much as 29 mmHg following opening of the chest despite administration of normal saline fluid boluses, resulting in pressures that were markedly less than the target range of 80 to 90 mmHg used previously by our colleagues in the United States in similar experiments (Figure 7.1). In experiments 3, 4 and 5, the change in surgical approach and a more aggressive intraoperative fluid management policy using synthetic colloid resulted in improved blood pressure stability (Figures 7.2 and 7.3).

7.4.5.2 Blood pressure manipulation for coronary blood flow measurements
In order to calculate coronary conductance, manipulation of mean aortic blood pressure was required to allow measurement of coronary blood flow at a range of pressures above and below baseline. In experiments 1 and 2, blood pressure was manipulated by use of cotton ties placed around the aorta and inferior vena cava (IVC), with extrinsic constriction of these vessels resulting respectively in an increase or decrease in mean aortic pressure (Figure 7.1). However, small changes in blood pressure were difficult to achieve with this technique. Furthermore, the change from midline sternotomy to left thoracotomy for experiment 3 impeded access to the IVC, making it difficult to place the tie around this vessel. Instead, intrinsic occlusion of the IVC with balloon and urinary catheters inserted via the left femoral vein was attempted. This was unsuccessful as the balloons on these devices were not large enough to occlude the IVC adequately. Decremental blood pressure reduction was eventually achieved in experiment 3 by extrinsic constriction of the superior vena cava (SVC), although the blood pressure reduction possible with this technique was less than with the use of IVC constriction in prior experiments (Figure 7.2). In experiment 4,
a larger balloon catheter was inserted via the right femoral vein for a further attempt at intrinsic IVC occlusion, although this was again unsuccessful. Therefore, further effort was made to access the IVC and a tie was successfully placed around this vessel allowing application of extrinsic IVC constriction in experiments 4 and 5. This resulted in improved blood pressure reduction, with a lower limit of mean aortic blood pressure of 30 mmHg in experiment 5 (Figure 7.3). However, fine control of blood pressure manipulation remained difficult and Prof Thornburg recommended use of specific vascular occluders with inflatable cuffs (In Vivo Metric, California, USA) for future experiments.
Figure 7.1: Changes in aortic pressure, right atrial pressure, left ventricular pressure, mean aortic pressure and mean coronary blood flow in response to adenosine infusion, experiment 2

Note baseline mean aortic pressure of 50 mmHg prior to commencement of adenosine infusion at 22:45 (marker 11). Marker 12 indicates first coronary blood flow recording at 25:30 and markers 13 to 19 indicate time of subsequent recordings. Aortic pressure increases at approximately 26:00 (marker 13) in response to aortic constriction, and decreases at approximately 26:40 (marker 14) in response to IVC constriction, resulting respectively in an increase and decrease in mean coronary blood flow. Total duration of adenosine infusion for this experiment was 8 minutes and 30 seconds.
Figure 7.2: Changes in aortic pressure, right atrial pressure, left ventricular pressure, mean aortic pressure and mean coronary blood flow in response to adenosine infusion, experiment 3

Note baseline mean aortic pressure of 75 mmHg prior to commencement of adenosine infusion at 10:45 (marker 4). Increased aortic pressure in response to aortic constriction with a concomitant increase in mean coronary blood flow can be seen from 12:45. Decreased aortic pressure was induced by SVC constriction in this experiment, with lowest pressure achieved approximately 50 mmHg. Note upper limit of left ventricular pressure monitoring (150 mm Hg) was exceeded between 12:50 and 14:40. Total duration of adenosine infusion for this experiment was 6 minutes and 50 seconds.
Figure 7.3: Changes in aortic pressure, right atrial pressure, left ventricular pressure, mean aortic pressure and mean coronary blood flow in response to adenosine infusion, experiment 5

Note baseline mean aortic pressure of 85 mmHg prior to commencement of adenosine infusion at 40:35 (marker 7). Increases in aortic pressure and coronary blood flow in response to aortic constriction can be seen from 42:45 and decreases in aortic pressure and coronary blood flow in response to IVC constriction can be seen from 00:15. Brief interruption to monitoring at 45:10 resulted in recommencement of timing. Note upper limit of left ventricular pressure monitoring (150 mm Hg) was exceeded between 44:30 and 00:10. Total duration of adenosine infusion for this experiment was 8 minutes.
7.4.6 Measurement of Coronary Blood Flow

7.4.6.1 Flow probe position
Coronary blood flow was measured from the diagonal branch of the left anterior descending artery in experiments 1 and 2, as this vessel was most easily accessible via midline sternotomy. However, the mass of myocardial tissue supplied by this vessel was small, and quantification of myocardial tissue mass was difficult (see section 7.4.7 below), thus increasing the potential for error in measurement of coronary blood flow. Fortunately, with the change in surgical approach in experiment 3, the circumflex artery was accessible for flow probe placement. It was also easier to dissect this much larger vessel away from the pericardial tissue, reducing the chance for surgical disruption of the vessel wall. Although placement of the coronary flow probe remained a delicate procedure, the time taken to achieve this reduced from 45 minutes in experiment 1 to 15 minutes in experiment 5. Once in place, care was taken to ensure that the position of the flow probe remained unchanged for measurement of coronary blood flow at rest and with adenosine infusion.

7.4.6.2 Assessment of coronary blood flow at rest and with adenosine-induced stress
Prior to undertaking these experiments, we were uncertain how long it would take for coronary blood flow to stabilise following blood pressure manipulation. In addition, in experiments 1 and 2, we found it difficult to maintain blood pressure stability following aortic or IVC constriction and in these experiments each coronary blood flow measurement took 30 to 50 seconds. However, as the length of time available for collection of coronary blood flow data with stress was limited by the duration of the adenosine infusion, this time frame needed to be shortened if data collection was to be maximised. Review of the blood pressure and coronary blood flow data recorded during experiments 1 and 2 revealed that coronary flow stabilised rapidly once blood pressure was stable, with accurate measurement possible within seconds. Thus, in subsequent experiments we attempted to shorten the time taken to achieve blood pressure stability following IVC or aortic manipulation and thus acquire coronary blood flow measurements more rapidly, particularly during adenosine infusion. This was successfully achieved, with twice as many mean aortic pressure and coronary blood flow data pairs acquired per minute of adenosine infusion in experiments 4 and 5 than in experiments 1 and 2.

7.4.6.3 Changes to adenosine infusion
For all experiments, adenosine was delivered at a dose of 0.14 mg.kg\(^{-1}\).min\(^{-1}\), in keeping with the dose utilised in the Fetal Anaemia Study. The maximum volume of the adenosine infusion was 50 ml, predetermined by the maximum syringe size the infusion pump was
capable of accepting. In experiment 1, the infusion was formulated using the standardised adenosine dose chart developed for the Fetal Anaemia Study, administered at a rate of 10 ml.min$^{-1}$, resulting in an infusion time of 5 minutes. However, as it took 2 to 3 minutes to achieve maximal vasodilation and cardiovascular stability following commencement of the infusion, this left inadequate time for manipulation of blood pressure and collection of pressure and flow data. Therefore, in experiment 2 the concentration of the infusion was increased and administration rate decreased to provide an infusion time of 8 minutes 30 seconds (Figure 7.1). Although this provided more time for collection of pressure and flow data, 70% more adenosine was required to achieve this formulation, resulting in a marked increase in drug costs. Therefore, in experiment 3 the concentration and administration rate were again altered to provide an infusion time of 6 minutes 50 seconds (Figure 7.2). However, following this experiment it was clear that an infusion time of at least 8 minutes was required in order to obtain adequate pressure and flow data during adenosine-induced stress. Therefore, a new standardised adenosine dose chart with an infusion time of 8 minutes was developed and this was used successfully in experiments 4 and 5.

7.4.7 Quantification of Myocardial Mass
Quantification of the mass of myocardial tissue supplied by the coronary artery from which flow was measured was necessary in order to calculate coronary blood flow per gram of myocardial tissue. In all experiments, following post mortem excision of the heart, a cannula was placed in the artery at the position at which flow was measured. The artery was tied off immediately proximal to this point and perfused with blue ink to mark the LV territory supplied by this vessel. The area of left ventricle marked by ink was then dissected away from the rest of the heart and the resulting tissue weighed. However, extravasation of ink onto the surface of the ventricle made it difficult to precisely define the outer margin of ventricular muscle supplied by the perfused vessel, which may have caused overestimation of myocardial tissue mass. In addition, concern was raised that blood may clot in the smaller arterial branches before these vessels could be perfused with ink, leading to underestimation of the myocardial tissue mass. Therefore, in experiment 5, the animal was heparinised prior to euthanasia and the heart was perfused with Krebs solution as soon as possible post mortem in an effort to clear the circulation of blood, prior to perfusion with ink. The heart was then left for 30 minutes before dissection, to allow the ink to completely perfuse into the distal vessels. The resulting myocardial mass measured was much larger than that of experiments 3 and 4, and the possibility that myocardial mass had been overestimated in this experiment was considered, especially given that this animal was the smallest of the
five. Assoc Prof LeGrice and Dr Sands planned to undertake further investigation of alternative techniques to improve the accuracy of this aspect of the experiment.

Total heart mass was not measured in these experiments, thus the proportion of myocardium supplied by the artery from which flow was measured could not be calculated. This was added to the experimental protocol following experiment 5. However, the ratio of the mass of myocardial tissue supplied by the artery from which flow was measured to total body weight was calculated for all experiments.

7.5 Results

7.5.1 Baseline Parameters
Weight ranged from 52.1 kg for ewe 5 to 79.3 kg for ewe 2, and body surface area from $1.19 \text{ m}^2$ to $1.57 \text{ m}^2$ in the same animals (Table 7.1). Haematocrit was available for ewes 1, 2 and 5 and ranged from 18 to 25%. Mean aortic pressure at baseline was less than the target range of 80 to 90 mmHg in ewes 1, 2 and 3 but within the target range in ewes 4 and 5. In ewe 1, baseline heart rate was 118 beats per minute, compared to 73 to 88 beats per minute in ewes 2 to 5.

7.5.2 Intraoperative Management
Procedure duration decreased from 440 minutes for experiment 1 to 230 minutes for experiment 5 (Table 7.1). In experiments 1 and 2, a total of $4.1 \text{ ml.kg}^{-1}.\text{h}^{-1}$ of fluid was administered during the procedure, including maintenance and bolus fluids. With adoption of a more aggressive fluid management policy, decreased procedure duration and lighter animals, the amount of fluid administered in subsequent experiments increased steadily to a maximum of $10.0 \text{ ml.kg}^{-1}.\text{h}^{-1}$ in experiment 5.

Mean respiratory rate was similar in all animals (Table 7.1). However, mean tidal volume ranged from 267 ml for ewe 5 to 531 ml for ewe 1, and consequently, minute ventilation ranged from 4.0 to 8.5 l.min$^{-1}$ in the same animals. Mean end tidal carbon dioxide and inspired isoflurane concentrations were similar in all sheep.
7.5.3 Left Ventricular Dimensions and Function
Echocardiographic assessment of LV dimensions and function was undertaken for sheep 2 to 5 (Table 7.1). EDD and ESD were indexed to body surface area to normalise for body size.

7.5.4 Cardiovascular Parameters Prior to Coronary Flow Assessments
In experiments 1 and 2 mean aortic pressures decreased from baseline values of 78 mmHg and 72 mmHg to 58 mmHg and 45 mmHg prior to assessment of coronary blood flow at rest, with this decrease mostly coincident with opening of the chest by sternotomy. In subsequent experiments, a target range of 80 to 90 mmHg was adopted and mean aortic pressures within this range were achieved for the majority of experiment 4 and the entirety of experiment 5 (Table 7.1). Heart rate was similar in all animals prior to assessment of coronary blood flow at rest and during stress.

7.5.5 Myocardial Mass Assessed
The mass of myocardial tissue assessed was less in experiments 1 and 2 than in subsequent experiments as coronary blood flow was measured from the diagonal branch of the left anterior descending artery (Table 7.1). Although myocardial mass was less for ewe 1 than ewe 2, body weight was also less for ewe 1, thus the ratio of myocardial mass to body weight was similar for these animals. In later experiments, coronary blood flow was assessed from the larger circumflex artery; consequently myocardial mass was increased in these experiments. However, the largest myocardial mass was obtained from ewe 5, the smallest animal studied, and the ratio of myocardial mass to body weight for ewe 5 was 3.17 g.kg\(^{-1}\), compared to 1.44 and 1.55 g.kg\(^{-1}\) for ewes 3 and 4 respectively.

7.5.6 Coronary Blood Flow, Perfusion Reserve and Conductance
Coronary blood flow at rest, interpolated at 90 mmHg, was highest in ewes 1 and 2 (Table 7.1 and Figure 7.4a). In comparison, in ewes 3 and 4 resting flow was approximately half, and in ewe 5 less than one fifth, that found in ewes 1 and 2. During adenosine-induced stress, coronary flow changed proportionately less in ewes 1 and 2 than in the other 3 animals and surprisingly was less than resting flow in ewe 1, resulting in a negative coronary perfusion reserve. In ewes 3, 4 and 5, coronary flow during stress was approximately 2.5 times greater than resting flow, and coronary perfusion reserve equated to approximately 60% of maximal flow for these three animals.
Table 7.1: Body size, haematocrit and baseline cardiovascular parameters, procedure duration, fluid administration and ventilation and anaesthesia details, echocardiography findings, cardiovascular parameters prior to assessment of coronary blood flow at rest and during adenosine-induced stress, myocardial tissue mass and coronary blood flow, perfusion reserve and conductance for sheep 1 to 5

