

Developmental programming of allergic susceptibility

Amy Louise Wooldridge, BSc (Hons)

Early Origins of Health and Disease Research Group

Robinson Research Institute

Department of Obstetrics and Gynaecology

Adelaide School of Medicine

Faculty of Health Sciences

University of Adelaide

South Australia

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THE UNIVERSITY

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"The real voyage of discovery consists not in seeking new landscapes, but in having new eyes."

-Marcel Proust

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ABSTRACT

Allergic susceptibility is associated with early life exposures, including intrauterine growth restriction and maternal allergy. Epidemiological and animal model studies suggest that restricted growth before birth is protective against later allergy development, whilst maternal allergy is generally associated with increased allergy risk in progeny. Causality and mechanisms mediating these associations are poorly understood, and I therefore investigated immune and allergic responses in ovine models following these prenatal exposures.

The first aim of study one (**chapter 2**) was to determine the effects of intrauterine growth restriction, due to placental restriction (PR), on allergic susceptibility. The second aim (**chapter 3**) was to determine the effects of maternal dietary methyl donor and cofactor supplementation during late pregnancy on allergic susceptibility of PR progeny, since methyl donors can regulate gene methylation via the one-carbon pathway. Placental restriction was induced by pre-pregnancy surgical reduction of placental attachment sites and its effects on progeny immune function and underlying mechanisms were investigated. Allergen-induced antibody and cutaneous hypersensitivity responses were measured in progeny from control and PR pregnancies following sensitisation to house dust mite and ovalbumin allergens. Effects of PR on cutaneous hypersensitivity responses did not correspond with effects on allergen-specific IgE responses. Delayed-phase cutaneous responses to ovalbumin were reduced in PR compared to control singletons, consistent with reports of epidemiological studies where low birth weight or poor fetal growth are generally protective against allergy, and despite no loss of IgE antibody response. Delayed-phase cutaneous responses to house dust mite were normal in PR singletons, despite enhanced IgE responses. Maternal dietary methyl donor and cofactor supplementation decreased antibody responses to allergens in some subgroups, but not those in which PR reduced cutaneous responses. This discord between antibody and cutaneous hypersensitivity responses suggests that mast cell function or other factors contribute to prenatally programmed regulation of allergy.

The aim of study two (**chapter 4**) was to investigate the effects of maternal allergic asthma on the fetal immune system in an ovine model. Maternal allergic asthma reduced relative fetal size and lung development in late gestation, but did not alter fetal immune tissue weights. In late gestation we detected an increase in thymocyte CD44 expression in fetuses from allergic compared to control ewes, suggestive of increased thymocyte activation.

In conclusion, maternal dietary supplementation with methyl donors and cofactors partially reversed the protective effects of restricted fetal growth against allergy, consistent with an epigenetic mechanism contributing to prenatal programming of allergic phenotype. Further research should include direct measures of one-carbon metabolism and methylation of immune-regulatory genes after PR and methyl donor supplementation, and of mast cell function as a potential mechanism for altered skin inflammatory responses to allergens. Results in the ovine model of maternal allergic asthma suggest that altered immune development may contribute to associations between maternal asthma and increased risk of allergy in progeny observed in human cohorts. The findings in this thesis provide direct evidence that allergic susceptibility can be programmed before birth.

STATEMENT OF ORIGINALITY AND AUTHENTICITY

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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The author acknowledges that copyright of published works contained within this thesis (as listed below) resides with the copyright holder(s) of those works. The following manuscripts have been accepted/published from this work:

- **Wooldridge AL**, Bischof RB, Meeusen EN, Liu H, Heinemann GK, Hunter DS, Kind KL, Owens JA, Clifton VL & Gatford KL. (2014) Placental restriction of fetal growth reduces cutaneous responses to antigen after sensitization in sheep, published by Am J Physiol Regul Integr Comp Physiol 306(7):R441-6. doi: 10.1152/ajpregu.00432.2013 (Appendix 1)

- Clifton VL, Moss TJ, **Wooldridge AL**, Gatford KL, Liravi B, Kim D, Mühlhäusler BS, Morrison JL, Davies A, De Matteo R, Wallace MJ, Bischof RJ. (2016) Development of an experimental model of maternal allergic asthma during pregnancy, published by J Physiol 594(5):1311-1325. doi: 10.1113/JP270752 (Appendix 2)
- **Wooldridge AL**, McMillan M, Marshall HS, Gatford KL. (2016) Systematic review protocol: relationship between fetal growth rate and postnatal allergy, published by JBI Database System Rev Implement Rep 14(11):11-20. doi: 10.11124/JBISRIR-2016-003177 (Appendix 3)
- Gatford KL, **Wooldridge AL**, Bischof RJ, Clifton VL, Kind KL. Pre-birth origins of allergy and asthma, accepted by J Reprod Immunol since PhD submission (Appendix 4)

Signed,

Amy Louise Wooldridge

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I am forever thankful to my parents for housing and feeding me throughout much of my PhD, in addition to my whole family's helpful sending of memes related to finishing a PhD at the age of 90 years. I hereby proclaim that it is now safe to ask me whether I've finished writing my thesis.

I am very grateful to have received an Australian Postgraduate Award and a Healthy Development Adelaide and Channel 7 Children's Research Foundation supplementary scholarship to support me during my PhD candidacy. I am also very grateful to have been awarded the Robert Seamark Scholarship in Obstetrics and Gynaecology from the University of Adelaide, in addition to a travel grants from Healthy Development Adelaide, the School of Paediatrics and Reproductive Health/Robinson Research Institute, the Perinatal Society of Australia and New Zealand and the Endocrine Society of Australia. Without these travel awards, presenting the research in this thesis at conferences would not have been possible, my research CV would have been poor, and my confidence at networking would still be sorely lacking. My involvement in the publications listed in the following pages that do not form a part of this thesis has given me considerable confidence and skills in collaborative work, more experience in the peer-review and publication processes, and has helped me greatly to expand my professional network and reading outside of my primary field. The skills that I have gained through these other studies have already proven to be useful.

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TABLE OF ABBREVIATIONS

ABC Study	Auckland Birthweight Collaborative Study
AF647	Alexa Fluor® 647
AGA	Adequate size for gestational age
ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage
CD	Cluster of differentiation
cf.	<i>Confer</i>
CON	Control
dGA	Days gestational age
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
Et1	Endothelin-1
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
FSC	Forward-scatter
HBSS	Hank's buffered saline solution
HBW	High birth weight
HDM	House dust mite
HMD	High methyl donor and cofactors diet
HRP	Horseradish peroxidase
IgA	Immunoglobulin type A

IgE	Immunoglobulin type E
IgG	Immunoglobulin type G
IgG₁	Immunoglobulin type G ₁
IgM	Immunoglobulin type M
ISAAC	International Study of Asthma and Allergies in Childhood
IU	International units
IUGR	Intrauterine growth restriction
LBW	Low birth weight
LIFT Study	Loire Infant Follow-Up Study
LMD	Low methyl donor and cofactors diet
LPS	Lipopolysaccharide
M:F	Male:female
mAbs	Monoclonal antibodies
MB	Multiple birth (twin or triplet)
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class I
mo	Months old
NBW	Normal birth weight
NTD	Neural tube defect
OR	Odds ratio
OVA	Ovalbumin
PAULA Study	Perinatal Asthma and Environment Long-term Allergy Study
PE	Phycoerythrin
PR	Placental restriction or placentally-restricted