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ewe 1</th>
<th>Ewe 2</th>
<th>Ewe 3</th>
<th>Ewe 4</th>
<th>Ewe 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67.1</td>
<td>79.3</td>
<td>74.4</td>
<td>58.6</td>
<td>52.1</td>
</tr>
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<td>Body surface area (m^2)</td>
<td>1.41</td>
<td>1.57</td>
<td>1.50</td>
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<tr>
<td>Haematocrit (%)</td>
<td>25</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>Mean aortic pressure (mmHg)</td>
<td>78</td>
<td>72</td>
<td>76</td>
<td>87</td>
<td>90</td>
</tr>
<tr>
<td>Mean right atrial pressure (mmHg)</td>
<td>8</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Heart rate (beats.min^{-1})</td>
<td>118</td>
<td>73</td>
<td>77</td>
<td>88</td>
<td>84</td>
</tr>
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<td><strong>Intraoperative management</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Procedure duration (min)</td>
<td>440</td>
<td>370</td>
<td>380</td>
<td>310</td>
<td>230</td>
</tr>
<tr>
<td>Total fluid administered (ml.kg^{-1}.h^{-1})</td>
<td>4.1</td>
<td>4.1</td>
<td>5.3</td>
<td>7.3</td>
<td>10.0</td>
</tr>
<tr>
<td>Mean respiratory rate (breaths.min^{-1})</td>
<td>16</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Mean tidal volume (ml)</td>
<td>531</td>
<td>459</td>
<td>458</td>
<td>403</td>
<td>267</td>
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<tr>
<td>Minute ventilation (l.min^{-1})</td>
<td>8.5</td>
<td>6.9</td>
<td>6.9</td>
<td>6.0</td>
<td>4.0</td>
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<tr>
<td>Mean end tidal carbon dioxide (mmHg)</td>
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<td>39.0</td>
<td>37.5</td>
<td>38.3</td>
<td>37.5</td>
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<tr>
<td>Mean isoflurane concentration (%)</td>
<td>3.4</td>
<td>3.5</td>
<td>3.7</td>
<td>3.6</td>
<td>3.5</td>
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<td><strong>Left ventricular dimensions and function</strong></td>
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<td></td>
<td></td>
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<tr>
<td>End diastolic diameter (mm)</td>
<td>-</td>
<td>51.0</td>
<td>54.0</td>
<td>52.0</td>
<td>49.0</td>
</tr>
<tr>
<td>End diastolic diameter/BSA (mm.m^{-2})</td>
<td>-</td>
<td>32.4</td>
<td>36.0</td>
<td>40.5</td>
<td>41.3</td>
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<tr>
<td>End systolic diameter (mm)</td>
<td>-</td>
<td>30.0</td>
<td>33.0</td>
<td>33.0</td>
<td>31.0</td>
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<tr>
<td>End systolic diameter/BSA (mm.m^{-2})</td>
<td>-</td>
<td>19.1</td>
<td>22.0</td>
<td>25.6</td>
<td>26.1</td>
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<tr>
<td>Fractional shortening (%)</td>
<td>-</td>
<td>42</td>
<td>38</td>
<td>31</td>
<td>36</td>
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<tr>
<td>Ejection fraction (%)</td>
<td>-</td>
<td>73</td>
<td>68</td>
<td>67</td>
<td>69</td>
</tr>
<tr>
<td><strong>Parameters prior to rest CBF measurement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean aortic pressure (mmHg)</td>
<td>58</td>
<td>45</td>
<td>71</td>
<td>81</td>
<td>84</td>
</tr>
<tr>
<td>Mean right atrial pressure (mmHg)</td>
<td>9</td>
<td>10</td>
<td>6</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Heart rate (beats.min^{-1})</td>
<td>109</td>
<td>70</td>
<td>67</td>
<td>78</td>
<td>94</td>
</tr>
<tr>
<td><strong>Parameters prior to stress CBF measurement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean aortic pressure (mmHg)</td>
<td>68</td>
<td>43</td>
<td>74</td>
<td>75</td>
<td>84</td>
</tr>
<tr>
<td>Mean right atrial pressure (mmHg)</td>
<td>5</td>
<td>10</td>
<td>9</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Heart rate (beats.min^{-1})</td>
<td>88</td>
<td>69</td>
<td>70</td>
<td>76</td>
<td>85</td>
</tr>
<tr>
<td><strong>Myocardial mass</strong> (g)</td>
<td>13.0</td>
<td>22.6</td>
<td>107.0</td>
<td>91.0</td>
<td>165.0</td>
</tr>
<tr>
<td>Myocardial mass/ body weight ratio (g.kg^{-1})</td>
<td>0.19</td>
<td>0.28</td>
<td>1.44</td>
<td>1.55</td>
<td>3.17</td>
</tr>
<tr>
<td><strong>CBF and coronary perfusion reserve</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting CBF (ml.min^{-1}.(100g)^{-1})</td>
<td>302.0</td>
<td>322.2</td>
<td>141.5</td>
<td>121.1</td>
<td>55.8</td>
</tr>
<tr>
<td>Stress CBF (ml.min^{-1}.(100g)^{-1})</td>
<td>296.8</td>
<td>419.3</td>
<td>319.3</td>
<td>310.2</td>
<td>149.0</td>
</tr>
<tr>
<td>Coronary perfusion reserve (ml.min^{-1}.(100g)^{-1})</td>
<td>-5.2</td>
<td>97.1</td>
<td>177.8</td>
<td>189.1</td>
<td>93.2</td>
</tr>
<tr>
<td><strong>Coronary conductance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting (ml.min^{-1}.(100g)^{-1}.mmHg^{-1})</td>
<td>3.46</td>
<td>3.67</td>
<td>0.84</td>
<td>0.94</td>
<td>0.54</td>
</tr>
<tr>
<td>Stress (ml.min^{-1}.(100g)^{-1}.mmHg^{-1})</td>
<td>3.04</td>
<td>5.06</td>
<td>1.88</td>
<td>3.57</td>
<td>1.20</td>
</tr>
</tbody>
</table>

BSA=body surface area, CBF=coronary blood flow. * BSA=weight(kg)^{0.67}x0.084 (Evgenov, Evgenov et al. 2002); † Mass of myocardial tissue supplied by coronary artery from which flow was measured. ‡ Interpolated at 90 mmHg. - Data not available.
At rest, coronary conductance was highest for ewes 1 and 2, in keeping with higher resting flows in these animals (Table 7.1 and Figure 7.4b). Coronary conductance at rest was similar in ewes 3 and 4, with values approximately twice that of ewe 5. With stress, coronary conductance decreased for ewe 1, consistent with decreased flow during stress in this animal. Coronary conductance with stress increased for all other ewes, with this increase proportionately smallest for ewe 2 and largest for ewe 4.

7.5.7 Arterial Blood Gases

Results from arterial blood gas analysis were available for ewes 1, 2 and 5. Ewe 1 had a moderate respiratory acidosis prior to assessment of coronary flow at rest, but this resolved prior to assessment of coronary flow during stress. Arterial blood gas parameters for ewe 2 were stable throughout the procedure, demonstrating a mild respiratory acidosis with metabolic compensation. Ewe 5 had evidence of a mild respiratory acidosis without compensation prior to assessment of resting flows, which worsened over the course of the procedure (Table 7.2).

Table 7.2: Intraoperative arterial blood gas results for sheep 1, 2 and 5

<table>
<thead>
<tr>
<th></th>
<th>Ewe 1</th>
<th>Ewe 2</th>
<th>Ewe 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prior to rest coronary blood flow</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.13</td>
<td>7.35</td>
<td>7.30</td>
</tr>
<tr>
<td>pO₂ (mmHg)</td>
<td>90.3</td>
<td>67.2</td>
<td>234.8</td>
</tr>
<tr>
<td>pCO₂ (mmHg)</td>
<td>94.6</td>
<td>58.0</td>
<td>56.3</td>
</tr>
<tr>
<td>Bicarbonate (mmol.l⁻¹) *</td>
<td>23.3</td>
<td>28.7</td>
<td>-</td>
</tr>
<tr>
<td>Base excess (mmol.l⁻¹)</td>
<td>-1.3</td>
<td>4.8</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Prior to stress coronary blood flow</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.39</td>
<td>7.35</td>
<td>7.25</td>
</tr>
<tr>
<td>pO₂ (mmHg)</td>
<td>97.6</td>
<td>57.2</td>
<td>88.5</td>
</tr>
<tr>
<td>pCO₂ (mmHg)</td>
<td>48.0</td>
<td>58.5</td>
<td>64.7</td>
</tr>
<tr>
<td>Bicarbonate (mmol.l⁻¹) *</td>
<td>27.2</td>
<td>28.9</td>
<td>-</td>
</tr>
<tr>
<td>Base excess (mmol.l⁻¹)</td>
<td>3.1</td>
<td>5.1</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>At conclusion of experiment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.34</td>
<td>7.37</td>
<td>7.23</td>
</tr>
<tr>
<td>pO₂ (mmHg)</td>
<td>83.7</td>
<td>70.5</td>
<td>101.3</td>
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<tr>
<td>pCO₂ (mmHg)</td>
<td>53.8</td>
<td>53.8</td>
<td>68.3</td>
</tr>
<tr>
<td>Bicarbonate (mmol.l⁻¹) *</td>
<td>26.3</td>
<td>28.6</td>
<td>-</td>
</tr>
<tr>
<td>Base excess (mmol.l⁻¹)</td>
<td>2.1</td>
<td>4.6</td>
<td>-0.4</td>
</tr>
</tbody>
</table>

pO₂=partial pressure oxygen. pCO₂=partial pressure carbon dioxide. * Data not available for ewe 5.
Figure 7.4: Coronary flow at rest and with stress, coronary perfusion reserve interpolated at 90 mmHg, (left) and coronary conductance at rest and with stress (right) for ewes 2, 3 and 5.

Ewe 2

- Rest
- Stress
- Coronary Reserve

Ewe 3

- Rest
- Stress
- Coronary Reserve

Ewe 5

- Rest
- Stress
- Coronary Reserve

CBF = coronary blood flow
7.6 Discussion

This chapter describes the process of optimising assessment of cardiovascular outcomes in adult sheep, including measurement of coronary blood flow. The methodology for our initial pilot experiments was based on previous experiments undertaken in sheep by our colleagues in the United States (Davis, Roullet et al. 2003). However, our group had not previously undertaken these experiments and we encountered a number of difficulties, with adjustments to the experimental method required to counter these.

Intraoperative monitoring of cardiac output was one such early difficulty. Assessment of cardiac output at baseline and during measurement of coronary flow at rest and with stress was included in the original protocol to facilitate detection of intraoperative haemodynamic instability. The only technique available to us for measurement of cardiac output was bolus thermodilution, in which a bolus of fluid of known volume and temperature is injected into the right atrium via the proximal port of a pulmonary artery catheter and the resulting change in blood temperature is detected by a thermistor at the distal tip of the catheter, located in the pulmonary artery. Cardiac output is then calculated from the area under the thermodilution curve and is proportional to the change in blood temperature as a function of time (Nishikawa and Dohi 1993; Reuter, Huang et al. 2010). Although considered the gold standard, this technique requires placement of a pulmonary artery catheter, which can be difficult and may be associated with serious complications such as tricuspid incompetence, pulmonary artery rupture, cardiac arrhythmias and pneumothorax (Harvey, Harrison et al. 2005). In addition, as 2 to 4 bolus injections are required with cardiac output recorded as the mean of these measurements, this technique is time consuming and can only be performed intermittently (Ambrisko, Coppens et al. 2011). Thus, while feasible at baseline, measurement of cardiac output using bolus thermodilution was not practical during coronary blood flow assessment.

A number of other methods for measurement of cardiac output exist. For example, the thermodilution principal can be used to monitor cardiac output continuously using a pulmonary artery catheter equipped with a thermal filament which is intermittently heated (Ambrisko, Coppens et al. 2011). Ultrasonic flow probes placed on the ascending aorta or proximal pulmonary artery can also be used to provide continuous cardiac output monitoring (Dean, Jia et al. 1996). However, both these techniques require specialised equipment which was not available to us during the course of these experiments. Transpulmonary thermodilution, in which a thermistor placed in the aorta detects a change
in blood temperature following central venous injection of cold saline, has also been used to monitor cardiac output (Lemson, de Boode et al. 2008). Although this technique avoids placement of a pulmonary artery catheter, 2 to 4 bolus injections are still required rendering it impractical for use during coronary blood flow assessment. Other less invasive techniques for monitoring cardiac output are also possible, including measurement of carbon dioxide elimination (Peyton, Venkatesan et al. 2006), and various methods to derive cardiac output from analysis of arterial pressure waveform (Morgan, Al-Subaie et al. 2008; Saxena, Durward et al. 2013). However, these techniques are not well validated and were therefore not suitable for use in our study protocol, even if the required equipment had been available. Thus, measurement of cardiac output was omitted after experiment 2. If appropriate equipment was available in future experiments, the real time data provided by continuous cardiac output monitoring could help inform the effect of intravenous fluid challenges given for the management of haemodynamic instability.

Maintenance of intraoperative blood pressure was a further challenge during early experiments. Baseline recordings of mean arterial pressure were taken following placement of vascular access and prior to opening of the chest cavity, with subsequent recordings made prior to coronary blood flow assessments. In experiments 1 and 2, the marked decrease in mean aortic pressure from baseline to resting coronary blood flow assessment was in stark contrast to the findings in similar experiments of Davis et al, who report mean arterial pressures of 90 to 95 mmHg at baseline and decreases of 6 to 10 mmHg prior to assessment of resting coronary flow (Davis, Roullet et al. 2003). This discrepancy, and the effect it may have had on coronary blood flow measurements, raised concern. Thus, based on the findings of Davis et al, a target range for intraoperative blood pressure of 80 to 90 mmHg was agreed, and in order to maintain blood pressure within this range, a number of changes to the experimental protocol were implemented. The first of these changes was adoption of a more aggressive approach to fluid management, with a minimum of two litres of synthetic colloid administered throughout experiments 3, 4 and 5, and fluid boluses used as required to maintain blood pressure stability. This approach resulted in improved blood pressure control, especially in experiments 4 and 5, although this outcome was likely assisted by shorter procedure duration and smaller animal size. As blood pressure control was within the target range throughout the entire procedure only in experiment 5, we recommend a minimum fluid administration rate of 10 ml.kg\(^{-1}\).h\(^{-1}\) in future experiments.
Surgical approach was the second aspect of the protocol changed in order to improve intraoperative blood pressure. In experiments 1 and 2, the decrease in arterial pressure was temporally associated with opening of the chest cavity, suggesting that midline sternotomy was not well tolerated. The change to left thoracotomy, as used by Davis et al, successfully ameliorated the decline in blood pressure observed with chest opening in experiments 1 and 2, with decreases of 5 to 6 mmHg from baseline to resting coronary blood flow assessment in subsequent experiments. To our knowledge, this difference in blood pressure effects with differing surgical approaches has not previously been described in sheep. We postulate two factors that may explain these findings. Firstly, blood loss was likely greater with sternotomy than thoracotomy. Although we could find no studies in animals comparing blood loss with thoracotomy versus sternotomy, in humans, minimally invasive cardiac surgical techniques utilising thoracotomy in favour of sternotomy have been shown to result in lower blood loss and postoperative transfusion requirements and improved haemodynamic stability (Braxton, Higgins et al. 1996; Modi, Hassan et al. 2008; Falk, Cheng et al. 2011).

Secondly, as the supine position was required in order to perform sternotomy in experiments 1 and 2, it is possible that opening of the chest, pleurae and pericardium led to posterior translocation of the heart, resulting in compression of the superior and/or inferior vena cavae. In contrast, in experiments 3, 4 and 5, sheep were placed in the right lateral position, which may have mitigated the potential for caval compression. No studies investigating the effect of surgical position on venous return and haemodynamic parameters in animals could be found to support this hypothesis. There is also minimal evidence in humans of improved intraoperative blood pressure control during cardiac surgery performed by thoracotomy and lateral positioning versus sternotomy and supine positioning, with only one study reporting decreased intraoperative inotrope requirement with thoracotomy versus sternotomy for mitral valve repair (Srivastava, Garg et al. 1998). However, compression of the inferior vena cava, with consequent decreases in venous return, cardiac output and blood pressure, is known to occur in humans in association with increased abdominal pressure, most commonly due to intra-abdominal pathology or the gravid uterus (Scott 1968; Kron, Harman et al. 1984; Kinsella and Lohmann 1994; Lee, Khaw et al. 2012).