PR+METHYL, PR+M	Placentally-restricted, maternal dietary methyl donor and cofactor-supplemented
RBC	Red blood cell
RR	Risk ratio
RUNX3	Runt-related transcription factor 3
SAGE	Study of Asthma Genes and the Environment
SB	Singleton birth
SE	Standard error of the mean
SGA	Small for gestational age
SPT	Skin prick test
SSC	Side-scatter
Th1	Type 1 T helper
Th2	Type 2 T helper
TMB	3',3',5',5'-tetramethyl-benzidine dihydrochloride hydrate
Total Ig	Total immunoglobulin
Treg	Regulatory T cell
WBC	White blood cell
yo	Years old

MANUSCRIPTS ARISING FROM PhD

Work directly related to this thesis:

Published manuscripts:

Wooldridge AL, Bischof RB, Meeusen EN, Liu H, Heinemann GK, Hunter DS, Kind KL, Owens JA, Clifton VL & Gatford KL. (2014) Placental restriction of fetal growth reduces cutaneous responses to antigen after sensitization in sheep, *Am J Physiol Regul Integr Comp Physiol* 306(7):R441-6. doi: 10.1152/ajpregu.00432.2013 (Appendix 1)

Clifton VL, Moss TJ, **Wooldridge AL**, Gatford KL, Liravi B, Kim D, Mühlhäusler BS, Morrison JL, Davies A, De Matteo R, Wallace MJ, Bischof RJ. (2016) Development of an experimental model of maternal allergic asthma during pregnancy, *J Physiol* 594(5):1311-1325. doi: 10.1113/JP270752 (Appendix 2)

Wooldridge AL, McMillan M, Marshall HS, Gatford KL. (2016) Systematic review protocol: relationship between fetal growth rate and postnatal allergy, *JBI Database System Rev Implement Rep* 14(11):11-20. doi: 10.11124/JBISRIR-2016-003177 (Appendix 3)

Accepted manuscripts:

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Kaur M, **Wooldridge AL**, Wilkes M, Pitchford WS, Hynd PI, McConell GK, Gatford KL. (2016) Placental restriction in multi-fetal pregnancies increases spontaneous ambulatory activity during daylight hours in young adult female sheep, *J Dev Orig Health Dis* 7(5):525-537. doi: 10.1017/S2040174416000283 (Appendix 7)

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CONFERENCE ABSTRACTS ARISING FROM PhD

Gatford KL, **Wooldridge AL**, Kind KL, Bischof RJ, Clifton VL. (2016). Pre-birth origins of allergy and asthma, *Inflammation in Reproduction, Pregnancy and Development, Cairns, Australia*
(invited symposium presentation, KL Gatford)

Wooldridge AL, Gatford KL, Moss TJ, McDonald C, Clifton VL, Bischof RJ. (2016). Effects of maternal asthma on the fetal immune system, *Fetal and Neonatal Workshop, Magnetic Island, Australia*
(oral presentation, AL Wooldridge)

Gatford KL, **Wooldridge AL**, Kaur M, Rice J, Le Ber A, Falcao-Tebas F, Kerris J, Marin E, McConell GK. (2016). The metabolic response to exercise is sex-dependent and altered in sheep offspring of placentally-restricted multi-fetal pregnancies, *Fetal and Neonatal Workshop, Magnetic Island, Australia*
(oral presentation, KL Gatford)

Wooldridge AL, Bischof RJ, Liu H, Heinemann GK, Hunter DS, Giles LC, Kind KL, Owens JA, Clifton VL, Gatford KL. (2015). Late pregnancy maternal dietary methyl donor supplementation partially reverses effects of placental restriction on cutaneous hypersensitivity, *International Society for the Developmental Origins of Health and Disease Conference, Cape Town, South Africa*
(oral-poster presentation, AL Wooldridge)

Bischof RJ, Clifton VL, **Wooldridge AL**, Gatford KL, Liravi B, Kim D, Muhlhausler BS, Morrison JL, Davies A, De Matteo R, Wallace MJ, Moss TJ. (2015). A sheep model to examine the effects of maternal asthma on fetal outcomes, *Fetal and Neonatal Physiological Society, Vancouver, Canada*
(oral presentation, RJ Bischof)

Kaur M, **Wooldridge AL**, Wilkes MJ, Hynd PI, McConell GK, Gatford KL. (2015). Restricted placental growth does not reduce spontaneous activity in the adolescent or young adult sheep, *Endocrine Society of Australia Annual General Meeting, Adelaide, Australia*
(oral presentation, M Kaur)

Clifton VL, Moss TJ, **Wooldridge AL**, Gatford KL, Liravi B, Mühlhäusler BS, Morrison JL, Davies A, De Matteo R, Wallace MJ, Bischof RJ. (2015). Development of an experimental model of maternal allergic asthma during pregnancy, *Australian Society for Medical Research South Australian Scientific Meeting, Adelaide, Australia*
(poster presentation, AL Wooldridge)

Kaur M, **Wooldridge AL**, Wilkes MJ, McConell GK, Hynd PI, Gatford KL. (2015). Daily patterns of spontaneous activity in the adolescent sheep – effects of restricted fetal growth, sex and temperature, *Australian Society for Medical Research South Australian Scientific Meeting, Adelaide, Australia*
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Wooldridge AL, Bischof RJ, Liu H, Heinemann GK, Hunter DS, Giles LC, Kind KL, Owens JA, Clifton VL, Gatford KL. (2015). Late pregnancy maternal dietary methyl donor supplementation partially reverses effects of placental restriction on cutaneous hypersensitivity, *Florey International Postgraduate Research Conference, Adelaide, Australia*
(poster presentation, AL Wooldridge)

Clifton VL, Moss TJ, **Wooldridge AL**, Gatford KL, Liravi B, Kim D, Mühlhäusler BS, Morrison JL, Davies A, De Matteo R, Wallace MJ, Bischof RJ. (2015). Effect of maternal allergic asthma with exacerbations on fetal morphology. *Perinatal Society of Australia and New Zealand, Melbourne, Australia*
(poster presentation, AL Wooldridge)

Clifton VL, Moss TJ, **Wooldridge AL**, Gatford KL, Liravi B, Kim D, Mühlhäusler BS, Morrison JL, Davies A, De Matteo R, Wallace MJ, Bischof RJ. (2014). Effect of maternal allergic asthma with exacerbations on fetal morphology. *Robinson Institute Research Symposium, Adelaide, Australia*
(poster presentation, AL Wooldridge)

Wooldridge AL, Bischof RJ, Liu H, Heinemann GK, Hunter DS, Giles LC, Kind KL, Owens JA, Clifton VL, Gatford KL. (2014). Effect of placental restriction and late maternal methyl supplementation on immune functional outcomes in sheep. *28th Fetal and Neonatal Workshop, Yanchep, Australia*
(oral presentation, AL Wooldridge)

Wooldridge AL, Bischof RJ, Liu H, Heinemann GK, Hunter DS, Giles LC, Kind KL, Owens JA, Clifton VL, Gatford KL. (2014). Effect of placental restriction and late maternal methyl supplementation on immune functional outcomes in sheep. *Australian Society for Medical Research South Australian Scientific Meeting, Adelaide, Australia*
(oral presentation, AL Wooldridge)

Wooldridge AL, Bischof RJ, Meeusen EN, Liu H, Heinemann GK, Hunter DS, Kind KL, Owens JA, Clifton VL, Gatford KL. (2013). Does late pregnancy methyl donor supplementation reverse effects of placental restriction on immune function in sheep? *Endocrine Society of Australia Annual General Meeting, Sydney, Australia*
(oral presentation, AL Wooldridge)

Wooldridge AL, Bischof RJ, Meeusen EN, Liu H, Heinemann GK, Hunter DS, Kind KL, Owens JA, Clifton VL, Gatford KL. (2013). Does late pregnancy methyl donor supplementation reverse effects of placental restriction on immune function in sheep? *Robinson Institute Research Symposium, Adelaide, Australia*
(poster presentation, AL Wooldridge)