To further optimise blood pressure control, depth of anaesthesia was monitored closely in experiments 3, 4 and 5, in an attempt to titrate the concentration of isoflurane to the lowest dose possible. The cardiovascular depressant effect of isoflurane in humans has been well
known for over 40 years (Stevens, Cromwell et al. 1971). Subsequent studies in cats, sheep and mice have documented lower mean arterial pressures with increased alveolar concentration of isoflurane (Palahniuk and Shnider 1974; Hodgson, Dunlop et al. 1998; Okutomi, Whittington et al. 2009; Constantinides, Mean et al. 2011). Thus, by closely monitoring the depth of anaesthesia and adjusting the concentration of isoflurane delivered to the animal, we hoped to mitigate the possibility of isoflurane-related cardiovascular instability. As end tidal monitoring was not available, titration of isoflurane was only possible by adjustment of the vapouriser concentration. However, in our experiments, anaesthesia was administered using a closed circuit incorporating a carbon dioxide absorber and low fresh gas flow rates. In such a system, the majority of gas in the circuit is recycled exhaled gas. Thus fresh gas flow isoflurane is diluted when the high volume recycled exhaled gas mixes with the low volume fresh gas flow, resulting in a lower inspired concentration of isoflurane than that dialled on the vapouriser (Gowrie-Mohan, Chakrabarti et al. 1995; Park, Kim et al. 2005). Furthermore, as the isoflurane content of the exhaled gas is determined by the amount of gas taken up by the animal, which is in turn dependent upon multiple variables including cardiac output, minute ventilation, and body size and composition, the quantity of isoflurane in the exhaled gas is unpredictable (Hendrickx and De Wolf 2008). Thus, from the data we have available, it is not possible to determine if the quantity of isoflurane each animal received in later experiments was lower than that delivered in experiments 1 and 2. In future experiments, use of end tidal isoflurane monitoring would allow more meaningful comparison of this across animals.

The use of a nitrous oxide-isoflurane inhaled gas mix for maintenance of anaesthesia has been shown to result in improved intraoperative blood pressure control in humans and cats (Dolan, Stevens et al. 1974; Pypendop, Ilkiw et al. 2003), although this effect has not been specifically demonstrated in sheep. However, use of nitrous oxide as an anaesthetic agent has been described in sheep, in combination with halothane (Dueck, Prutow et al. 1991; Clutton, Murison et al. 1998), as well as fentanyl and ketamine (Davis, Roullet et al. 2003). Thus, in experiments conducted following these pilot procedures, nitrous oxide was added to the circuit at a ratio of 1:1 with oxygen, resulting in improved haemodynamic stability.

Findings for coronary flow, perfusion reserve and conductance varied considerably between animals in our experiments. These differences were most marked on comparison of results from experiments 1 and 2 with those from experiments 3, 4 and 5. Our findings also differed to those of Davis et al from similar experiments (Davis, Roullet et al. 2003). Specifically, in
comparison to our findings, those authors reported lower resting coronary blood flow and higher stress coronary blood flow, perfusion reserve and conductance with adenosine. A number of factors may explain these differences. Firstly, abovementioned difficulties with blood pressure control may have influenced coronary flow in our experiments. Coronary flow is dependent on coronary perfusion pressure, which is the difference between aortic diastolic and LV end diastolic pressures (Ramanathan and Skinner 2005). Thus, hypotension decreases coronary perfusion pressure and, if adequately severe, may decrease coronary blood flow resulting in myocardial ischaemia. However, coronary blood flow is also dependent on vasomotor tone, which is in turn largely determined by tissue oxygenation (Wieneke, von Birgelen et al. 2005). Thus, persistent hypotension in experiments 1 and 2 likely resulted in myocardial ischaemia, with the resulting tissue hypoxia inducing compensatory coronary vasodilation and increased coronary flow. Indeed, in experiment 1, coronary blood flow at rest was higher than that found with adenosine, suggesting that myocardial ischaemia and consequent vasodilation may have been present in the resting state in this experiment. In addition, this animal was more acidotic and hypotensive prior to assessment of resting coronary flow, supporting the suggestion that myocardial tissue hypoxia was greatest at this time. In keeping with these findings, in lambs hypoxia has been shown to result in greater vasodilation and higher coronary blood flow than adenosine infusion, and in adult sheep, severe hypoxia has been shown to increase resting coronary blood flow so markedly that coronary reserve is abolished (Lehot, Leone et al. 1991; Oskarsson, Pesonen et al. 2004).

Secondly, use of isoflurane for the maintenance of anaesthesia in our experiments may have influenced coronary blood flow findings independent of the effect of isoflurane on blood pressure, as isoflurane causes coronary vasodilation in addition to its cardiovascular depressant effect (Ramanathan and Skinner 2005). Thus, higher resting coronary flows in our experiments may, at least in part, be due to coronary vasodilation secondary to isoflurane. Davis et al used halothane for maintenance of anaesthesia in the early phase of their experiments (Davis, Roullet et al. 2003). Although halothane has a similar negative inotropic effect to isoflurane, it causes less coronary vasodilation (Conzen, Habazettl et al. 1992; Hohner, Nancarrow et al. 1994). In addition, in order to mitigate any potential cardiovascular effects, these authors changed from halothane to intravenous fentanyl and ketamine plus nitrous oxide for ongoing maintenance of anaesthesia prior to coronary blood flow measurements. Adoption of a similar approach in our future experiments may result in reduced coronary blood flow at rest.
Thirdly, measurement error may have influenced coronary blood flow and conductance results in experiments 1 and 2, in which coronary flow was measured from the diagonal branch of the left anterior descending artery. This vessel is much smaller than the circumflex artery from which flow was measured in later experiments. Thus, as flow in this vessel and the mass of myocardial tissue it supplied were small, measurement error was proportionately more important in these earlier experiments. For example, in experiment 1, the mass of myocardial tissue supplied by the artery from which flow was measured was 13 g. However, if this value was underestimated by only 2 g, coronary blood flow and conductance would increase by 15.4%. By comparison, in experiment 3, in which the mass of myocardial tissue was 107 g, an underestimation of the same magnitude increases coronary blood flow and conductance by only 1.9%.

Although inaccurate measurement of the mass of myocardial tissue supplied by the vessel from which flow was measured was proportionately more important in experiments 1 and 2, this difficulty may also have influenced the results of later experiments if more substantial under- or overestimation of myocardial mass occurred. Prior to undertaking these experiments, we had limited knowledge of the expected myocardial mass supplied by either of the vessels from which flow was measured. Some guidance was provided by the report of Davis et al, in which the LV mass of control sheep was 75.8±14.4 g (mean±SD) (Davis, Roullet et al. 2003). However, this value was for total LV weight, rather than the mass of tissue supplied by a single artery, and these sheep were smaller than ours (mean weight±SD: 45.0±5.6 kg). Furthermore, we found it difficult to precisely define the myocardial territory of the vessel from which flow was measured, due to leakage of dye onto the surface of the myocardium at the time of vessel infusion and tissue dissection. These uncertainties resulted in considerable variation in myocardial mass. For example, in the last 3 experiments, in which flow was measured from the circumflex artery, myocardial mass was substantially greater for ewe 5 than ewes 3 and 4, and the ratio of myocardial mass to total body weight of ewe 5 was more than twice that of ewes 3 and 4. Thus, it is possible that the true mass of myocardial tissue supplied by the circumflex artery for ewe 5 was only half that measured. If so, the true values of coronary blood flow, perfusion reserve and conductance in this experiment would have been twice those calculated; findings much more in keeping with those from ewes 3 and 4.

Given these difficulties, following the pilot experiments a new technique was devised to allow more accurate quantification of myocardial tissue mass using a liquid radio-opaque
rubber polymer (Microfil, Flow Tech Inc., Massachusetts, USA). This substance has a low viscosity, allowing it to completely fill the arterial tree with little resistance, and its hydrophobic properties ensure that it is retained within the vascular compartment (Zagorchev, Oses et al. 2010). It is primarily used for visualisation of vascular networks by micro-computed tomography, with several studies investigating microvascular networks in mice, rats and pigs (Kwon, Sangiorgi et al. 1998; Bentley, Ortiz et al. 2002; Wischgoll, Choy et al. 2009; Zagorchev, Oses et al. 2010; Vasquez, Gao et al. 2011). The vascular casts created by perfusion of this substance have also been used to quantify the mass of myocardial tissue supplied by the left anterior descending, left circumflex and right coronary arteries in pigs (Le, Wong et al. 2008). This more precise technique has been used in the 16 experiments undertaken by others in our group following our pilot procedures, with the mass of myocardial tissue supplied by the circumflex artery in these experiments ranging from 68.1 to 128.1 g (mean±SD: 95.4±17.9 g) and the ratio of myocardial mass to total body weight ranging from 0.9 to 1.4 g.kg⁻¹ (1.1±0.2 g.kg⁻¹). These findings are in keeping with the results for myocardial mass obtained in pilot experiments 3 and 4, suggesting that coronary blood flow, perfusion reserve and conductance data from these experiments are unlikely to have been adversely influenced by inaccuracies in measurement of myocardial mass. In addition, these findings also support the hypothesis that myocardial mass was overestimated in experiment 5, with the true value likely to be approximately half that measured, as suggested above.

The accuracy of coronary blood flow measurements in our experiments may have also been influenced by difficulties encountered with blood pressure manipulation. This was largely achieved by placement of cotton ties around the aorta and IVC to enable extrinsic compression of these vessels with consequent increase or decrease in blood pressure. However, placement of these ties was technically difficult following the change to left thoracotomy, and production of small, stable decrements in blood pressure following IVC compression proved particularly challenging. To counter this difficulty, in the subsequent 16 experiments undertaken by others in our group, an inflatable vascular occluder has been used to compress the IVC, resulting in considerable improvement in fine control and stability of blood pressure. This device has previously been used successfully in experiments similar to ours (Davis, Roullet et al. 2003), and has also been used to compress the umbilical cord (Gardner, Fletcher et al. 2001), pulmonary artery, and ductus arteriosus (Jaillard, Larrue et al. 2006) in fetal sheep, and the pulmonary and bronchoesophageal arteries in adult sheep (Ashley, Herndon et al. 1992). Given the success of this device for
IVC compression, we plan to obtain a second inflatable occluder to enable compression of the aorta in this manner in future experiments.

The possibility that maximal vasodilation with adenosine was not achieved in our experiments is raised by our findings of lower stress coronary blood flow, perfusion reserve and conductance in comparison to those of Davis et al (Davis, Roullet et al. 2003). This could occur if an inadequate dose of adenosine was administered. However, all animals in our experiments received adenosine at a dose of 0.14 mg.kg\(^{-1}\).min\(^{-1}\), in keeping with the dose used in humans in the Fetal Anaemia Study. By comparison, the mean dose of adenosine used by Davis et al to achieve maximal vasodilation was 0.07 mg.kg\(^{-1}\).min\(^{-1}\) (Davis, Roullet et al. 2003). Thus, it is unlikely that the dose used in our experiments was inadequate. Alternatively, given the short half-life of adenosine, it is possible that maximal pharmacological effect was not obtained in our earlier experiments in which adenosine was administered via a catheter in the left internal jugular or right femoral vein. Therefore, in order to eliminate this potential problem, in experiment 5 adenosine was administered directly into the left atrium, in keeping with the method used by Davis et al (Davis, Roullet et al. 2003). However, there was no increase in stress coronary blood flow, perfusion reserve or conductance in experiment 5 as a result of this change. Thus, it is likely that the differences in stress coronary flow, perfusion reserve and conductance are due to intrinsic differences between animals used in our experiments and those of Davis et al.

The main intrinsic differences between these two groups of animals were that our sheep were heavier (mean±SD: 66.2±11.1 kg versus 45.0±5.6 kg) and older (4 to 5 years versus 7 months). We can find no evidence to suggest that animal weight affects coronary blood flow. However, with regard to age, total and regional resting myocardial blood flow in newborn lambs is approximately twice that found in adult sheep (Fisher, Heymann et al. 1982; Koehler, Traystman et al. 1985). Although the magnitude of this difference decreases in the neonatal period, at 10 weeks of age LV blood flow at rest and at maximal vasodilation with adenosine remains significantly higher than that found in adult sheep (Flanagan, Aoyagi et al. 1994). Thus, it is plausible that the difference in age between our sheep and those of Davis et al contributed to our findings of decreased coronary blood flow with adenosine.

Assessment of LV dimensions and function was also undertaken in our experiments. References ranges for these echocardiographic parameters have recently been reported from a study of 51 adult sheep with a weight of 74±13 kg (mean±SD) (Hallowell, Potter et al.
Our sheep were similar in size to these animals and findings for the 4 sheep in our series who underwent echocardiography fall within the reference ranges determined by these authors, suggesting that the echocardiographic techniques utilised in our experiments were appropriate.

7.7 Summary
Cardiovascular outcomes of interest, including coronary blood flow, can be assessed in adult sheep provided appropriate equipment and surgical expertise are available. With improvements to the methodology and increasing familiarity with the study protocol, experiment duration decreased and more consistent data were produced. However, these experiments require close attention to maintenance of haemodynamic stability and the anaesthetic agents used should have minimal cardiovascular effect. In addition, precise quantification of the mass of myocardial tissue supplied by the vessel from which flow is assessed is essential to ensure measures of coronary blood flow, perfusion reserve and conductance are accurate. The knowledge and experience gained from undertaking these pilot experiments will allow the influence of preterm anaemia on cardiovascular outcome in adulthood to be investigated in future experiments.
Chapter 8: Conclusions

8.1 Long-Term Effects of Fetal Anaemia
The technique of IUT was pioneered in New Zealand by William Liley in 1963. Thus, the Fetal Anaemia Study cohort was recruited from the oldest and largest single group of IUT recipients in the world. Outcomes for babies with severe fetal anaemia secondary to RhHD improved dramatically thanks to Liley’s work, but the health of survivors of fetal anaemia and IUT in adulthood has not previously been investigated. Thus, 50 years on from the first IUT, it is fitting that we have had the opportunity to follow up Liley’s “rhesus babies”.

However, the value of this work lies not just with the knowledge gained regarding health outcomes for the small group of individuals studied. As the management of fetal anaemia was established in New Zealand and widely accepted internationally as the standard of care until the mid 1980s, our findings should be applicable to almost all adult survivors of IUT. Thus, the outcomes reported from this historical cohort are of relevance to the thousands of IUT survivors throughout the world who are currently in adulthood, as well as the physicians caring for them now and in the future.

The primary question the Fetal Anaemia Study set out to investigate was simply whether exposure to intrauterine anaemia and transfusion is associated with any detectable changes in health in adulthood, in particular cardiovascular status. There is sound physiological evidence on which to basis an a priori hypothesis that fetal anaemia may influence cardiovascular development. In anaemic fetal sheep, stroke volume, heart rate and cardiac output increase by approximately 50% and resting MBF increases nearly six-fold (Davis and Hohimer 1991). In humans, if left untreated, fetal anaemia results in high-output cardiac failure and may lead to fetal demise (Lindenburg, van Klink et al. 2013). IUT ameliorates cardiovascular compromise, but the efficacy of this treatment is dependent on the timing and severity of anaemia at presentation and the extent to which anaemia is resolved.

Prior to the Fetal Anaemia Study, there had been only one small follow up study of cardiovascular outcome following fetal anaemia, which reported lower LV mass and left atrial area, but similar LV function in 25 IUT recipients aged 3 to 16 years compared to age and sex-matched controls (Dickinson, Sharpe et al. 2010). In addition, several studies in sheep have suggested that exposure to fetal anaemia may result in abnormal cardiomyocyte maturation, alterations to coronary vessel architecture and increased susceptibility to
myocardial damage following ischaemic insult (Davis, Roullet et al. 2003; Yang, Hohimer et al. 2008; Jonker, Giraud et al. 2010; Jonker, Scholz et al. 2011). Collectively, these studies provide experimental evidence to support the suggestion that fetal anaemia may play a role in the programming of cardiovascular disease.