Wooldridge AL, Bischof RJ, Meeusen EN, Liu H, Heinemann GK, Hunter DS, Kind KL, Owens JA, Clifton VL, Gatford KL. (2013). Does late pregnancy methyl donor supplementation reverse effects of placental restriction on immune function in sheep? *Faculty of Health Sciences Postgraduate Conference, Adelaide, Australia*
(poster presentation, AL Wooldridge)

Wooldridge AL, Bischof RJ, Meeusen EN, Liu H, Heinemann GK, Hunter DS, Kind KL, Owens JA, Clifton VL, Gatford KL. (2013). Does late pregnancy methyl donor supplementation reverse effects of placental restriction on immune function in sheep? *27th Fetal and Neonatal Workshop, Barossa Valley, Australia*
(oral presentation, AL Wooldridge)

Chapter 1: INTRODUCTION/LITERATURE REVIEW

1.1 Overview

Sections 1.4.2, 1.4.3, 1.5.1.1, 1.5.2.1 and 1.5.3.1 are taken directly from a submitted review that I co-authored (Appendix 4, Gatford et al. J Reprod Immunol; accepted since thesis submission), and the content of these sections is unchanged, as per University of Adelaide guidelines. Due to the timing of the review, these include some discussion of work published in the experimental chapters. Some of the concepts developed in this chapter have been incorporated in a review of *in utero* programming of allergy, of which I am a co-author (Appendix 6, 68). This work also relates in part to a published review of animal models in developmental origins of health and disease (DOHaD) research, which I also contributed to as co-author (Appendix 8, 49), although the content of this review has not been directly included within this thesis. The discussion and initial review of studies investigating programming of allergy in humans identifies the need for a systematic review in this area to comprehensively assess available evidence for the currently accepted paradigm that poor fetal growth protects against allergy. I have developed a systematic review protocol for this evidence synthesis, which has been published (Appendix 3, 204), however the systematic review itself is beyond the scope of this thesis. The rationale for the systematic review under the “Gaps in knowledge” subheading in section 1.4.1 and parts of the definitions of allergy in section 1.3.4 and are taken from this protocol.

1.2 Statements of authorship for Chapter 1

1.2.1 Systematic review protocol: relationship between fetal growth rate and postnatal allergy

Note: the manuscript mentioned in the signed Statement of Authorship forms below has been **published** since thesis submission (JBI Database System Rev Implement Rep 14(11):11-20).

Statement of Authorship	
Title of Paper	Relationship between birth weight or fetal growth rate and postnatal allergy: a systematic review protocol
Publication Status	<input type="checkbox"/> Published <input checked="" type="checkbox"/> Accepted for Publication <input type="checkbox"/> Submitted for Publication <input type="checkbox"/> Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript number: JBISRR-2016-003177 Woodridge A.L., McMillan M., Marshall, H.S. & Gafford K.L. (2016). Relationship between birth weight or fetal growth rate and postnatal allergy: a systematic review protocol. Accepted for publication by JBI Database of Systematic Reviews and Implementation Reports, 9 June 2016.
Principal Author	
Name of Principal Author (Candidate)	Amy Woodridge
Contribution to the Paper	Conception and design of research; drafted manuscript; edited and revised manuscript; approved final version of manuscript
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 26/10/16
Co-Author Contributions	
By signing the Statement of Authorship, each author certifies that:	
I. the candidate's stated contribution to the publication is accurate (as detailed above); II. permission is granted for the candidate to include the publication in the thesis; and III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.	
Name of Co-Author	Mark McMillan
Contribution to the Paper	Conception and design of research; drafted manuscript; edited and revised manuscript; approved final version of manuscript
Signature	Date 28/10/2016
Name of Co-Author	Helen Marshall
Contribution to the Paper	Conception and design of research; drafted manuscript; edited and revised manuscript; approved final version of manuscript
Signature	Date 31 st October 2016

Name of Co-Author	Kathryn Galford		
Contribution to the Paper	Conception and design of research; drafted manuscript; edited and revised manuscript; approved final version of manuscript		
Signature		Date	28/10/2016

1.2.2 Pre-birth origins of allergy and asthma

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PhD Candidate's Contribution

Name of Candidate	Amy Wooldrige
Contribution to the Paper	Conception and design of research described within manuscript; performed experiments described within manuscript; analysed data described within manuscript; interpreted results of experiments described within manuscript; edited and revised manuscript; approved final version of manuscript
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Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- I. the candidate's stated contribution to the publication is accurate (as detailed above);
- II. permission is granted for the candidate to include the publication in the thesis; and
- III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Kathryn Gattford
Contribution to the Paper	Conception and design of research described within manuscript; performed experiments described within manuscript; analysed data described within manuscript; interpreted results of experiments described within manuscript; prepared figures; drafted manuscript; edited and revised manuscript; approved final version of manuscript
Signature	Date 28 October 2016

Name of Co-Author	Karen Kind		
Contribution to the Paper	Conception and design of research described within manuscript; performed experiments described within manuscript; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript		
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Name of Co-Author	Robert Bischof		
Contribution to the Paper	Conception and design of research described within manuscript; performed experiments described within manuscript; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript		
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Name of Co-Author	Vicki Clifton		
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1.3 Introduction

1.3.1 General introduction and scope of literature review

Allergy is a non-communicable disease requiring preventative research. The incidence of allergy is continually increasing (139), affecting quality of life, healthcare and placing pressure on public healthcare services. The annual overall economic cost of food allergy alone in the United States of America is almost \$25 billion, or over \$4,000 per allergic child (71). Therefore, it is not feasible to continue treating the symptoms of this condition without also identifying preventative treatments. To develop preventative treatments, however, we must first understand the mechanisms that predispose or protect individuals to later development of allergy. Because the age group with the apparent greatest increase in allergy rates is the youngest (0-4 years, 139), this suggests that early life factors affect allergy susceptibility, hence it is important to determine the effects of a range of early life factors on later allergy susceptibility. Birth weight (190) and maternal asthma (157) have both been identified as factors that alter subsequent allergy risk. The developmental origins of health and disease (DOHaD) are broadly defined as events in early life causing epigenetic changes that result in altered phenotype in later life, for better or worse. Research in this field holds great potential in preventing non-communicable diseases before they occur at the population-level, through the development of sound guidelines and preventative treatments targeted towards improving conceptus, fetal or infant health. There remain many challenges within the field, such as the sheer number of early life factors now identified that may affect later health and disease outcomes, the different outcomes of fetal insults at different times during gestation and the ethical limitations of research in humans.

This literature review introduces the concepts related to developmental programming of postnatal susceptibility to allergy, with a focus on restricted fetal growth and maternal allergy. It then summarises prior research and gaps in knowledge from studies of developmental programming of allergy in humans and experimental models, discusses the potential role of methyl donors in

developmental programming of allergy and concludes with a summary of the scope, aims and hypotheses of the studies described in this thesis.

1.3.2 Developmental origins of health and disease (DOHaD) concepts

1.3.2.1 Introduction to DOHaD concepts

Developmental programming describes the effects of exposures during ‘critical windows’ of development, which have a lasting impact through to adult health (114). David Barker is the most recognisable name in DOHaD history. Barker was director of the Medical Research Council Environmental Epidemiology Unit in Southampton in the United Kingdom (40). This unit investigated mortality trends throughout England and Wales. As the unit mapped cardiovascular deaths, Barker noticed that areas of greatest infant mortality in 1910 also had the greatest rates of cardiovascular deaths 60 years later. Associations like this were found with a range of adult-onset diseases (13, 14, 64), and this forms the basis of the ‘predicted adaptive response’ hypothesis. The basis of this hypothesis is that exposure to sub-optimal environments causes the conceptus or fetus to alter its phenotype to be better suited to a predicted sub-optimal early postnatal environment at the cost of potentially compromising the long-term health of the individual (61, 62). The potential for phenotypic change brought about by exposures to different environments during these critical windows is known as ‘plasticity’, or ‘adaptive plasticity’. The terms used to describe this research field and the underlying concepts have progressed over the years to encompass a greater variety of exposures (such as prenatal over-nutrition as well as under-nutrition), and incorporate the understanding that exposures continue to occur and impact development across the lifespan and include preconceptional and early postnatal exposures. This theory is now broadly referred to as the “developmental origins of health and disease” or DOHaD hypothesis.