The results reported in this thesis suggest exposure to fetal anaemia and IUT is indeed associated with cardiovascular changes in adulthood. The most important findings of the Fetal Anaemia Study can be divided into three categories: the good news, the potentially bad news and the puzzling news. Dealing with the good news first, we have that found that exposure to fetal anaemia is not associated with altered body size, smoking status, alcohol use, fasting plasma cholesterol concentration or glucose tolerance in adulthood; all important risk factors for cardiovascular disease. These findings will be reassuring for adult survivors of IUT. However, given the difference in gestational age at birth between affected and unaffected participants, some of these findings are surprising. Insulin resistance, decreased alcohol and tobacco use, and reduced physical activity have been reported in previous investigations of outcomes in adulthood following preterm birth (Dalziel, Parag et al. 2007; Hille, Dorrepaal et al. 2008; Clemm, Roksund et al. 2012), but we found no evidence of a relationship between these outcomes and gestational age in our study. This may be because our study was inadequately powered to detect the relatively small differences in these outcomes previously reported between preterm and term born infants. Alternatively, as most of these previously reported preterm cohorts were born considerably more preterm than our affected participants, it is also possible that the gestational age at birth of our affected participants was not low enough to influence these outcomes.

Our finding of similar blood pressure in affected and unaffected participants is particularly interesting. An inverse relationship between gestational age and blood pressure has been well documented by others, with this association present even for those born in the last few weeks before term (Lawlor, Hubinette et al. 2007). In addition, an increase in blood pressure of 3 to 4 mmHg has been reported in adulthood following moderate preterm birth, compared to term born controls (Dalziel, Parag et al. 2007). The preterm participants of that study were very similar to our affected participants, being born at National Women’s Hospital between 1969 and 1974, with a median gestational age of 33.7 weeks. However, in this, and other investigations of blood pressure following preterm birth, early delivery occurred for a variety of reasons, whereas all our affected participants were born preterm due to RhHD. This raises the intriguing possibility that exposure to fetal anaemia and IUT may have
programmed our affected participants for lower blood pressure than they may otherwise have had.

It is also possible that use of unaffected siblings as our comparator group minimised the impact of social, familial and genetic diversity on our findings, better controlling for these factors that may confound the outcomes of interest. This might be particularly important for blood pressure, which can be influenced by factors such as maternal body size, familial dietary and exercise habits and genetic influences on metabolic and cardiovascular health (Lawlor, Mortensen et al. 2011). Given the potential importance of these factors, and the difficulties inherent in adjusting for them in multivariate analysis, surprisingly few studies investigating outcome following unfavourable perinatal events have utilised siblings as the comparison group. However, a large Swedish family-based study of subjects born between 1973 and 1984 found that the previously reported inverse relationship between blood pressure and gestational age is not explained by confounding from shared familial factors (Lawlor, Hubinette et al. 2007). Thus, it is unlikely that use of siblings in our study prevented detection of a significant difference in blood pressure between groups, or that the increase in blood pressure in adults born preterm previously reported by others can be explained by comparison to unrelated term born controls.

Continuing the good news story, we found similar educational achievement, socioeconomic status, general health and medication use in affected and unaffected participants of the Fetal Anaemia Study. These outcomes have not previously been reported in adult survivors of fetal anaemia and IUT. However, several studies report favourable neurological outcome in children up to 17 years old following IUT for fetal anaemia, with rates of neurodevelopmental impairment similar to that seen in the general population (Farrant, Battin et al. 2001; Weisz, Rosenbaum et al. 2009; Lindenburg, Smits-Wintjens et al. 2011). This should provide valuable reassurance to the families of children with fetal anaemia, and the clinicians caring for them. Similar reassurance has been provided by follow up studies of children affected by other perinatal illnesses, such as congenital heart disease and congenital diaphragmatic hernia (Menahem, Poulakis et al. 2008; Loup, von Weissenfluh et al. 2009; Danzer, Gerdes et al. 2013). Furthermore, in a cohort of adults born moderately preterm who were very similar to our affected participants, socioeconomic status and psychological functioning was reported to be similar to that of term born controls (Dalziel, Lim et al. 2007). However, if the sickest infants from our cohort died in the perinatal period, our findings may be due to the fact that those babies who survived were less severely
compromised and thus more likely to do well in the long-term; the so-called survivor advantage. It is possible that advances in neonatal care over the last three to four decades and consequent improved survival of more severely unwell infants may mean that educational achievement and socioeconomic status in adulthood may be worse in more modern cohorts of IUT survivors.

We must now turn to the bad news, or at least that which may potentially signify increased risk of cardiovascular disease. We found that affected participants had lower concentrations of HDL. As decreased levels of this protein are associated with increased rates of cardiovascular disease, this finding implies that exposure to fetal anaemia and IUT may result in increased risk of cardiovascular disease (Rahilly-Tierney, Bowman et al. 2008). Secondly, on assessment of heart rate variability, affected participants had evidence of altered cardiac autonomic tone, with predominance of sympathetic activity, which is also a marker of increased cardiovascular risk (Buccelletti, Gilardi et al. 2009). Thirdly, lower LV volumes, radius, and possibly mass together with increased relative wall thickness in affected participants indicate that exposure to fetal anaemia alters cardiac size and function. Finally, lower MBF at rest and with cold pressor stress suggests that fetal anaemia may lead to impaired endothelial function, which may be mediated through increased sympathetic tone (Davel, Wenceslau et al. 2011).

These findings are unlikely to be of clinical significance in early to mid adulthood. However, they may signify that adult survivors of fetal anaemia are at risk of earlier onset of cardiovascular disease, or at greater risk of cardiovascular disease with ageing. A growing body of evidence suggests that unfavourable early life events may not only programme individuals for the occurrence of specific diseases in later life, but also influence the age of onset of these illness, accelerate ageing and shorten survival. For example, recent examination of over 600 birth cohorts from the 19th and 20th centuries suggests that poor nutrition in early life, exposure to infection and infant death rates are associated with higher mortality at age 40 and acceleration of mortality with subsequent ageing (Beltran-Sanchez, Crimmins et al. 2013). Evidence of accelerated ageing has also been found in animals subjected to prenatal nutrient restriction and/or postnatal dietary excess, and in humans with a history of poor early growth (Sayer and Cooper 2002). Furthermore, adults conceived during the Dutch famine have both earlier onset, and a higher cumulative incidence of coronary artery disease, independent of size at birth; findings which may be mediated at least in part through higher LDL/HDL cholesterol ratio and impaired glucose tolerance.
(Ravelli, van der Meulen et al. 1998; Roseboom, van der Meulen et al. 2000; Painter, de Rooij et al. 2006). However, the processes by which adverse early life events result in earlier onset of disease, accelerated ageing and decreased longevity remain unclear, with hypotheses including defective cellular regeneration and repair, alterations to hormonal axis programming, endothelial dysfunction and impaired immune function (Sayer and Cooper 2002; Painter, de Rooij et al. 2006; Feltes, de Faria Poloni et al. 2011).

Given that all affected participants of the Fetal Anaemia Study were less than 50 years of age, we did not expect to find evidence of earlier onset or increased rates of cardiovascular disease. However, our findings may have clinical utility for these individuals, particularly at their current age, prior to the onset of cardiovascular disease. Calculation of an individual’s risk of cardiovascular disease can be made using sex, age, blood pressure and lipid findings, and a number of “risk calculators” are in common clinical use for this purpose (Allan, Nouri et al. 2013). We used the findings of our study to estimate cardiovascular risk following fetal anaemia using the University of Edinburgh Cardiovascular risk calculator, based on the Framingham cohort (cvrisk.mvm.ed.ac.uk/calculator/calc.asp). Similar results were obtained using two New Zealand specific cardiovascular risk calculators (www.knowyournumbers.co.nz and www.health.govt.nz/publication/new-zealand-cardiovascular-risk-charts) although the absolute quantification of risk provided by these tools was less precise than that obtained from the Edinburgh calculator.

Table 8.1: 10-year cardiovascular risk of affected and unaffected Fetal Anaemia Study participants at 40, 60 and 75 years of age

<table>
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<tr>
<th></th>
<th>10-year cardiovascular risk (%)</th>
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<tr>
<td></td>
<td>At 40 years</td>
<td>At 60 years</td>
<td>At 75 years</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Affected</td>
<td>2.8</td>
<td>2.0</td>
<td>12.6</td>
<td>7.5</td>
<td>23.2</td>
</tr>
<tr>
<td>Unaffected</td>
<td>2.6</td>
<td>1.8</td>
<td>11.9</td>
<td>7.0</td>
<td>22.3</td>
</tr>
<tr>
<td>% change in risk†</td>
<td>7.7</td>
<td>11.1</td>
<td>5.9</td>
<td>7.1</td>
<td>4.0</td>
</tr>
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* Risk estimated assuming systolic blood pressure of 125mmHg using the University of Edinburgh Cardiovascular Risk Calculator, with data obtained from the Framingham cohort. † % change in risk = risk in affected–risk in unaffected/risk in unaffected

Our estimates of 10-year cardiovascular disease risk are increased following fetal anaemia in both men and women (Table 8.1). Moreover, the relative increase in risk is greatest when younger, and for women. However, the relative increase in baseline risk of 7.7% in men and 11.1% in women at 40 years is very small in absolute terms (0.2%). Indeed, this absolute difference may fall within the limits of error of the risk calculator. Thus, the change in
cardiovascular risk which results from the observed decrease in HDL is unlikely to be of any clinical relevance at this age. Even at 75 years, when cardiovascular disease risk is greatest, the relevance of an increase in risk from 22.3% to 23.2% in men, and from 12.8% to 13.5% in women is questionable. However, these future risk estimates are based on current plasma lipid measurements, and it is not possible to predict whether exposure to fetal anaemia may result in an increasingly unfavourable lipid profile with advancing age. Furthermore, it is not possible to predict whether other risk factors for cardiovascular disease may be altered with increasing age in adult survivors of fetal anaemia. Thus, while these calculations indicate that the decrease in HDL observed in affected participants is unlikely to alter cardiovascular risk for the ensuing ten years, it is more difficult to be certain that this will remain true with increasing age.

Using our finding of altered autonomic tone in affected participants to quantify cardiovascular risk is not straightforward, as previous investigations have reported increased coronary heart disease risk only in association with decreased HRV parameters. For example, in the Framingham Heart Study cohort, a one standard deviation reduction in any HRV parameter except for LF/HF ratio was associated with increased incidence of new cardiac events, with a hazard ratio of approximately 1.4 for each parameter (Tsuji, Larson et al. 1996). In a further large population-based cohort study, reduced HF power was associated with increased 3-year coronary artery disease incidence (RR 1.72, 95% CI 1.17 to 2.51) (Liao, Cai et al. 1997). Thus, reduced normalised HF power in affected participants in our study is suggestive of an increase in coronary heart disease risk of as much as 70%, but increased normalised LF power and LF/HF ratio are not in keeping with increased risk, and could even suggest that coronary heart disease risk is decreased in affected participants. However, this seems unlikely as, when taken together, our HRV findings indicate sympathetic predominance in affected participants, which is widely accepted to be associated with increased cardiovascular risk (Buccelletti, Gilardi et al. 2009; Seravalle, Dimitriadis et al. 2013).

Increased sympathetic tone may also be associated with our findings of lower resting and cold pressor MBF in affected participants. Chronic sympathetic overactivity is thought to play an important role in the aetiology of endothelial dysfunction (Davel, Wenceslau et al. 2011), which is an independent risk factor for cardiovascular events (Lerman and Zeiher 2005; Shechter, Issachar et al. 2009). A recent meta-analysis of over 5,000 patients reported that endothelial dysfunction, assessed as a decrease of one standard deviation in flow-
mediated dilation of the brachial artery, was associated with a 22% increase in the risk of future cardiovascular events (Inaba, Chen et al. 2010). Thus, it is possible that our finding of sympathetic predominance in affected participants confers increased cardiovascular risk to adult survivors of fetal anaemia due to its effect on endothelial function. If this were the case, endothelial dysfunction following fetal anaemia may not confer any additional cardiovascular risk above that of increased sympathetic tone.

The question of what advice should be given to adult survivors of fetal anaemia is not straightforward. The estimates of cardiovascular risk discussed above are based on group effects, whereas an individual’s cardiovascular risk is based on their individual risk factors of age, blood pressure, lipid status and so on. Thus, the most important advice for adult survivors of fetal anaemia might simply be to ensure they follow routine advice around maintenance of a healthy lifestyle and clinical monitoring of cardiovascular risk factors. This is, of course, important advice to any individual heading towards middle-adulthood. While our findings might suggest that this may be relatively more important for adult survivors of fetal anaemia, we do not have enough evidence to suggest monitoring should be undertaken earlier or more frequently in these individuals.

Finally, two findings from this study provide some puzzling news. Firstly, higher haemoglobin concentration and haematocrit were observed in affected participants compared to unaffected siblings. These unexpected findings suggest that exposure to fetal anaemia may alter the set point at which tissue hypoxia is detected by erythropoietin secreting cells, thereby stimulating increased production of red blood cells and haemoglobin. Why exposure to fetal anaemia may result in these changes is uncertain, especially as increased haematocrit and consequent hyperviscosity are usually associated with the negative sequelae of decreased cardiac output and impaired systemic blood flow (Ergun-Cagli, Ileri-Gurel et al. 2011). However, small increases in haematocrit (< 20% of baseline) have recently been shown to result in paradoxical increases in cardiac output and microvascular flow, with these findings mediated through increased production of endothelium-dependent nitric oxide resulting from increased shear wall stress secondary to increased blood viscosity (Martini, Carpentier et al. 2005; Martini, Tsai et al. 2006). Thus, taken together with our findings of decreased LV volumes and resting MBF in adult survivors of fetal anaemia, we postulate that these haematological changes may be a compensatory response aimed at restoring both cardiac output and MBF. It is possible that
without these haematological changes, LV function and resting MBF may have been even lower than we observed.

Our finding of similar hyperaemic MBF in affected and unaffected participants is the second piece of puzzling news to emerge from this study. Previous investigation of MBF in adult sheep exposed to fetal anaemia reported marked increase in flow with adenosine-induced hyperaemia, suggesting expansion of the coronary vasculature which persisted into adult life (Davis, Roullet et al. 2003). However, we found no evidence of a similar “coronary supertree” in humans exposed to fetal anaemia. Instead, our findings indicate that the coronary microcirculation appears unchanged in humans following exposure to fetal anaemia. However, lower MBF at rest and with cold pressor stress and increased sympathetic tone suggest that endothelial function may be impaired following exposure to fetal anaemia, in keeping with reports of increased susceptibility to ischaemic myocardial injury in previously anaemic sheep (Yang, Hohimer et al. 2008). Furthermore, exposure to fetal anaemia in sheep alters cardiomyocyte maturation, resulting in fewer and larger cardiomyocytes; observations which could help explain our findings of decreased LV volumes and size in adult survivors of fetal anaemia (Jonker, Giraud et al. 2010; Jonker, Scholz et al. 2011). Thus, we propose a pathway by which previously reported morphological findings following fetal anaemia might be related to the outcomes observed in our study, culminating in increased cardiovascular risk for adult survivors of fetal anaemia (Figure 8.1).

Further follow up of the Fetal Anaemia Study cohort with increasing age will help determine the clinical significance of the findings reported in this thesis. Reassessment of this cohort in 10 to 20 years time would help determine whether adult survivors of fetal anaemia have an earlier onset, or increased cumulative incidence of cardiovascular disease, similar to that found for individuals born during the Dutch Famine (Painter, de Rooij et al. 2006).