The field of DOHaD research has grown rapidly, with the study of almost all organ systems, paternal health, preconceptional health, and intergenerational effects; supported by the development of a

large range of animal models and better understanding of how to conduct quality DOHaD research. Many well-described experimental animal models, both small and large, have now been developed to investigate various early life exposures, which is aiding in a combined approach to move research from phenotypic studies to mechanistic and intervention studies (Appendix 8, 49). One of the most well-known hypotheses in the field of developmental programming is the hygiene hypothesis, which proposes that reduced exposure to environmental microbes is a causal factor for increasing rates of allergic disease. This idea is expanded upon by the microflora hypothesis, which proposes that the human microbiota is of particular importance as a causal factor, as demonstrated by concurrent studies of gut microflora and subsequent lung phenotype (reviewed in 84, 187). The most-studied outcomes within DOHaD research are cardiovascular and metabolic health; there have not been as many studies investigating allergy.

1.3.2.2 Application of DOHaD concepts to improve human health

Whilst the study of DOHaD themes enhances our understanding of organogenesis and the mechanisms underlying non-communicable diseases in general, it also provides other benefits. Some of the most costly diseases to first-world countries are non-communicable diseases (34). With a growing and aging global population, it is increasingly important to prevent chronic non-communicable diseases in order to protect economies from the costs associated with healthcare for these conditions. Additionally, first-world countries are capable of providing assistance to help developing countries reach their potential faster; early life interventions are likely to play a key role in this. As pregnancy only lasts for nine months, it is more cost-effective and practical on a population-scale to provide advice or preventative treatments during the critical windows within these nine months than to provide lifetime preventative or therapeutic treatments to individuals. Some interventions can be applied without requiring costly and sometimes ineffective education and active uptake. One successful example is the folate fortification of bread-making flour, which reduces the incidence of neural tube defects in fetuses and infants (101, 183). A cost-benefit analysis

has estimated that this fortification saves an estimated annual direct cost of over \$88 million, with an economic benefit of over \$312 million in the United States of America alone (69).

1.3.3 Intrauterine growth restriction (IUGR) definitions and incidence

Intrauterine growth restriction (IUGR) affects 6-12% of pregnancies in developed countries, and a greater percentage of pregnancies within poorer populations (92). These infants are unable to reach their genetic growth potential *in utero*. Many studies, particularly older studies, use low birth weight (LBW, birth weight <2500 g) as a marker of IUGR, although this measure does not take gestational age into account. Infants born at a weight below the 10th percentile for gestational age are classified as being born at a small size for their gestational age (SGA).

Causes of IUGR include fetal genetic conditions, maternal factors such as low maternal bodyweight, age or parity, maternal under-nutrition, alcohol consumption or smoking, and other causes such as pre-eclampsia, intrauterine infections and placental dysfunction (175). The majority of IUGR in developed countries is associated with poor placental development and/or function (175). The placenta transports oxygen and nutrients from mother to fetus, synthesises hormones that influence these transfer rates, and metabolises key nutrients for the fetus (144, 202). Thus, inadequate placental size or function restricts fetal growth (14) and can result in infants being born at low birth weight (20).

IUGR is detrimental to both short- and long-term health. Short-term complications in IUGR or LBW infants include an increased risk of obstetric complications (106), increased susceptibility to infection (1, 181) and greater likelihood of being hospitalised with viral gastroenteritis (149). Long-term effects of IUGR include greater risks of metabolic and cardiovascular diseases (11). However, the longer term effects of IUGR on immune function are largely unknown.

1.3.4 Allergy definitions and incidence

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Wooldridge AL, McMillan M, Marshall HS, Gatford KL. (2016) Systematic review protocol: relationship between fetal growth rate and postnatal allergy, JBI Database System Rev Implement Rep 14(11):11-20. doi: 10.11124/JBISRIR-2016-003177 (Appendix 3)

Allergy is a common non-communicable disease worldwide that is estimated to affect 30-40% of the world's population (205) and is increasing in prevalence, particularly in young children (139). Allergic conditions, including anaphylaxis and asthma can become life-threatening if not well-managed. Common allergic conditions include eczema (atopic dermatitis), hay fever (allergic rhinitis), allergic asthma and anaphylaxis. This section gives a brief description of clinical allergic conditions in preparation for later review sections discussing evidence for prenatal programming of allergic susceptibility.

Atopy is associated with the tendency to mount strong Immunoglobulin E (IgE) responses in response to common environmental allergens and have a greater susceptibility to allergic inflammation, although allergic reactions can also be IgE independent. IgE is an antibody type that binds to a particular allergen, and is produced by plasma cells (which mature from B lymphocytes) in lymph nodes and sites of allergic inflammation (2). After IgE has been produced upon an initial exposure, re-exposure to the same allergen causes an allergic response including inflammation. Allergen-dependent inflammation is driven primarily by mast cells, which are bone marrow-derived cells that localise and differentiate within vascularised tissues (2). These cells contain granules of histamine, heparin and other chemicals; the Fc region on IgE can bind to high-affinity FcεRI receptors on the mast cell surface, sensitising them to degranulate upon subsequent encounters with their

specific target antigen (2). Histamine from granules acts on vasculature to increase the permeability of capillaries to white blood cells and some proteins, resulting in the localised heat, redness, pain and swelling characteristic of inflammation; this localised inflammation enables white blood cells to quickly migrate to areas where they are needed (2). The most commonly-reported clinical allergic outcomes are eczema, hay fever, allergic asthma, anaphylaxis, and food allergy.

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Eczema, also known as atopic dermatitis, is an inflammatory skin condition induced by sensitisation and subsequent cutaneous exposure to allergen/s (Appendix 3, 204, 205). Hay fever, also known as allergic rhinitis, is an allergic condition resulting from IgE-mediated inflammation of the nasal mucosa and produces symptoms including watery eyes, a runny nose and sneezing (Appendix 3, 204, 205). The term asthma is used to describe a complex chronic inflammatory disease syndrome affecting the airways, characterised by bronchial hyperresponsiveness, and progressive airway remodelling and loss of function (3, Appendix 3, 204). Allergy is one of the causes of asthma (25, 204, Appendix 3); this review (Appendix 3) addresses allergic asthma but not asthma or wheezing that are because of other causes such as exercise-induced asthma or non-specified causes.

Anaphylaxis is a severe, potentially fatal, systemic allergic reaction which is rapid in onset and occurs suddenly after contact with an allergy-causing substance (174, Appendix 3, 204). Clinical allergy diagnosis involves consideration of medical history and any current symptoms, identifying an association between symptoms and allergen exposure, followed by confirmation with either positive

intradermal allergen challenge or direct measurement of high circulating IgE antibody concentrations (205).

1.3.5 Incidence of maternal allergy and asthma

In Australian women of reproductive age, asthma affects 11.7% of women aged 15-24 years, 12.3% of women aged 25-34% and 10.1% of women aged 35-44 years (9). The maternal immune system undergoes suppression in order to prevent rejection of a semi-foreign fetus (172). This may contribute to the fact that over 50% of asthmatic women experience worsening asthma symptoms during pregnancy (141), particularly towards late gestation, with 20% of asthmatic women undergoing exacerbations requiring medical intervention (140, 141). Around 8-65% of pregnant asthmatic women experience exacerbations (141), or acute episodes of shortness of breath, wheezing or chest tightness. Thus, fetuses of asthmatic women are thought to be exposed to both hypoxia as well as inflammatory signals (140, 178).

1.4 Developmental programming of allergic disease in humans

1.4.1 Associations between low birth weight or poor fetal growth and offspring allergy

This section compares and discusses key human studies investigating associations between low birth weight or poor fetal growth and three of the most common allergic conditions (eczema, hay fever and food allergy). Because the majority of human studies do not differentiate allergic and non-allergic asthma, programming of asthma is not reviewed in this section. The available evidence from human studies suggests that programming affects allergic disease risk at different ages, and allergic outcomes in key studies are therefore discussed below based on age groups. These key papers were selected based on coverage of different age groups and allergic conditions and have also been restricted to studies reporting physician-diagnosed allergy to ensure correct diagnosis (Appendix 3, 204).

Infancy: Allergy in this age group may be transient, and only a limited number of well-powered studies correcting for confounding factors such as gestational age at birth are available (Table 1.1). Varying associations of size at birth with eczema have been reported, including no change in risks in LBW groups (110), no association between birth weight for gestational age and risk of eczema (133), and a positive association between risk of eczema in infancy and birth weight (159). One study reported a trend for infants with a birth weight of at least 4,000 g to have an increased risk of eczema compared to infants with a birth weight between 3,500-4,000 g (110). LBW infants have also been reported as having a lower risk of eczema compared to NBW infants (76), and very LBW infants (z-score <-2) as having a lower risk of eczema compared to infants with a z-score of 0-2 (10). There has been one study directly investigating the relationships between fetal growth patterns and allergic outcomes in infants. AlMakoshi *et al.* (5) reported that although fetal size measures in the second and third trimesters did not predict eczema in infancy, faster relative fetal growth rates between the second and third trimesters appeared to be protective. This suggests that patterns of growth, not only the final size at delivery, are likely important determinants of allergy in infants. A single study reported lower food allergy prevalence in LBW infants compared to NBW infants (Table 1.1, 76).

Childhood: The most studied allergic outcome in childhood is eczema. Many studies did not find an association between size at birth and risk of childhood eczema (Table 1.1, 10, 23, 60, 76, 116, 151, 169). In those studies that reported an association with change in risk, however, SGA or LBW populations consistently had a lower risk of eczema (Table 1.1). Similarly, some evidence suggests that a heavier birth weight predisposes to a greater risk of childhood eczema (104, 115, 151, 162). In a large multi-country report from the International Study of Asthma and Allergies in Childhood (ISAAC) Phase III Study, based on outcomes for >160,000 children across 26 countries, LBW was associated with a decreased risk of having ever experienced eczema, particularly in affluent countries (132). This study was part of the broader ISAAC study of allergy, and the questionnaire and

allergy diagnostic methods within this study have been widely used in the field, providing a semi-standardised approach allowing comparisons between studies (8). The strongest evidence, due to the reduction in genetic and environmental confounding, comes from a Swedish twin study, which included both monozygotic and dizygotic twins (115). In that study, for each 500 g increase in birth weight, the risk of eczema increased by 62% after adjustment for early life factors overall, and in same-sex twin pairs was increased four-fold (Table 1.1, 115). Overall, of those studies that identified an association between size at birth and risk of childhood eczema, small size at birth was associated with reduced risk of eczema.

In contrast to these reports of protective effects of restricted growth *in utero* against childhood eczema, the majority of studies have found no effect of LBW or SGA on risk of hay fever (Table 1.1), although the ISAAC study of 26 countries found that having a birth weight greater than 2.5 kg was associated with an increased risk of current (within the last 12 months) hay fever and current rhinoconjunctivitis (104). The above described multi-national ISAAC study found no association between LBW and a physician diagnosis of rhinoconjunctivitis at any age by 6-7 years of age (132). Interestingly, this multi-national study found no association between LBW and current or severe rhinoconjunctivitis overall and within affluent countries, but when analysed within non-affluent countries LBW was associated with increased risks for current and severe childhood rhinoconjunctivitis (132). There was no statistically significant difference in the risk of current or severe rhinoconjunctivitis between affluent versus non-affluent countries, however (132). A study from the UK reported on direct fetal growth measures, and whilst they observed no effect of absolute fetal size during first or second trimesters on risk of childhood eczema or hay fever, they found that growth acceleration between first and second trimesters was associated with increased risk of eczema but not hay fever, and that growth deceleration during this time was associated with reduced risk of hay fever but not associated with risk of eczema (192). Risk of food allergy is a less reported outcome; however, the few studies published have reported that size of birth does not

appear to be associated with risk of childhood food allergy (76, 107). Overall, these studies suggest that restricted growth *in utero* may protect against childhood allergy, with the strongest evidence for eczema protection, and some variation in outcomes possibly reflecting use of different statistical models and corrections for different confounders.

Adolescence/adulthood: Most studies in older groups have been carried out in adolescents, with limited data available regarding programming of allergic outcomes in cohorts comprised only of adults (Table 1.1). Although the majority of studies in this age group have not found associations between size at birth and adolescent or adult eczema after correction for confounders including gestational age (87, 102, 109), there is a study with contrasting effects to those seen at younger ages (Table 1.1). Steffensen *et al.* (186) reported that within a term-born male subgroup of adults, LBW was associated with a 5-fold increase in odds ratio (OR) for adult eczema.

Studies of hay fever outcomes likewise report inconsistent relationships between incidence and size at birth (Table 1.1), including no association between size at birth and adjusted risk of hay fever (87, 102, 109). In two studies, lower OR for hay fever were observed in individuals with smaller size at birth (27), and greater OR for hay fever were observed in heavier compared to lighter twins (170). However, another study reported that although birth weight was not associated with risks of hay fever by 15-25 years of age, higher rates of current hay fever were observed in term-born LBW 15-25 year olds, compared to matched controls of unspecified birth weight (75). Based on these studies, evidence for developmental programming of allergy in adolescents and adults is currently weak, possibly reflecting greater impacts of variable postnatal environments.

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Gaps in knowledge: It is important to define *in utero* pathways and mechanisms behind the developmental programming of susceptibility to allergic disease in order to then identify targets for interventions. It is also unclear whether relationships between birth weight or fetal growth and later allergy in humans are consistent between different allergic diseases, populations and ages. No recent or underway systematic reviews of this topic were found when searching the *JBISRIR Database of Systematic Reviews and Implementation Reports*, Cochrane Library, PubMed, CINAHL and Prospero. Some detailed literature review articles describing the association between size at birth and allergic disease exist. However, no reviews published on this topic have used systematic review methodology, except for one meta-analysis of the relationship between categories of absolute birth weight, uncorrected for gestational age (LBW compared to normal birth weight, NBW, and HBW compared to NBW, 158) and atopic dermatitis (158). The final meta-analysis included 10 studies of a total of ~111,000 study participants (158). Based on this set of evidence, the authors concluded that LBW is protective against occurrence of atopic dermatitis when compared to with study participants of NBW [I^2 85.90, 95% confidence interval (CI) 75.93-91.74] and that HBW represents a risk factor for atopic dermatitis (I^2 58.38, 95% CI 0-83.1)(158). It is not considered that this meta-analysis identified all the relevant literature, as the databases searched were limited to PubMed, Cochrane Library and Web of Knowledge only, and eczema was not included as a search term (158). In addition, the analysis may be confounded by a lack of precision in the outcome measure, as studies where outcomes were parent- and patient-reported allergy were included in addition to studies reporting

clinical diagnosis (158). This may have been exacerbated by a lack of stratification of outcomes by age, with outcomes in infants, children and adolescents grouped together and potential recall bias (158). Furthermore, no correction was made for gestational age, included in analysis of data from very LBW cohorts, where low birth weight probably reflected prematurity (29), and the authors acknowledge the need for additional studies of this relationship that take gestational age into account (158). Finally, because of poor figure quality in the published manuscript (158), interpretation of results is difficult. It is therefore considered that a more comprehensive analysis of the relationship between birth weight or fetal growth, reflecting fetal under and overgrowth, is required, in addition to analysis of relationships between these exposures and other atopic diseases.