Further investigations could also be undertaken in the near future on this cohort. Since our findings of lower MBF at rest and with cold pressor stress are suggestive of endothelial dysfunction, which may be associated with increased sympathetic tone, this could be formally tested, for example by assessing flow-mediated vasodilatation of the brachial artery (Lerman and Zeiher 2005), or direct measurement of sympathetic activity in skeletal muscle using microneurography (Seravalle, Dimitriadis et al. 2013). Given the
We hypothesise that fetal anaemia compromises cardiovascular function \textit{in utero} and alters cardiomyocyte development before birth, resulting in decreased cardiomyocyte endowment in the mature myocardium (Thornburg, Jonker et al. 2011). These developmental alterations manifest clinically in early to mid-adulthood as increased sympathetic tone, decreased myocardial blood flow at rest and with cold pressor stress indicative of endothelial dysfunction, and lower left ventricular function, and may culminate in increased cardiovascular risk in later life.
apparent value of endothelial dysfunction for prediction of cardiovascular disease, even in healthy individuals (Shechter, Issachar et al. 2009; Inaba, Chen et al. 2010), together with the reported relationship between sympathetic overactivity and cardiovascular disease (Grassi 2010), such investigation might help to quantify cardiovascular risk in adult survivors of fetal anaemia. In addition, assessment of exercise tolerance may help clarify whether our finding of lower LV function is of any functional consequence. Such investigations have previously been undertaken in adults born preterm, with most reporting no difference in exercise capacity, but reduced participation in exercise compared to term born controls (Rogers, Fay et al. 2005; Kajantie, Strang-Karlsson et al. 2010; Clemm, Roksund et al. 2012; Kaseva, Wehkalampi et al. 2012). We found no difference between our affected participants and unaffected siblings for reported participation in exercise, but did not perform functional assessment of exercise capacity.

Given the changing nature of fetal anaemia over the last 20 to 30 years due to advances in diagnostic and therapeutic strategies, follow up of more recent IUT cohorts will also be important to assess whether these improvements in care translate into both short and long-term improvements in outcomes, including neurological and psychological function, educational achievement and socioeconomic status, as well as cardiovascular disease. In particular, our observation of lower MBF in those born following the introduction of intravascular IUT suggests that cardiovascular risk may be greatest in those born later. Further investigation of more recent IUT survivors, together with ongoing follow up of our cohort, will be required to determine the veracity of this speculation.

As modern diagnostic techniques can provide detailed information regarding the severity of fetal anaemia, cardiovascular compromise and growth, as well as the response of the fetus to treatment, it should also be possible to correlate factors such as lowest in utero haemoglobin concentration, presence of fetal hydrops and volume of blood transfused with short and long-term outcomes in future cohorts. Such information may help clarify appropriate transfusion thresholds and optimal timing of intervention for future IUT recipients. However, due to the widespread implementation of anti-D prophylaxis, fetal anaemia has become much less common in recent years, and the once frequent procedure of IUT is now performed only occasionally in most centres. Therefore, it is likely that multicentre studies will be required to generate future cohorts of sufficient size to allow investigation of outcomes following IUT.
Greater understanding of the influence of fetal anaemia on the programming of cardiovascular disease may also be gained from further work in sheep. For example, assessment of cardiovascular outcomes in older sheep may provide more relevant information with which to compare our findings in humans, especially as the influence of fetal anaemia may be more apparent with increasing age. In addition, earlier induction of anaemia and repeated IUT of fetal sheep may better mimic the real-life clinical scenario of fetal anaemia and its treatment in humans.

Finally, given the accumulating evidence regarding the role of fetal anaemia in cardiovascular programming, it is likely that fetal and neonatal polycythaemia may also have long-term cardiovascular implications. The clinical relevance of this lies in the condition of neonatal polycythaemia-hyperviscosity syndrome, which occurs in 1 to 2% of live births and is most often caused by chronic fetal hypoxia, for example due to maternal diabetes, hypertension, hyperthyroidism or smoking (Sarkar and Rosenkrantz 2008). In addition, twin anaemia-polycythaemia syndrome, a recently described variant of twin-to-twin transfusion syndrome in which the recipient twin becomes polycythaemic, rather than polyuric and hypervolaemic, can also cause polycythaemia-hyperviscosity syndrome (Slaghekke, Kist et al. 2010). Polycythaemia results in decreased cardiac output and increased pulmonary vascular resistance secondary to hyperviscosity, which manifest clinically as cardiomegaly, cyanosis, tachycardia and tachypnoea (Fouron and Hebert 1973; Murphy, Reller et al. 1986; Swetnam, Yabek et al. 1987). A small number of follow up studies investigating short-term cardiovascular outcome following fetal and neonatal polycythaemia have shown that these findings resolve with haematocrit reduction (Fesslova, Villa et al. 1998; Sarkar and Rosenkrantz 2008; Maschke, Diemert et al. 2011), although an increased incidence of pulmonary stenosis has been reported in recipient survivors of twin-to-twin transfusion syndrome (Herberg, Gross et al. 2006). Moreover, while partial exchange transfusion may reduce haematocrit and lower blood viscosity, resulting in improved neonatal cardiovascular function, this technique has associated risks and does not result in improved neurodevelopmental outcome (Ozek, Soll et al. 2010). Thus, considerable controversy exists regarding optimum neonatal management of polycythaemia. To our knowledge, no investigations have been undertaken of long-term cardiovascular outcomes following fetal and neonatal polycythaemia. Such investigation may not only provide further insight into cardiovascular programming, but may also help inform the future management of polycythaemic babies.
8.2 Long-Term Effects of Preterm Anaemia

Birth prior to 37 weeks’ gestation accounts for up to 12% of all live births (Hamilton, Martin et al. 2010). Infants born preterm frequently become anaemic in the first few weeks after birth, at a similar gestational age to fetuses exposed to anaemia due to RhHD. However, it is not known whether cardiovascular changes similar to those seen in adults who were anaemic in utero also occur when anaemia occurs ex utero following preterm birth. In addition, there is considerable controversy regarding the management of anaemia in preterm infants, and data are sparse regarding the long-term effects of liberal versus restrictive transfusion policies.

The Preterm Anaemia Study set out to establish a cohort of lambs born preterm in whom anaemia was induced prior to TEA. We found that anaemia can be successfully induced in preterm lambs, without compromising survival at 12 months. This work produced two important findings. Firstly, we have shown that anaemia prior to TEA may have long-term effects on growth. However, growth impairment was apparent only in those sheep exposed to the most severe anaemia, in whom haematocrit was decreased to 30% of baseline. A reduction in haematocrit of this magnitude is not typical in human infants born preterm (Widness 2008). However, other than lactate concentration at TEA, we did not record any measures of the functional effects of anaemia in our preterm sheep, such as heart rate, respiratory rate, oxygen saturations, reticulocyte count or erythropoietin concentration. Thus, we cannot be certain that the physiological implications of a specified reduction in haematocrit are the same in preterm sheep and preterm infants. Investigation of these parameters in future studies could help determine whether equivalent declines in haematocrit result in equivalent physiological responses in preterm sheep and infants.

Secondly, we found higher haemoglobin concentrations in both groups of anaemic preterm lambs at 12 months of age. These observations were unexpected, and remarkably consistent with our findings of increased haematocrit and haemoglobin concentration in adult survivors of fetal anaemia, indicating that exposure to anaemia prior to TEA, either in or ex utero, may influence haematological parameters in later life.

Given these similarities in haematological findings, it is possible that preterm anaemia may also result in decreased LV function and resting MBF in adulthood. In order to explore this further, investigation of cardiovascular outcome will be undertaken in our preterm anaemic sheep cohort within the next 12 months. However, for the time being, these haematological
findings provide the first experimental evidence to suggest that preterm anaemia has long-term cardiovascular effects, perhaps even for infants born moderately to late preterm. In addition, as haematological changes were not only evident in the most severely anaemic preterm sheep, but also in the group subjected to more moderate anaemia, these findings may have implications for the many thousands of adults throughout the world who were born preterm.

Intriguingly, a recent investigation of cardiovascular outcome following preterm birth reports decreased LV volumes in adulthood (Lewandowski, Augustine et al. 2013). The authors of this study hypothesise that their findings may be attributable to preterm birth resulting in exposure of the immature heart to the haemodynamic changes of the postnatal circulation. However, the findings of both the Fetal Anaemia and Preterm Anaemia Studies prompt us to speculate that anaemia prior to TEA might contribute to these outcomes.

A reliable and reproducible experimental protocol is required in order to investigate cardiovascular outcome following preterm anaemia. Thus, the final component of the Preterm Anaemia Study was to develop and refine this protocol. The experiments were successfully piloted, and some valuable lessons were learned, particularly the importance of maintaining intraoperative haemodynamic stability and accurately quantifying the mass of myocardial tissue supplied by the vessel from which blood flow was measured. The experience gained from piloting these procedures will assist with the acquisition of accurate cardiovascular data when sheep from the Preterm Anaemia Study are assessed.

The methodology devised offers exciting opportunities for future investigation of both short and long-term effects of preterm anaemia, for example, on ventilatory and oxygen requirements, feed tolerance, glucose homeostasis, growth factors, stress hormone responses and endothelial function. Taken together with our planned investigations of cardiovascular outcome following preterm anaemia, the knowledge gained from this work will further elucidate the influence of preterm anaemia on long-term health, and may help inform the future management of anaemia in preterm babies.

8.3 Summary

The Fetal Anaemia Study is the first investigation of long-term outcomes in humans following fetal anaemia and IUT. We have found that adult survivors of fetal anaemia have an unfavourable lipid profile, augmented sympathetic tone, lower LV function and
decreased MBF at rest and with cold pressor stress in comparison to unaffected siblings. Thus, this study provides the first evidence in humans that exposure to fetal anaemia may confer increased risk of cardiovascular disease in later life.

The Preterm Anaemia Pilot Study is the first report of long-term outcomes following preterm birth and anaemia prior to term equivalent age. This work is experimentally unique, and the methodology offers new opportunities for further investigation of the effects of preterm anaemia. Our findings suggest that anaemia prior to term equivalent age, whether in or ex utero, may have similar implications for long-term cardiovascular status. The experimental protocol for investigation of cardiovascular outcomes in adult sheep devised and refined as part of this work will enable further testing of this hypothesis.
Appendices

Appendix 1: Invitation letters to potential Fetal Anaemia Study participants, their mothers and siblings
Dear <Participant’s name>

Re: The Long Term Effects of Fetal Anaemia Study

We are researchers from The Liggins Institute at The University of Auckland. We are involved in a study of the health of people who suffered from anaemia as an unborn baby and received a blood transfusion for this before birth at National Women’s Hospital.

The most common cause for severe anaemia before birth is rhesus disease. This occurs when there is a difference in particular blood groups (called rhesus groups) between a mother and her baby. This can be life-threatening. A method was developed at National Women’s Hospital in 1963 to treat affected babies before birth by giving them blood transfusions. This pioneering treatment is now standard practice around the world. Our records indicate that you may have been one of the babies who received this treatment at National Women’s Hospital.

We now know that events before birth can change the risk of developing certain diseases as adults. Recent studies have shown that animals that were anaemic and were treated with blood transfusions before birth have altered structure of their heart and blood vessels as adults. We do not know if this occurs in humans, or what it might mean for their later health. The best way to answer this question is to see whether the heart structure and health of adults who were treated for anaemia before birth is different from those of their brothers or sisters who were not, which is why we have contacted you. We would like to study both you and one of your brothers or sisters, to compare your heart structure and health with that of your brother or sister.

The information we have about you is printed on the enclosed form. We would be very grateful if you would check this information to confirm that you did receive a blood transfusion before you were born. If you are the person we are trying to locate, we would like to invite you to take part in a study which will look at your health now as an adult, particularly with regard to your heart. We would like to ask a number of questions about your health and the health of your brother or sister and organise for you both to undergo a cardiac MRI scan and some blood tests. A leaflet containing more detailed information about the study is enclosed with this letter. A second copy is also included for your brother or sister.

If you are the person we are trying to locate, we would be very grateful if you would fill in and return the attached form indicating your interest in finding out more about this study. We will then contact you to discuss the study in more detail. There is no obligation for you to take part and, if you do agree to participate, you may withdraw from the study at any time. If you have any questions about the proposed study or the original treatment, please do not hesitate to contact us on (0800) 500-194 or email to fetalanaemiastudy@auckland.ac.nz.

Thank you for your time, we look forward to hearing back from you.

Yours faithfully

Dr Alexandra Wallace  Professor Jane Harding
Liggins Institute  Liggins Institute
Dear <Mother's name>

Re: The Long Term Effects of Fetal Anaemia Study

We are researchers from the Liggins Institute at The University of Auckland. We are involved in a study of the health of people who suffered from anaemia as an unborn baby and received a blood transfusion for this before birth at National Women's Hospital.

The most common cause for severe anaemia before birth is rhesus disease. This occurs when there is a difference in particular blood groups (called rhesus groups) between a mother and her baby. This can be life-threatening. A method was developed at National Women's Hospital in 1963 to treat affected babies before birth by giving them blood transfusions. This pioneering treatment is now standard practice around the world. Our records indicate that you may have been one of the mothers who received this treatment at National Women's Hospital.

We now know that events before birth can change the risk of developing certain diseases as adults. Recent studies have shown that animals that were anaemic and were treated with blood transfusions before birth have altered structure of their heart and blood vessels as adults. We do not know if this occurs in humans, or what it might mean for their later health. The best way to answer this question is to see whether the heart structure and health of adults who were treated for anaemia before birth is different from those of their brothers or sisters who were not, which is why we have contacted you. We are trying to locate <name of child> and were wondering if you could help us with this task. We would like to invite both <name of child> and one of <his/her> brothers or sisters to take part in a follow-up study to compare their heart structures and health.

The reason for sending you this letter is to see if you can help us locate your <son/daughter>, <name of child>. If you are happy and able to, we would be very grateful if you could fill in and return the attached form, with their current contact address and/or telephone number. We will then contact <name of child> and ask them if they are interested in being involved in this study.

If you have any questions about the proposed study or the original treatment that you received, please do not hesitate to contact the study investigators on (0800) 500-194 or fetalanaemiastudy@auckland.ac.nz. We have enclosed an information sheet about this research for your information.

Thank you for your time. We look forward to hearing back from you.

Yours faithfully

Dr Alexandra Wallace
Liggins Institute

Professor Jane Harding
Liggins Institute
Dear <Mother's name>

**Re: The Long Term Effects of Fetal Anaemia Study**

We are researchers from the Liggins Institute at The University of Auckland. We are involved in a study of the health of people who suffered from rhesus disease as an unborn baby and received a blood transfusion for this before birth at National Women’s Hospital. Our records indicate that you may have had a baby who received this treatment at National Women’s Hospital before he or she was born.

The most common cause for severe anaemia before birth is rhesus disease. This occurs when there is a difference in particular blood groups (called rhesus groups) between a mother and her baby. This can be life threatening. A method was developed at National Women’s Hospital in 1963 to treat affected babies before birth by giving them blood transfusions. This pioneering treatment is now standard practice around the world.

We now know that events before birth can change the risk of developing certain diseases as adults. Recent studies have shown that animals that were anaemic and were treated with blood transfusions before birth have altered structure of their heart and blood vessels as adults. We do not know if this occurs in humans, or what it might mean for their later health. The best way to answer this question is to see whether the heart structure and health of adults who were treated for anaemia before birth is different from those of their brothers or sisters who were not anaemic before birth.