Therefore, even within the existing literature, a clear conclusion regarding the relationship between poor fetal growth and allergic outcomes has not yet been reached. A systematic review of this literature is warranted and is currently in progress (Appendix 3, 204), but is beyond the scope of the thesis itself.

Table 1.1 Summary of key studies for effects of weight/size at birth on allergic diseases.

INFANCY (0-2 years of age)			
Study region (cohort name) and reference	Study design, participants and outcome measures	Effect of birth weight/fetal growth on allergic outcomes	Study strengths & limitations
Denmark (Danish National Birth Cohort) Linneberg <i>et al.</i> 2006 (110)	Prospective study Pregnant women attending first pregnancy consultation with physician were recruited ($n=34,793$). Parental questionnaire specifying physician-diagnosed eczema was completed when infant was 18 mo, plus prior questionnaires during pregnancy (12 and 30 weeks gestation) and at 6 mo. Inclusion limited to pairs with all questionnaires complete.	Infants with birth weight ≥ 4 kg tended to have greater OR for eczema by 6 mo (OR 1.12, 95% CI 1.00-1.25) than infants in reference birth weight category (3,000-3,500 g). OR for eczema in other birth weight categories (<2,500 g, 2,500-3,000 kg and 3,500-4,000 kg) not different from reference category.	Strengths: - Allergy based on medical diagnosis/criteria - Allergy status from questionnaire responses confirmed by clinical examination in a subset - Fetal size/birth data obtained from medical records - Representative population sample Weaknesses: - No correction for gestational age - Parent-report of medical diagnosis, potential misunderstanding or recall failure - 65% loss to follow-up (largely due to incomplete questionnaire completion in this national birth cohort)
Massachusetts, United States of America (Project Viva) Moore <i>et al.</i> 2004 (133)	Prospective study Women ≤ 22 weeks gestational age and with singleton pregnancies at first prenatal clinical appointment who were planning to give birth in either Brigham and Women's Hospital or Beth Israel Deaconess Medical were recruited. 172 infants with eczema and 833 infants without eczema participated at 6 mo. Parental questionnaire specifying physician-diagnosed eczema completed when infant was 6 mo.	Birth weight for gestational age did not affect risk of eczema at 6 mo of age.	Strengths: - Gestational age included implicitly by use of z-scores - Allergy based on medical diagnosis/criteria - Fetal size/birth data obtained from medical records - Only 22% loss to follow-up from eligible singleton-bearing women by 6 mo interview (~20% due to incomplete covariate data) - Representative population sample Weaknesses: - Parent-report of medical diagnosis, potential misunderstanding or recall failure
Bergamo, Lombardy, Northern Italy Parazzini <i>et al.</i> 2014 (159)	Prospective study 796 infants born in obstetric departments and resident in the Bergamo area were recruited shortly after birth. Parental questionnaire specifying physician-diagnosed eczema conducted when infant was 12 mo.	Birth weight was slightly positively associated with risk of eczema at 12 mo: RR 1.04 (95% CI 1.00-1.08, $P=0.0412$).	Strengths: - Gestational age included in statistical model - Allergy based on medical diagnosis/criteria - Only 26% loss to follow-up - Representative population sample Weaknesses: - Parent-report of medical diagnosis, potential misunderstanding or recall failure - Parent-reported birth weight, potential recall failure

<p>Fukuoka, Japan Hikino <i>et al.</i> 2001 (76)</p>	<p>Prospective study</p> <p>All infants/children in Fukuoka were eligible for the study, performed by paediatricians during well-baby checks at 4 mo, 18 mo and 3 yo (85-90% of infants attended 18 mo check-up). Parental questionnaire for physician-diagnosed food allergy and physician examination for eczema of 18 mo infants ($n=21,766$) and 3 yo infants ($n=4,378$) who had also attended a 4 mo check.</p>	<p>At 18 mo, LBW infants had a lower prevalence of eczema (1.2%) compared to NBW (2.3%, $P=0.0041$). In multivariate analysis, OR for eczema in LBW was 0.59 (95% CI 0.37-0.97, $P=0.0219$).</p> <p>At 18 mo, LBW infants had a lower prevalence of food allergy (8.1%) compared to the rest of the infants (NBW: 11.2%, $P=0.0002$). In multivariate analysis, OR for food allergy in LBW was 0.81 (95% CI 0.66-0.97, $P=0.0216$).</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age corrected for within statistical analyses - Eczema and food allergy based on medical diagnosis - Only 10-15% population were not recorded as attending 18 mo and 3 yo check-ups (although the same children were not necessarily included at both age groups due to later-enrolled infants not reaching 3 yo by the study end time) - Representative population sample <p>Weaknesses:</p> <ul style="list-style-type: none"> - Parent-report of medical diagnosis for food allergy, potential misunderstanding or recall failure - Parent-reported birth weight, potential recall failure
<p>France (Epipage and Loire Infant Follow-up cohort studies) Barbarot <i>et al.</i> 2013 (10)</p>	<p>Prospective study</p> <p>Based on the original Epipage cohort, which included all births from 22-32 completed weeks of gestation in almost all of the maternity wards in nine regions of France. This was a follow-up study of 1836 preterm infants from the original cohort plus 346 term (39-40 weeks of gestation) infants recruited at a rate of every 4 births in the same regions as the original cohort.</p> <p>Parental questionnaire specifying physician-diagnosed eczema was completed when the infant/child was aged 2 yo (Epipage cohort).</p>	<p>Very LBW (z-score <-2) was associated with lower risk of eczema at 2 yo in the Epipage cohort relative to a z-score of 0-2.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Within Epipage study, a sample of full-term infants were compared with preterm infants - Allergy based on medical diagnosis - Fetal size/birth data obtained from medical records <p>Weaknesses:</p> <ul style="list-style-type: none"> - Parent-report of medical diagnosis, potential misunderstanding or recall failure - Epipage cohort study had 26% or 48% loss to follow-up or incomplete data for preterm or term infants, respectively - Population sample was not representative (most were born at <35 weeks of gestation)
<p>Riyadh, Saudi Arabia AlMakoshi <i>et al.</i> 2015 (5)</p>	<p>Prospective study</p> <p>Pregnant women attending an antenatal clinic in King Fahad Medical City Hospital in Riyadh were recruited. 1063 infants participated in the study.</p> <p>Parental questionnaires specifying physician-diagnosed eczema were completed when infant was 1 and 2 yo.</p>	<p>Fetal size in 2nd and 3rd trimester and infant birth size were not associated with risk of eczema by 2 yo.</p> <p>Faster relative fetal growth (greater increase in abdominal circumference z-score) between 2nd and 3rd trimester was associated with reduced risk of eczema by 2 yo (OR 0.65 per z-score increase, 95% CI 0.48-0.89, $P=0.007$).</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age included implicitly by use of z-scores - Allergy based on medical diagnosis/criteria - Fetal size/birth data obtained from medical records - Only 23% loss to follow-up by 2 yo <p>Weaknesses:</p> <ul style="list-style-type: none"> - Parent-report of medical diagnosis, potential misunderstanding or recall failure

CHILDHOOD (3-12 years of age)

Study region (cohort name) and reference	Study design, participants and outcome measures	Effect of birth weight/fetal growth on allergic outcomes	Study strengths & limitations
Fukuoka, Japan Hikino <i>et al.</i> 2001 (76)	(See above)	At 3 yo, LBW and NBW had similar incidences of food allergy and eczema.	(See above)
Auckland, New Zealand (Auckland Birthweight Collaborative Study; ABC Study) Purvis <i>et al.</i> 2005 (169)	Prospective study Singleton New Zealand SGA infants and a random sample of all AGA infants ($n=744$ at 12 mo and $n=550$ at 3.5-4 yo) born in the Waitemata Health or Auckland Healthcare regions, without congenital anomalies likely to affect birth weight and later growth and without home births. Only those of European ethnic groups were included in the final analyses. Parental questionnaire for symptoms of eczema completed when infant was 12 mo (however, analysis of these outcomes and size at birth were not conducted). Examination for evidence of eczema when children were 3.5-4 yo.	Birth weight not associated with eczema by 4 y of age.	Strengths: - Gestational age included implicitly by use of size for gestational age - Direct clinical diagnosis of eczema at 3.5-4 yo - Fetal size/birth data obtained from medical records - Representative population sample Weaknesses: - Not direct clinical diagnosis of eczema at 12 mo - Parent-report of medical symptoms, potential misunderstanding or recall failure - Non-European ethnic groups excluded from study due to >50% loss to follow-up. European subjects had 15% and 47% loss to follow-up at 1 and 3.5 yo, respectively
France (Epiage and Loire Infant Follow-up (LIFT) cohort studies) Barbarot <i>et al.</i> 2013 (10)	(See above) Prospective study The LIFT cohort included 493 infants born at <35 full weeks gestation and hospitalised at Nantes University Hospital in Western France. Parental questionnaire specifying physician-diagnosed eczema was completed when the infant/child was aged 5 yo (LIFT cohort)	Very LBW was not associated with risk of eczema at 5 yo in the LIFT cohort.	(See above) Strengths: - Within LIFT cohort study, gestational age included implicitly by use of z-scores Weaknesses: - LIFT cohort study had 41% loss to follow-up or incomplete data - Population sample not representative (all preterm)
Turku, Finland Luoma <i>et al.</i> 1983 (116)	Prospective study Mothers and infants ($n=543$) with and without family history of allergic disease (asthma, hay fever or eczema) recruited in the delivery unit of the Women's Clinic of the University Central Hospital of Turku. Hay fever and eczema by parental questionnaires (periodically from 1 mo to 5 yo; however, analysis of these outcomes and size at birth were not conducted), plus clinical examination in 3/8 of children at 1 yo and ~2/3 of children at 5 yo.	LBW (not defined) compared to rest of cohort had similar incidences of eczema and hay fever by 5 yo.	Strengths: - Allergy status from questionnaire responses confirmed by clinical examination in a subset - Fetal size/birth data obtained from medical records Weaknesses: - No correction for gestational age - 19% loss to follow-up by 1 yo of age; of the remaining participants there was a further 43% loss to follow-up by 5 yo

<p>Munich, Germany (German ISAAC Phase II Surveys) Bolte <i>et al.</i> 2004 (23)</p>	<p>Prospective study</p> <p>Cross-sectional survey of school beginners randomly selected for questionnaires; random subset invited for physician examination. 741 school beginners had complete perinatal data available.</p> <p>Parental questionnaire specifying physician-diagnosed hay fever and eczema was completed when children were aged 5-7 yo.</p>	<p>Hay fever and eczema at 5-7 yo not associated with birth weight category (5 categories).</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Allergy based on medical diagnosis - Fetal size/birth data obtained from medical records - Random sampling of population - Representative population sample <p>Weaknesses:</p> <ul style="list-style-type: none"> - No correction for gestational age - Parent-report of medical diagnosis, potential misunderstanding or recall failure - Of those invited for examination, 40% loss to follow up or incomplete data
<p>Aarhus, Denmark Olesen <i>et al.</i> 1997 (151)</p>	<p>Retrospective study followed by prospective subgroup questionnaire specifying physician-diagnosed eczema.</p> <p>Data from 7,862 singleton children born within a selected period at the Aarhus Maternity Hospital was analysed. A subgroup analysis with an overrepresentation of preterm children (19% preterm, 54% term, 27% post-term) was conducted to further investigate the effects of preterm birth on eczema.</p> <p>Children with a recorded physician diagnosis of eczema had a mean age of 6.3 (5.5-8.5) yo at time of diagnosis.</p>	<p>First study ($n=7,862$): Birth weight difference from expected calculated birth weight not associated with risk of eczema at 5.5-8.5 yo.</p> <p>Second study (subgroup analysis of $n=985$: Birth weight ≥ 500 g greater than expected calculated birth weight had a greater risk of eczema at 5.5-8.5 yo (RR 1.59, 95% CI 1.05-2.42) using the reference group with birth weight less than 200 g from expected calculated birth weight. No other birth weight categories were associated with risk of eczema.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age adjusted for within statistical analyses - Allergy based on medical diagnosis - Fetal size/birth data obtained from medical records - Only 7% of subgroup did not return questionnaire - Representative population sample in first study <p>Weaknesses:</p> <ul style="list-style-type: none"> - Parent-report of medical condition in second study, potential recall failure - Second study was not a representative population sample (overrepresentation of preterm children)
<p>UK Peters <i>et al.</i> 1987 (162)</p>	<p>Prospective study</p> <p>Of all children born between 5-11th April 1970 in the UK, 11,920 singletons with recorded status of eczema development were included.</p> <p>Development of eczema at 5 yo was assessed by parental questionnaire specifying the condition by name only.</p>	<p>Increasing birth weight category (7 categories) was associated with increasing eczema prevalence by 5 yo of age in unadjusted χ^2 analyses ($P<0.01$), however this was not significant after adjustment for labour variables (opiates, duration of second stage of labour).</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Fetal size/birth data obtained from medical records - Only 4% loss to follow-up of singletons after exclusion of the 20% of the original cohort that lived in Northern Ireland - Representative population sample <p>Weaknesses:</p> <ul style="list-style-type: none"> - No correction for gestational age - Allergy not based on medical diagnosis or symptoms but based on name of medical condition; potential misunderstanding - Parent-report of medical condition, potential misunderstanding or recall failure