The reason for sending you this letter is to ask if you can confirm whether your baby did receive a blood transfusion before they were born and if so, if you can help us locate your child now. Unfortunately we have not been able to establish when or where your baby was born or if they were born alive. Therefore we apologise sincerely if this letter causes you any distress by rekindling painful memories. However, we would be very grateful if you could provide us with information about your child by completing and returning the attached form. If appropriate, we will then contact your child and ask them if they are interested in being involved in this study.

If you have any questions about the proposed study or the original treatment that you received, please do not hesitate to contact the study investigators on (0800) 500-194 or fetalanaemia@auckland.ac.nz. We have enclosed an information sheet about this research for your information.

Thank you for your time. We look forward to hearing back from you.

Yours faithfully

Dr Alexandra Wallace  
Liggins Institute

Professor Jane Harding  
Liggins Institute
Dear <Sibling’s name>

Re: The Long Term Effects of Fetal Anaemia Study

We are researchers from the Liggins Institute at The University of Auckland. We are involved in a study of the health of people who suffered from anaemia as an unborn baby and received a blood transfusion before birth at National Women’s Hospital. Our records indicate that your brother/sister XXXX [insert name if known] may have received this treatment at National Women’s Hospital before he or she was born.

The most common cause for severe anaemia before birth is rhesus disease. This occurs when there is a difference in particular blood groups (called rhesus groups) between a mother and her baby. This can be life threatening. A method was developed at National Women's Hospital in 1963 to treat affected babies before birth by giving them blood transfusions. This pioneering treatment is now standard practice around the world.

We now know that events before birth can change the risk of developing certain diseases as adults. Recent studies have shown that animals that were anaemic and were treated with blood transfusions before birth have altered structure of their heart and blood vessels as adults. We do not know if this occurs in humans, or what it might mean for their later health. The best way to answer this question is to see whether the heart structure and health of adults who were treated for anaemia before birth is different from those of their brothers or sisters who were not anaemic before birth.

So far we have been unable to find any contact details for your brother/sister. Therefore, the reason for sending you this letter is to ask if you can help us locate XXXX [or “your brother/sister” if name unknown]. We would be very grateful if you could provide us with information about your brother/sister by completing and returning the attached form. We will then contact them and ask if they are interested in being involved in this study.

If you have any questions about this study, please do not hesitate to contact the study investigators on (0800) 500-194 or fetalanaemiastudy@auckland.ac.nz. We have enclosed an information sheet about this research for your information.

Thank you for your time. We look forward to hearing back from you.

Yours faithfully

Dr Alexandra Wallace
Liggins Institute

Professor Jane Harding
Liggins Institute
PARTICIPANT INFORMATION SHEET

You are invited to take part in a study of

The Long-Term Effects of Fetal Anaemia

Thank you for taking the time to read this information leaflet.
Please read this leaflet carefully so that you can decide whether to take part in this study

Principal Investigator:
- Dr Alexandra Wallace, The Liggins Institute, University of Auckland, Private Bag 92019, Auckland, phone (0800) 500-194 or (027) 281-9766

Supervising Investigators:
- Professor Jane Harding, The Liggins Institute, University of Auckland, Private Bag 2019, Auckland, phone 09 373 7599
- Dr Stuart Dalziel, Auckland District Health Board, Park Road, Grafton, Private Bag 92024, Auckland, phone 09 307 4949
Introduction
You are invited to take part in a study of the effects in adults of having anaemia and blood transfusions before birth.

The most common cause for severe anaemia before birth is rhesus disease. This occurs when there is a difference in particular blood groups (called rhesus groups) between a mother and her baby. Because this can be life-threatening, a method was developed at National Women’s Hospital in Auckland in 1963 to treat affected babies before birth by giving them blood transfusions. This pioneering treatment is now standard practice around the world. You or your brother/sister received this treatment in Auckland and are therefore now one of the oldest people in the world to have been treated. Please read this brochure carefully and think about whether you would like to take part this study. Whether you take part or not is entirely your own decision.

Why do a follow-up study of adults?
We now know that events before birth can change the risk of developing certain diseases as adults. Recent studies have shown that animals that were anaemic and were treated with blood transfusions before birth have altered structure of their heart and blood vessels as adults. We do not know if this occurs in humans, or what it might mean for their later health. The best way to answer this question is to see whether the heart structure and health of adults who were treated for anaemia before birth is different from those of their brothers or sisters who were not anaemic.

Why have I been contacted?
We have contacted you because you or your brother or sister received a blood transfusion to treat anaemia before you or they were born. We would like to study both of you, to compare your heart structure and health with that of your brother or sister.

Do I have to take part?
No, your participation in this study is entirely voluntary (your choice). You do not have to take part in this study, and if you choose not to take part this will not affect your future health care in any way. If you do agree to take part you are free to withdraw from the study at any time, without having to give a reason and this will in no way affect your future health care. Please feel free to talk to others (e.g. Whanau/family, friends, your GP) about this study before agreeing to take part.

Who can take part?
We wish to study up to 160 people (80 sibling pairs) who:
- Received a blood transfusion before birth for fetal anaemia OR
- Is a brother or sister (preferably the same sex as them) who did not have anaemia before birth and who is also willing to take part.

If I decide to take part, what do I have to do?
Please discuss this study with your brother or sister and show them the extra copy of this brochure. If you both decide to take part, simply complete the enclosed form and return it to us by reply paid post. We will then contact you both to arrange an appointment that is convenient for you. If you live outside of Auckland, we will arrange and pay for transport to Auckland and accommodation for the night before the tests if this is required. You and your brother or sister can be seen together or separately; this is entirely your choice.
Where will this appointment be, and how long will it take?
The visit will take place at the Centre for Advanced MRI in the Radiology Department at Auckland City Hospital, Park Road, Grafton, Auckland. If you would like to be seen with your brother or sister, we will arrange for you both to come on the same day. It will take four hours in total for both you and your sibling to complete the study, including the MRI. Most visits will occur in the morning, from 8:00am to 1:30pm, although alternative arrangements can be made if this time does not suit.

What will happen at this appointment?
If you agree to take part in the study, you will be asked to:
- Sign the consent forms to take part in the study.
- Fill in a questionnaire about your health and lifestyle.
- Be weighed and have your height and blood pressure measured.
- Have an ECG tracing of your heart rhythm.
- Have some blood tests.
- Undergo an MRI (magnetic resonance imaging) scan of your heart.

Questionnaire. The questions will cover a variety of topics, such as your age, ethnicity, exercise levels, whether you smoke or drink alcohol, recreational drug use, reproductive history, any past or current illnesses, the health of your mother and father, and any medications you are currently taking. You do not have to answer all the questions, you may stop at any time, and all information collected will be treated as STRICTLY CONFIDENTIAL.

ECG. This involves recording the electrical rhythm of your heart using ECG leads placed on your chest. It is completely painless and takes only a few seconds.

Blood tests. You will be asked to have some blood tests to assess liver and kidney function, blood cholesterol levels and your body’s ability to manage sugar (a glucose tolerance test). If you do not know your blood group and it is not recorded in your medical notes, we will also check this. If the scan is done in the morning, these tests can be done on the same day as the scan, at the Liggins Institute. For the glucose test, blood needs to be drawn 3 times over 120 minutes. As you will require an intravenous cannula (a “drip”) for the MRI scan, we will aim to take the bloods from the cannula. This means you should only need one needle prick. If it is not possible to do the blood tests on the same day as the scan, you will be asked to return to have your bloods taken on another day or, if you live outside of Auckland, we will arrange for these tests to be done at a local laboratory.

Cardiac MRI scan. We will ask you not to consume caffeine (tea, coffee, chocolate, fizzy drinks etc) from the day before the MRI scan, and not to eat anything for 4 hours before the scan.

Before the scan we will ask you to have a “drip” placed in a vein in one arm. This will be used to give you a kind of dye, gadolinium, and another drug called adenosine during the scan, so that we are able to get the best images of your heart. These drugs are both licensed in New Zealand and are used daily in hospitals and for obtaining MRI scans. We will ask you to hold your breath for between 10 and 15 seconds for most of the pictures. We will also ask you to put one hand in iced water for 3 minutes before some of the pictures. The scan will take about 70 minutes, and you will be in contact with staff at all times. You are welcome to bring a CD of your choice that can be played during the scan.
All scans are performed by trained and registered MRI technologists, under the supervision of a cardiac radiologist, Director of the Centre or advanced trainee or researcher with specific experience in cardiac MRI imaging. Medically qualified and other experienced research staff will also available at the Centre for Advanced MRI. A qualified doctor will administer the drugs.

Unfortunately we cannot perform an MRI scan on people who:

- Are pregnant.
- Have known cardiac disease.
- Have a known allergy to medicines given during the MRI scan.
- Have active asthma.
- Are not able to go in the MRI scanner. This is particularly important if you have an implanted heart pacemaker or cochlear (ear) device. You will be required to fill out a check list on at least two occasions to ensure you do not have anything inside you that would prevent you from having an MRI scan.
- Suffer from claustrophobia.
- Are unable to hold their breath for the scan.

We will discuss all these things with you. If you agree to undergo an MRI scan but then cannot have the scan for any reason, your participation will still be useful to us in this study.

**Will any other information about me be collected?**
The investigators will need to access your medical (hospital) records in order to find out details about your mother’s pregnancy, the severity of your rhesus disease, the treatment you received for this as a baby and your subsequent health. We may also need to ask your GP for some of this information. We will discuss this further with you at the time of your appointment and ask your permission to do this on the consent form.

**What will happen to the information you collect about me?**
As New Zealand was the first place in the world where blood transfusions were given to babies before birth, you belong to the oldest group of people to have received this treatment. This means that the information collected in this study may be very important in the future. We therefore plan to store this information indefinitely, as it is possible we may want to contact you again in the future. All information will be kept confidential and stored securely.

**What are the benefits?**
The results will help future babies with anaemia who need blood transfusions before birth, as we will know more about the long-term safety of this treatment. We will also learn more about how events before birth can affect health in later life.

**What are the risks?**
**MRI.** MRI scanning has no known health risks. Participants who feel unwell or claustrophobic in the scanner will be able to press the emergency buzzer and be removed from the scanner. There is no exposure to radiation with an MRI scan.

**Adenosine infusion.** Adenosine is used worldwide for treating fast heart rates and for investigating angina in cardiac patients. It can produce chest discomfort (this is usually brief and mild), brief slowing of the heart and altered blood pressure. Adenosine stimulates breathing and approximately 14% of patients feel breathless and an urge to breathe deeply during adenosine infusion. Fewer than one person in a thousand experiences an important reaction to adenosine.
Gadolinium infusion. Gadolinium is used worldwide in millions of people each year and has an extremely low serious complication rate. In a combined MRI experience of greater than 20 years the Centre for Advanced MRI, staff have seen no serious side effects from it. However, short lasting and minor side effects (mild headache and nausea) are quite common.

In the event that a condition which is assessed to be a clinical abnormality is detected through performing a scan on you, you will be informed of this and will be advised to consult your general practitioner or other health professional of your choice.

Because the images are not routinely reviewed by a radiologist we are unable to perform diagnostic scans for medical purposes of areas where you have known abnormalities.

You should be aware that if a clinical abnormality is detected through performing a scan on you, this could affect your ability to obtain insurance whether or not you take the matter further.

Compensation - what will happen if I experience any ill effects?
In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation and Compensation Act. ACC cover is not automatic and your case will need to be assessed by ACC according to the provisions of the 2002 Injury Prevention Rehabilitation and Compensation Act. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators. If you have any questions about ACC, contact your nearest ACC office or the investigator.

Will I receive any payment for taking part in the study?
No. This is a voluntary study. There will be no costs to you as you will receive reimbursement for any travel expenses incurred. If you live in Auckland, you will receive a petrol voucher to help cover the costs of transport to the Centre for Advanced MRI. If you live outside of Auckland, the cost of transport to Auckland for participation in this study will be paid for by the study.

If I need an interpreter, can one be provided?
Yes. If you wish the appointment to be conducted in your own language an interpreter can be provided for this. Additionally, an interpreter can be provided to help you better understand this information sheet if you require one.

What will happen at the end of the study?
The cardiac MRI scans will be analysed by researchers at the Auckland MRI Research Group. In addition, some of the more technical MRI data will be analysed by doctors at the Heart Research Center, Oregon Health Sciences University, in Oregon, USA.

Data from this study may be used to develop new computer software and analysis methods for MRI scans of the heart. Data may also be included as part of larger international combined studies or used for educational purposes such as publication in journals, books, lectures or the internet. No data that might directly identify you will be used for these purposes.
At the end of the study, if you wish to receive it, you will be sent a brief summary of the study findings. The main results will be published in an international medical journal. The information collected in this study will be stored indefinitely because it is possible that further questions might arise in the future about the long-term effects of anaemia or blood transfusions before birth.

**How confidential will the information be?**
All information collected from you will be treated as STRICTLY CONFIDENTIAL and will be stored in a locked cabinet in a locked room. The information will be available to the small team of researchers at The University of Auckland. No material that could possibly identify you will be used in any of the reports of this study. You are free to have access to your information at any time.

**Will I get a copy of the information you collect about me?**
We will send you a copy of the results of your blood tests and body size data if you wish. A copy of the final results of the study can also be sent to you.

**Will my GP be told I am in the study?**
If you wish, your GP can be informed of your involvement with the study and sent the results of your blood tests and body size data. We will obtain your consent to do this at the time of the appointment.

**Where can I get more information about the study?**
If you have any problems or questions regarding this study you may contact one of the investigators on (0800) 500-194 or email fetalanaemiastudy@auckland.ac.nz.

You may have a friend or Whanau/family support to help you understand the risks and/or benefits of this study and any other explanation you may require.

If you have any queries or concerns regarding your rights as a participant in this study you may wish to contact an independent Health and Disability Advocate, telephone:
Free phone (NZ wide): 0800 555 050
Free Fax (NZ wide): 0800 2787 7678 (0800 2 SUPPORT)
Email (NZ wide): advocacy@hdc.org.nz

For Maori health support, or to discuss any concerns or issues regarding this study, please contact Mata Forbes RGON, Maori Health Services Co-ordinator / Advisor, 5th Level, GM Suite, Auckland City Hospital. Telephone 09 307 4949 extension 23939 or mobile 021 348 432.
Appendix 3: Fetal Anaemia Study questionnaire
The Long-term Effects of Fetal Anaemia
QUESTIONNAIRE
Date questionnaire completed: 1920

Demographic details

1. What is your date of birth?

2. How old are you

3. Sex (please tick box)
   - Male
   - Female

4. Which ethnic groups do you belong to? (answer all questions)
   - Yes
   - No
   4.1 ☐ ☐ New Zealand European
   4.2 ☐ ☐ Māori
   4.3 ☐ ☐ Samoan
   4.4 ☐ ☐ Cook Island Maori
   4.5 ☐ ☐ Tongan
   4.6 ☐ ☐ Niuean
   4.7 ☐ ☐ Chinese
   4.8 ☐ ☐ Indian
   4.9 ☐ ☐ Other (such as Dutch, Japanese, Tokelauan)

   If other, please specify: ___________________________________________

   Office use only
5. What is your marital status? (tick one only)
- Married or living with partner
- Divorced
- Separated
- Widowed
- Never married (single)

6. What is the highest level of education that you have received? (tick one only) If you are currently attending one of these institutions, please tick that box.
- Primary school / intermediate school
- High school / secondary school
  - How many years did you spend at high school (form 3/Year 9 onwards)?
- Polytechnic or similar
- University

7. Do you or your family own or rent the home you live in? If you have a mortgage on the home in which you live, please answer “Own” (tick one only)
- Own
- Rent

8. Do you own a car, van, truck or similar vehicle? (please do not include motorcycles) (tick one only)
- Yes
- No

9. Which of the following categories best describes your current work situation? (please answer all questions. Please be as specific as possible (eg. Fitter and turner, secretary, primary school teacher, lawyer)

9a. Yes No
   - A worker for pay
     - What is your main job? ____________________________
     - How many hours per week do you work in this job?

9b. Yes No
   - Self-employed
     - What is your main job? ____________________________
     - How many hours per week do you work in this job?
9c. Yes No A homemaker
What was your main job before becoming a homemaker?

| How many hours per week do you work in this job? |
|---|---|

| What is your spouse or partner’s main job now? |
|---|---|

| How many hours per week do they work in this job? |
|---|---|

9d. Yes No A student
What is (or was) your father’s main job?

| How many hours per week did/does he work in this job? |
|---|---|

| What is (or was) your mother’s main job? |
|---|---|

| How many hours per week did/does she work in this job? |
|---|---|

9e. Yes No Unemployed
What was your main job before becoming unemployed?

| How many hours per week did you work in this job? |
|---|---|

9f. Yes No Other
Please specify

| How many hours per week? |
|---|---|

10. What type of living arrangements do you currently have? **(tick one only)**

- Living on your own or in a flatting situation (where your income is not shared with the rest of the household)
- In a family situation or living with your partner (where, if you are working, your income is shared with the rest of the household)
11. What was your total personal income and your household’s total income before tax, during the past 12 months? (please tick one for yourself and one for your household)

Include income from all sources: spouse (if appropriate), children or boarders; wages, salary, commission, business or farming income (less expenses), income support and accident compensation regular payments; interest, dividends, rent, inheritance; Child support payments

<table>
<thead>
<tr>
<th>Yourself</th>
<th>Household</th>
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Smoking

12. Have you ever smoked cigarettes (not cigars/pipes) once a week or more? Cigarettes includes both manufactured and “roll your own” (tick one only)

| ○ Yes (continue to Q13) |
| ○ No (go to Q18) |

13. How old were you when you started smoking once a week or more?

| Years |

14. Do you smoke cigarettes (not cigars/pipes) now? (tick one only)

| ○ Yes (go to Q16) |
| ○ No (continue to Q15) |

15. How old where you when you stopped smoking cigarettes?

| Years -please go to Q17 |

16. What kind of cigarettes do you smoke? (answer all questions)

16.1 ○ ○ Manufactured
16.2 ○ ○ Hand-made (roll your own)
17. During the years that you have smoked (or did smoke), on average, how many manufactured cigarettes would you have smoked each day?

   Cigarettes each day

18. Do you currently smoke cigars once a week or more? (tick one only)
   - Yes (continue to Q19)
   - No (go to Q21)

19. How many cigars do you usually smoke each week?

   Cigars each week

20. How many years have you smoked cigars?

   Years

21. Do you currently smoke a pipe once a week or more? (tick one only)
   - Yes (continue to Q22)
   - No (go to Q24)

22. How many times a week do you usually smoke a pipe?

   Times per week

23. How many years have you smoked a pipe?

   Years

24. Does your husband/wife/partner smoke? (tick one only)
   - No
   - Yes
   - Not applicable

25. How many hours each week do you usually spend near someone who is smoking? (please include exposure at work, home and all other places. Put 0 if none)

   Hours per week
Your parents’ smoking habits

26. Did your father ever smoke regularly during your childhood? *(tick one only)*
- Yes
- No
- Don’t know

27. Did your mother ever smoke regularly during your childhood, or before you were born? *(tick one only)*
- Yes *(continue to Q28)*
- No *(go to Q29)*
- Don’t know *(go to Q29)*

28. When your mother was pregnant, in particular with you, did she: *(tick one only)*
- Stop smoking before pregnancy
- Cut down or stop during pregnancy
- Smoke as usual during pregnancy
- Don’t know

Alcohol

29. Have you ever drunk alcohol once a month or more? *(tick one only)*
- Yes *(continue to Q30)*
- No *(go to Q34)*

30. Do you currently drink alcohol once a month or more? *(tick one only)*
- Yes *(continue to Q31)*
- No *(go to Q34)*

31. About how often do you currently drink alcohol? *(tick one only)*
- 6-7 days a week
- 4-5 days a week
- 2-3 days a week
- once a week
- once every 2 weeks
- once a month
For the next two questions please refer to the following guide:

Drink equivalents:
1 drink = 1 can, small bottle or handle of beer or home brew
2 drinks = 1 quart/750ml bottle of beer
3 drinks = 1 jug of beer
6 drinks = 1 flagon/peter of beer
1 drink = 1 glass wine or sherry
6 drinks = 1 bottle of wine
1 drink = 1 double nip of spirits
1 drink = 1 premixed spirit-based drink (such as alcopops, ready to go drinks)

32. On an average day when you drink alcohol, how many drinks would you usually have in total?  
   Drinks

33. In the past 3 months, what is the largest number of drinks that you had on any one day?  
   Drinks

Recreational drug use

34. During the past 12 months, how often did you use marijuana (also known as grass, pot, cannabis, hashish, hash oil)?  (tick one only)
   ○ Not at all
   ○ Less than once a month
   ○ Once a month
   ○ Once every 2 weeks
   ○ Once a week or more often
35. During the past 12 months, how often did you use other illegal drugs (that is, those not prescribed by your doctor or bought from a chemist, such as cocaine, LSD, amphetamines, or speed, heroin, morphine, ecstasy etc)? (tick one only)

- Not at all
- Less than once a month
- Once a month
- Once every 2 weeks
- Once a week or more often

Exercise

36. In a normal week would you usually do any vigorous activity or exercise, either at work or away from work, that makes you breathe hard or sweat? (tick one only)

- Yes (continue to Q37)
- No (go to Q39)

37. On how many days a week would you usually do this activity?

☐ Day(s) out of 7

38. On the days you do this activity, about how long would you do it for?

☐ : ☐ hrs mins

39. In a normal week would you usually do any moderate activity or exercise, either at work or away from work, such as brisk walking, cycling or mowing the lawn? (tick one only)

- Yes (continue to Q40)
- No (go to Q42)

40. On how many days a week would you usually do this activity?

☐ Day(s) out of 7

41. On the days you do this activity, about how long would you do it for?

☐ : ☐ Hrs mins
# Medical history

42. **Has a doctor ever told you that you have high blood pressure?** *(tick one only)*
- Yes *(continue to Q43)*
- No *(go to Q45)*
- Don’t know *(go to Q45)*

43. **Do you currently have high blood pressure?** *(tick one only)*
- Yes *(continue to Q44)*
- No *(go to Q45)*
- Don’t know *(go to Q45)*

44. **What treatment are you currently having for your high blood pressure?** *(answer all questions)*
- Yes/No
- None
- Pills/other medication
- Diet change
- Change in lifestyle (eg. reduced alcohol consumption, more exercise)

45. **Has a doctor ever told you that you have high cholesterol?** *(tick one only)*
- Yes
- No
- Don’t know

46. **Has a doctor ever told you that you have had a heart attack or angina?** *(tick one only)*
- Yes
- No
- Don’t know

47. **Has a doctor ever told you that you have had a stroke?** *(tick one only)*
- Yes
- No
- Don’t know
48. Has a doctor ever told you that you have diabetes? *(tick one only)*

- Yes *(continue to Q49)*
- No *(go to Q52)*
- Don’t know *(go to Q52)*

49. How many years have you had diabetes?

[ ] Years

50. What type of diabetes do you have? *(tick one only)*

- Type 1 (Child onset)
- Type 2 (Adult onset)
- Don’t know

51. What treatment are you currently having for your diabetes? *(answer all questions)*

- Yes
- No

51.1 [ ] [ ] Pills
51.2 [ ] [ ] Diet
51.3 [ ] [ ] Insulin injections

Yes No

- Although you currently use insulin, in the past have you ever taken pills to control your diabetes?
52. **Do you have (or have you had) any other medical conditions not mentioned above?**

*Please write any condition down using block letters and be as specific as possible (e.g. Cancer of the breast, epilepsy etc.).*

- Yes (please complete below)
- No (go to Q53)

<table>
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<tr>
<th>Name of condition</th>
<th>Condition code</th>
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53. **Do you regularly take (one a week or more) any prescription or non-prescription medicine, pill or tablets?** Include injections, sprays (such as those used for asthma), patches, creams and oral contraceptives, and non-prescription treatments such as Disprin, Panadol and Quickeze. Do not include vitamins or other food supplements. (Tick one only)

- Yes (continue to Q54)
- No (go to Q55)
54. Please list all medications using block letters. Where possible copy names directly off packaging and include dosage). Include injections, sprays (such as those used for asthma) patches, creams and oral contraceptives, and non-prescription treatments such as Disprin Panadol and Quickezeze. Do not include vitamins or other food supplements.

<table>
<thead>
<tr>
<th>Name</th>
<th>How often do you take it? (eg. daily, weekly, when needed, etc.)</th>
<th>Dose/units</th>
<th>Was it prescribed (circle answer)</th>
<th>Medication code (Office use only)</th>
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<td>Yes / No</td>
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</table>

Respiratory

Wheeze and tightness in the chest

55. Have you ever had wheezing or whistling in the chest at any time in the last 12 months? (Tick one only)
- Yes (continue to Q56)
- No (go to Q58)

56. Have you been at all breathless when the wheezing noise was present? (Tick one only)
- Yes
- No

57. Have you had this wheezing or whistling when you did not have a cold? (Tick one only)
- Yes
- No
58. Have you woken up with a feeling of tightness in your chest at any time in the last 12 months? *(Tick one only)*
   - Yes
   - No

**Shortness of breath**

59. Have you had an attack of shortness of breath that came on during the day when you were at rest at any time in the last 12 months? *(Tick one only)*
   - Yes
   - No

60. Have you had an attack of shortness of breath that came on following strenuous activity at any time in the last 12 months? *(Tick one only)*
   - Yes
   - No

61. Have you been woken by an attack of shortness of breath at any time in the last 12 months? *(Tick one only)*
   - Yes
   - No

**Cough and phlegm from the chest**

62. Have you been woken by an attack of coughing at any time in the last 12 months? *(Tick one only)*
   - Yes
   - No

63. Do you usually cough first thing in the morning (or on getting up if you are a night shift worker) in the winter? *(Tick one only)*
   - Yes
   - No

64. Do you usually cough during the day, or at night, in the winter? *(Tick one only)*
   - Yes *(continue to Q65)*
   - No *(go to Q66)*
65. Do you cough like this on most days for as much as three months each year? *(Tick one only)*
- Yes
- No

66. Do you *usually* bring up any phlegm from your chest first thing in the morning (or on getting up if you are a night shift worker) in the winter? *(Tick one only)*
- Yes
- No

67. Do you *usually* bring up any phlegm from your chest during the day, or at night, in the winter? *(Tick one only)*
- Yes *(continue to Q68)*
- No *(go to Q69)*

68. Do you bring up phlegm like this on most days for as much as three months each year? *(Tick one only)*
- Yes
- No

### Breathing

69. Do you ever have trouble with your breathing? *(Tick one only)*
- Yes *(continue to Q70)*
- No *(go to Q71)*

70. Do you have this trouble? *(Tick one only)*
- Continuously, so that your breathing is never quite right?
- Repeatedly, but it always gets completely better?
- Only rarely?

71. Are you disabled from walking by a condition other than heart or lung disease? *(Tick one only)*
- Yes *(state condition and go to Q75)*
- No *(go to Q72)*
72. Are you troubled by shortness of breath when hurrying on level ground or walking up a slight hill?  *(Tick one only)*
   - Yes (continue to Q73)
   - No (go to Q75)

73. Do you get short of breath walking with other people of your own age on level ground?  *(Tick one only)*
   - Yes (continue to Q74)
   - No (go to Q75)

74. Do you have to stop for breath when walking at your own pace on level ground?  *(Tick one only)*
   - Yes
   - No

Asthma

75. Have you ever had asthma?  *(Tick one only)*
   - Yes (continue to Q76)
   - No (go to Q83)

76. Was this confirmed by a doctor?  *(Tick one only)*
   - Yes
   - No

77. How old were you when you had your first attack of asthma?
   - ___ Years

78. How old were you when you had your most recent attack of asthma?
   - ___ Years
79. Which months of the year do you usually have attacks of asthma?  
*(answer all questions)*

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<tr>
<th>Yes</th>
<th>No</th>
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<tbody>
<tr>
<td>79.1</td>
<td>January/February</td>
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<td>79.2</td>
<td>March/April</td>
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<td>79.3</td>
<td>May/June</td>
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<td>79.4</td>
<td>July/August</td>
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<td>79.5</td>
<td>September/October</td>
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<tr>
<td>79.6</td>
<td>November/December</td>
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</table>

80. Have you had an attack of asthma in the last 12 months? *(tick one only)*

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<thead>
<tr>
<th>Yes</th>
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<tbody>
<tr>
<td>Yes (continue to Q81)</td>
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<tr>
<td>No (go to Q82)</td>
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</table>

81. How many attacks of asthma have you had in the last 12 months?

<table>
<thead>
<tr>
<th>Number</th>
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82. Are you currently taking any medicines, including inhalers, aerosols or tablets, for asthma? *(tick one only)*

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<th>Yes</th>
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<td>Yes</td>
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Other allergy-related conditions

83. Do you have any nasal allergies, including hay fever? *(tick one only)*

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<tr>
<th>Yes</th>
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84. Have you ever had eczema or any kind of skin allergy? *(tick one only)*

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85. Are you allergic to any insect stings or bites? *(tick one only)*

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<tr>
<td>Yes (continue to Q86)</td>
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<td>No (go to Q87)</td>
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</table>
86. Which insect(s)?

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<thead>
<tr>
<th>Insect</th>
<th>Kind of reaction (tick all boxes)</th>
<th>Other (please specify)</th>
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<td>Breathing difficulty, feeling faint, nausea or fever</td>
<td>Yes No</td>
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87. Have you ever had any difficulty with your breathing after taking medicines? (Tick one only)

- o Yes (continue to Q88)
- o No (go to Q99)

88. Which medicines? (please list all medications in block letters)

<table>
<thead>
<tr>
<th>Name</th>
<th>Medication code (office use only)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Background of mother

89. What was your mother's date of birth (or age now, if you don’t know her date of birth)?

Date of birth

day month year

or

Age (years)
90. **Is your mother still alive? (tick one only)**

- Yes (go to Q94)
- No (continue to Q91)

91. **At what age did she die?**

___ Years

92. **What did she die of?**

Please specify: ____________________________ office use only

93. **If she died of cancer, please specify the main site of the cancer.**

Please specify: ____________________________ office use only

94. **Was your mother ever told by a doctor that she had high blood pressure? (tick one only)**

- Yes, but she only had it during pregnancy
- Yes, but it was unrelated to any pregnancies
- Yes, but I’m not sure if it was related to any pregnancies or not
- No
- Don’t know

95. **Was your mother ever told by a doctor that she had had a stroke? (tick one only)**

- Yes
- No
- Don’t know

96. **Was your mother ever told by a doctor that she had had a heart attack or angina? (tick one only)**

- Yes
- No
- Don’t know
97. **Was your mother ever told by a doctor that she had high cholesterol? (tick one only)**
   - Yes
   - No
   - Don’t know

98. **Was your mother ever told by a doctor that she had asthma? (tick one only)**
   - Yes
   - No
   - Don’t know

99. **Was your mother ever told by a doctor that she had diabetes? (tick one only)**
   - Yes, but she only had it during pregnancy (continue to Q100)
   - Yes, but it was unrelated to any pregnancies (continue to Q100)
   - Yes, but I’m not sure if it was related to any pregnancies or not (continue to Q100)
   - No (go to Q103)
   - Don’t know (go to Q103)

100. **How many years did she have diabetes? (answer one only)**
   - [ ] ___ Years
   - Don’t know

101. **What type of diabetes did she have? (tick one only)**
   - Type 1 (Child onset)
   - Type 2 (Adult onset)
   - Don’t know

102. **What treatment did she have for her diabetes? (answer all questions)**
   - Yes
   - No
   - Pills
   - Diet
   - Insulin injections
   - Don’t know
Background of father

103. What was your father’s date of birth (or age now, if you don’t know his date of birth)?

|   |   |   | Date of birth

or

|   |   | Age (years)

104. Is your father still alive? (tick one only)

○ Yes (go to Q108)
○ No (continue to Q105)

105. At what age did he die?

|   |   | Years

106. What did he die of?

Please specify: ___________________________________________ office use only

107. If he died of cancer, please specify the main site of the cancer.

Please specify: ___________________________________________ office use only

108. Was your father ever told by a doctor that he had high blood pressure? (tick one only)

○ Yes
○ No
○ Don’t know

109. Was your father ever told by a doctor that he had a stroke? (tick one only)

○ Yes
○ No
○ Don’t know
110. **Was your father ever told by a doctor that he had had a heart attack or angina? (tick one only)**

- Yes
- No
- Don’t know

111. **Was your father ever told by a doctor that he had high cholesterol? (tick one only)**

- Yes
- No
- Don’t know

112. **Was your father ever told by a doctor that he had asthma? (tick one only)**

- Yes
- No
- Don’t know

113. **Was your father ever told by a doctor that he had diabetes? (tick one only)**

- Yes (continue to Q114)
- No (go to Q117)
- Don’t know (go to Q117)

114. **How many years did he have diabetes? (answer one only)**

[ ] ______ Years

115. **What type of diabetes did he have? (tick one only)**

- Don’t know
- Type 1 (Child onset)
- Type 2 (Adult onset)

116. **What treatment did he have for his diabetes? (answer all questions)**

- 116.1 Yes: Pills
- 116.2 Yes: Diet
- 116.3 Yes: Insulin injections
- 116.4 Yes: Don’t know
Reproductive history *(women only - men go to Q141)*

117. **How old were you when you first began to have menstrual periods?** *(answer one only)*

|   | Years | Don’t know |

118. **Have you had a menstrual period in the last year?** *(tick one only)*

|   | Yes *(go to Q121)* | No *(continue to Q119)* |

119. **How old were you when you had your last natural period?**

|   | Years |

120. **Why did your periods end?** *(tick one only)*

|   | Because of early menopause (the change of life) | Because of a surgical operation | Because of another medical treatment (eg. Chemotherapy) | Breastfeeding | Pregnancy | Other *(please specify):* ____________________________ |

**Contraceptives**

121. **Have you ever used oral contraceptives (the pill)?** *(tick one only)*

|   | Yes *(continue to Q122)* | No *(go to Q126)* |

122. **How old were you when you started taking oral contraceptives?**

|   | Years |

123. **For how many years altogether have you used oral contraceptives?**

<p>|   | Years |</p>
<table>
<thead>
<tr>
<th>124.</th>
<th>Do you use oral contraceptives now? <em>(tick one only)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes <em>(continue to Q125)</em></td>
</tr>
<tr>
<td></td>
<td>No <em>(go to Q126)</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>125.</th>
<th>What brand do you use?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Please specify:* 

office use only

<table>
<thead>
<tr>
<th>126.</th>
<th>Have you ever had contraceptive injections (eg. Depo Provera)? <em>(tick one only)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes <em>(continue to Q127)</em></td>
</tr>
<tr>
<td></td>
<td>No <em>(go to Q130)</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>127.</th>
<th>How old were you when you started receiving contraceptive injections?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Years</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>128.</th>
<th>For how many years altogether have you received contraceptive injections?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Years</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>129.</th>
<th>Do you receive contraceptive injections now? <em>(tick one only)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>130.</th>
<th>Have you ever had the morning-after/emergency pill? <em>(tick one only)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes <em>(continue to Q131)</em></td>
</tr>
<tr>
<td></td>
<td>No <em>(go to Q132)</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>131.</th>
<th>How many times have you had the morning-after/emergency pill? <em>(answer one only)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Times</td>
</tr>
<tr>
<td></td>
<td>Don’t know</td>
</tr>
</tbody>
</table>
### Pregnancy

132. Are you currently pregnant? *(tick one only)*

- Yes
- No
- Don't know

133. Have you been pregnant before? *(tick one only)*

- Yes (continue to Q134)
- No (go to Q143)

134. How many times have you been pregnant (include all pregnancies of 20 weeks duration or more)?

- Number of pregnancies

135. Please list the details all babies you have given birth to (include stillbirths/miscarriages of 20 weeks duration or more. Please list one child per row)

<table>
<thead>
<tr>
<th>Child</th>
<th>Date of birth (day/month/year)</th>
<th>Sex (please circle one)</th>
<th>How many weeks gestation?</th>
<th>Birthweight (lb / kg)</th>
<th>Was the baby born alive? (please circle one)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
</tbody>
</table>
136. How many miscarriages (at less than 20 weeks pregnant) have you had?

Number of miscarriages

137. During your pregnancy did a doctor ever tell you that your blood pressure was high?  
(tick one only)

- Yes (continue to Q138)
- No (go to Q139)
- Don’t know (go to Q139)

138. What treatment did you receive for your high blood pressure you developed when you were pregnant? (answer all questions)

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>138.1</td>
<td></td>
</tr>
<tr>
<td>138.2</td>
<td></td>
</tr>
<tr>
<td>138.3</td>
<td></td>
</tr>
<tr>
<td>138.4</td>
<td></td>
</tr>
<tr>
<td>138.5</td>
<td></td>
</tr>
</tbody>
</table>

139. During your pregnancy did a doctor ever tell you that you had developed diabetes as a result of your pregnancy?  
(tick one only)

- Yes (continue to Q140)
- No (go to Q143)
- Don’t know (go to Q143)

140. What treatment did you receive for the diabetes you developed when you were pregnant?  
(answer all questions)

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>140.1</td>
<td></td>
</tr>
<tr>
<td>140.2</td>
<td></td>
</tr>
<tr>
<td>140.3</td>
<td></td>
</tr>
<tr>
<td>140.4</td>
<td></td>
</tr>
<tr>
<td>140.5</td>
<td></td>
</tr>
<tr>
<td>140.6</td>
<td></td>
</tr>
</tbody>
</table>
Reproductive history (men only - women go to Q143)

141. How many babies have you fathered (include all pregnancies of 20 weeks duration or more)

Number of babies

142. Please list the details of all babies you have fathered (include stillbirths/miscarriages of 20 weeks duration or more. Please list one child per row)

<table>
<thead>
<tr>
<th>Child</th>
<th>Date of birth (day/month/year)</th>
<th>Sex (please circle one)</th>
<th>How many weeks gestation?</th>
<th>Birthweight (lb / kg)</th>
<th>Was the baby born alive? (please circle one)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>M / F</td>
<td></td>
<td></td>
<td>Yes / No</td>
</tr>
</tbody>
</table>
Birth history

143. How much did you weigh at birth? *(answer one only)*

[ ] Kg or [ ] lb
[ ] Don’t know

144. How many weeks pregnant was your mother when you were born?

[ ] Number of weeks

Rhesus Disease, blood transfusions and treatment for jaundice

145. What is your blood group?

[ ] Blood group
[ ] Don’t know

146. Did you receive a blood transfusion before birth?

[ ] Yes *(continue to question 147)*
[ ] No *(go to question 151)*
[ ] Don’t know *(go to question 151)*

147. How many blood transfusions did you receive before birth?

[ ] Number of blood transfusions before birth
[ ] Don’t know

148. How many weeks pregnant was your mother when Rhesus Disease was diagnosed?

[ ] Number of weeks
[ ] Don’t know
149. How many weeks pregnant was your mother at the time of your first blood transfusion?

<table>
<thead>
<tr>
<th>Number of weeks</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

150. If you received more than one blood transfusion before birth, how many weeks pregnant was your mother at the time of your subsequent transfusions?

<table>
<thead>
<tr>
<th>Weeks at 2nd transfusion</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks at 3rd transfusion</td>
<td>Don’t know</td>
</tr>
<tr>
<td>Weeks at 4th transfusion</td>
<td>Don’t know</td>
</tr>
<tr>
<td>Weeks at 5th transfusion</td>
<td>Don’t know</td>
</tr>
<tr>
<td>Weeks at 6th transfusion</td>
<td>Don’t know</td>
</tr>
<tr>
<td>Weeks at 7th transfusion</td>
<td>Don’t know</td>
</tr>
<tr>
<td>Weeks at 8th transfusion</td>
<td>Don’t know</td>
</tr>
<tr>
<td>Weeks at 9th transfusion</td>
<td>Don’t know</td>
</tr>
</tbody>
</table>

(Go to question 152)

151. If you did not receive a blood transfusion before birth, were you still affected by Rhesus Disease?

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

152. Did you receive phototherapy after you were born?

<table>
<thead>
<tr>
<th>Yes</th>
<th>Number of days</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

153. Did you receive an exchange (blood) transfusion after you were born for the treatment of jaundice or rhesus disease?

<table>
<thead>
<tr>
<th>Yes</th>
<th>Number of exchange transfusions</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

154. Did you receive a top-up blood transfusion after you were born to treat a low blood count (anaemia)?

<table>
<thead>
<tr>
<th>Yes</th>
<th>Number of top up transfusions</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
General comments

155. Do you have any comments you would like to add regarding this study?

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

Thank you for taking the time to complete this questionnaire. Please bring it with you to your appointment and give to the research nurse or doctor.
Appendix 4: Forms and letters for dissemination of body size, blood pressure and blood results to Fetal Anaemia Study participants and their General Practitioners
Participant Assessment Results

<table>
<thead>
<tr>
<th>Name</th>
<th>NHI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study ID Number</td>
<td>DOB</td>
</tr>
<tr>
<td>Gender</td>
<td>Date assessed</td>
</tr>
</tbody>
</table>

The following readings were taken by a registered nurse for the Fetal Anaemia Study (MEC/09/04/037). Please note that these results were obtained for research purposes only.

**Blood pressure**

<table>
<thead>
<tr>
<th>Test</th>
<th>Reading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Systolic (mmHg)</td>
</tr>
<tr>
<td>BP reading 1</td>
<td></td>
</tr>
<tr>
<td>BP reading 2</td>
<td></td>
</tr>
<tr>
<td>BP reading 3</td>
<td></td>
</tr>
</tbody>
</table>

**Body size data**

<table>
<thead>
<tr>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>Head circumference (cm)</th>
<th>Body Mass Index (BMI)</th>
</tr>
</thead>
</table>

**Blood results** (Reference Ranges from LabPLUS Laboratory Test Guide)

<table>
<thead>
<tr>
<th>Test Name</th>
<th>Reference Range</th>
<th>Result</th>
<th>Test Name</th>
<th>Reference Range</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>135-145 mmol/L</td>
<td>Haemoglobin</td>
<td>115 - 154 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td>3.5-5.2 mmol/L</td>
<td>Haematocrit</td>
<td>0.36 – 0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>3.2-7.7 mmol/L</td>
<td>MCV</td>
<td>82-99 fL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td>45-90 mmol/L</td>
<td>MCH</td>
<td>27-33 pg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>38-52 mmol/L</td>
<td>Platelet count</td>
<td>150 - 400 x10^9/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Bilirubin</td>
<td>0-24 µmol/L</td>
<td>White Cell Count</td>
<td>4.1 - 11.7 x10^9/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamma GT</td>
<td>0-50 U/L</td>
<td>Neutrophils</td>
<td>1.9 - 7.9 x10^9/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>40-100 U/L</td>
<td>Lymphocytes</td>
<td>1.1 - 4.0 x10^9/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>0-45 U/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td>&lt;45 U/L</td>
<td>Oral Glucose Tolerance Test</td>
<td>Standard 75 gram glucose load given</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (fasting)</td>
<td>&lt;5.0 mmol/L</td>
<td>(fasting glucose)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>&gt;1.0 mmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chol/HDL ratio</td>
<td>&lt;4.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>&lt;3.4 mmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>&lt;2.0 mmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Dear XXXX

Thank you very much for taking part in the Fetal Anaemia Study. Without the participation of people like you this study could not take place.

If you requested that your GP receive your blood results, blood pressure and body size data these have been sent to your named GP. If you requested a copy of these results for yourself, they are included with this letter. If you would like to discuss these results further please contact your GP directly.

If you have requested the final study results, we will write to you when the study is finished, probably in late 2012.

It would be a great help if you could inform us of any change of address you might have in the future, so that we can keep in contact with you if required.

In the meantime, if there is anything further you would like to discuss about the study, please do not hesitate to contact us on (0800) 500-194 or at fetalanaemiastudy@auckland.ac.nz.

Once again, many thanks on behalf of the Fetal Anaemia Study team for your valuable contribution.

Yours sincerely

Dr Alexandra Wallace
Research Fellow
Liggins Institute

Ref: ID no: XXXX
Dear Dr <GP name>

Fetal Anaemia Study: Clinical results for <participant’s name> <DOB>

Your patient XXXX recently participated in the Fetal Anaemia Study. They requested that we send to you the results of their blood pressure, body size data and blood test results, and these are enclosed. These results have also been sent to XXXX at their request. However no interpretation of these results has been supplied, and we rely on you for any decisions about clinical management that may be indicated as a result of these tests.

The Fetal Anaemia Study is a follow up study to investigate the cardiovascular health of adults who received in utero transfusion for rhesus haemolytic disease. In 1963, the technique of in utero blood transfusion for babies affected by rhesus haemolytic disease was pioneered in Auckland by Sir William Liley. Since then, this treatment has become the standard of care around the world. Small follow up studies of recipients of in utero transfusion have demonstrated no effect on development into late childhood. However, there have been no follow up studies of later cardiovascular function.

This study is investigating the long-term effects of fetal anaemia on cardiac function using cardiac MRI scans, together with assessment of cardiovascular risk factors (body size, blood pressure, serum lipids, glucose tolerance), renal function and liver function. Unaffected siblings of affected individuals are being studied as a comparison group. Therefore, your patient, <Participant name>, either received in utero transfusion or is the unaffected sibling of an in utero transfusion recipient.

This study has received ethical approval from the Multi-Region Ethics Committee. Your patient gave written consent to participate and for us to send these results to you. Should you require any further details about the study, please feel free to contact me on (0800) 500-194 or fetalanaemiastudy@auckland.ac.nz.

Yours sincerely

Dr Alexandra Wallace

Principal Investigator, Fetal Anaemia Study
Liggins Institute
University of Auckland

Ref: ID no. <XXXX>
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