Study region (cohort name) and reference	Study design, participants and outcome measures	Effect of birth weight/fetal growth on allergic outcomes	Study strengths & limitations
26 countries worldwide (ISAAC Phase 3) Mitchell <i>et al.</i> 2014 (132)	<p>Prospective study</p> <p>162,324 students recruited from 60 schools in 26 countries</p> <p>Parental questionnaire for current and severe eczema and current and severe rhinoconjunctivitis based on symptoms and distribution was completed when children were aged 6-7 yo.</p>	<p>Overall, LBW was associated with a decreased risk of eczema ever (OR 0.88, 95% CI 0.82-0.96) at 6-7 yo. LBW was associated with a reduced risk of eczema ever in affluent countries (OR 0.83, 95% CI 0.75-0.92), but was not associated with risk of eczema ever in non-affluent countries.</p> <p>LBW not associated with current eczema and severe eczema at 6-7 yo.</p> <p>LBW not associated with rhinoconjunctivitis ever at 6-7 yo.</p> <p>Overall and within affluent countries, LBW was not associated with current or severe rhinoconjunctivitis at 6-7 yo. Within non-affluent countries, LBW was associated with a greater odds ratio for current (OR 1.12, 95% CI 1.01-1.25) and severe (OR 1.42, 95% CI 1.06-1.92) rhinoconjunctivitis. However, there was no significant difference in risk of affluent versus non-affluent countries for current or severe rhinoconjunctivitis.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Large global study: 60 centres in 26 countries with at least 1000 participants at each centre - Multi-country study: ability to analyse outcomes between countries - Allergy based on medical criteria - Countries included in study had response rates of ≥60% - Random sampling of population <p>Weaknesses:</p> <ul style="list-style-type: none"> - No correction for gestational age - Parent-report of medical diagnosis, potential misunderstanding or recall failure - Parent-reported birth weight, potential recall failure - 65 countries originally submitted data for the study, but 39 were excluded due to missing data, <1000 participants or response rates of <60%
Hong Kong (ISAAC Phase 3) Lee <i>et al.</i> 2004 (104)	<p>Prospective study</p> <p>4,448 school children from 25 primary schools in Hong Kong were randomly selected for recruitment into the study.</p> <p>Parental questionnaire for hay fever, rhinoconjunctivitis and eczema symptoms and distribution when children were 6-7 yo.</p>	<p>Birth weight >2.5 kg associated with increased odds for current hay fever (OR 1.17, 95% CI 1.02-1.35), current rhinoconjunctivitis (OR 1.26, 95% CI 1.05-1.51) and eczema ever (OR 1.23, 95% CI 1.01-1.41) at 6-7 yo.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Allergy based on medical diagnosis/criteria - >95% responded to survey - Random sampling of population - Representative population sample <p>Weaknesses:</p> <ul style="list-style-type: none"> - No correction for gestational age (which was non-significant in univariate analyses) - Parent-report of medical diagnosis, potential misunderstanding or recall failure - Parent-reported birth weight, potential recall failure

<p>Manitoba, Canada (Study of Asthma Genes and the Environment; SAGE) Liem <i>et al.</i> 2007 (107)</p>	<p>Retrospective study</p> <p>13,980 children (including 691 born with LBW) born locally in 1995 were divided into groups based on gestational age and birth weight.</p> <p>Healthcare records from the Manitoba Health Services Insurance Plan were analysed. These records included physician visits, hospitalisations and prescription drugs over the first 7 y of life. Food allergy was defined as an International Classification of Diseases, Ninth Revision, Clinical Modification code of 693 in medical claims or a prescription of injectable epinephrine, after exclusion of children using epinephrine treatment of venom allergy.</p>	<p>LBW and very LBW categories were not associated with risk of current or ever food allergy across the first 7 y of life, using a birth weight of $\geq 2,500$ g to $< 4,500$ g as a reference.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Allergy based on medical records of treatment received, no potential recall failure; method of diagnosis of food allergy validated by three methods - Fetal size/birth data obtained from medical records <p>Weaknesses:</p> <ul style="list-style-type: none"> - Unclear whether gestational age corrected for within statistical analyses
<p>Italy Girolomoni <i>et al.</i> 2003 (60)</p>	<p>Prospective study</p> <p>1,369 children attending 4th grade at randomly selected elementary schools in 7 Italian cities.</p> <p>Parental questionnaire asking about eczema incidence and examination by a trained dermatologist at 9 yo.</p>	<p>Birth weight not associated with eczema at 9 yo.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Direct clinical diagnosis of eczema - Only 29% did not return questionnaires - Random stratified sampling of population <p>Weaknesses:</p> <ul style="list-style-type: none"> - No correction for gestational age - Parent-reported birth weight, potential recall failure

Study region (cohort name) and reference	Study design, participants and outcome measures	Effect of birth weight/fetal growth on allergic outcomes	Study strengths & limitations
Sweden (Child and Adolescent Twin Study) Lundholm <i>et al.</i> 2010 (115)	<p>Prospective study</p> <p>Swedish Twin Register study, twins born in Sweden July 1992-June 1998. 10,896 twins with perinatal data available from the Medical Birth Register participated.</p> <p>Parental questionnaires for eczema and hay fever symptoms and distribution was completed when children were aged 9 and 12 yo; secondary analyses used a question specifying physician-diagnosis of eczema.</p>	<p>For each 500 g increase in birth weight, the OR for eczema at 9 or 12 yo (combined) was 1.43 (95% CI 1.17-1.74) after adjustment for child's age and sex and OR 1.62 (95% CI 1.27, 2.06) after adjustment for other additional early life factors. For each 1 SD increase in birth weight z-score, the OR for eczema at 9 or 12 yo (combined) was 1.07 (95% CI 1.01-1.13) adjusted for child's age and sex and OR 1.12 (95% CI 1.05-1.20) after adjustment for other additional early life factors.</p> <p>In same-sex twin pairs discordant for eczema, for each 500 g increase in birth weight, the OR for eczema ever at 9 or 12 yo (combined) was 3.93 (95% CI 1.55-9.98; monozygotic twin pairs OR 4.56, 95% CI 0.84-24.76; dizygotic twin pairs OR 3.69, 1.21-11.24). Similarly for each 500 g increase in birth weight, the OR for eczema at 9 or 12 yo (combined) was 6.52 (95% CI 1.94-21.86) in same-sexed twin pairs discordant for eczema (monozygotic twin pairs OR 10.02, 95% CI 0.87-115.8; dizygotic twin pairs OR 5.64, 95% CI 1.40-22.70).</p> <p>Hay fever at 9 or 12 yo (combined) not associated with birth weight overall or with birth weight difference in analyses of co-twins discordant for hay fever.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age accounted for by co-twin analyses - Allergy based on medical criteria - Fetal size/birth data obtained from medical records - Only 30% loss to follow-up or incomplete birth data - Twin cohort study: corrects for some genetic and early postnatal environmental factors <p>Weaknesses:</p> <ul style="list-style-type: none"> - Parent-report of medical diagnosis, potential misunderstanding or recall failure - Population sample not representative (twins)
Aberdeen, UK Turner <i>et al.</i> 2011 (192)	<p>Prospective study</p> <p>Healthy pregnant women recruited at their first antenatal clinic appointment at Aberdeen Maternity Hospital. Allergic outcomes followed up in singletons born locally ($n=1,840$ singletons born in Aberdeen with survey information available for 927, and various fetal size measures available for 350-584 of these). Parental questionnaire specifying physician-diagnosed hay fever and eczema when child was 10 yo.</p>	<p>Results for fetal growth were analysed using persistent high growth ($n=122$) as reference group. Fetal growth categories were based on change in CRL (1st trimester) and biparietal diameter (2nd trimester).</p> <p>Absolute fetal size during 1st or 2nd trimesters, or persistent low growth was not associated with risk of eczema or hay fever at 10 yo.</p> <p>Growth acceleration between 1st and 2nd trimesters was associated with greater risk of eczema at 10 yo ($n=65$, regression coefficient – effect size 2.52, 95% CI 1.21-5.26, $P=0.01$) but was not associated with risk of hay fever at 10 yo.</p> <p>Growth deceleration was not associated with eczema at 10 yo but was associated with lower risk of hay fever ($n=47$, regression coefficient – effect size 0.10, 95% CI 0.01-0.82, $P=0.03$).</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age corrected for within statistical analyses - Allergy based on medical diagnosis - Fetal size data obtained from medical records; fetal size and growth rates measured directly - Representative population sample <p>Weaknesses:</p> <ul style="list-style-type: none"> - Parent-report of medical diagnosis, potential misunderstanding or recall failure - 50% loss to follow-up based on survey responses

ADOLESCENCE/ADULTHOOD (≥ 13 years of age)

Study region (cohort name) and reference	Study design, participants and outcome measures	Effect of birth weight/fetal growth on allergic outcomes	Study strengths & limitations
<p>Sheffield, England Katz <i>et al.</i> 2003 (87)</p>	<p>Prospective study</p> <p>From secondary schools in the region, 3,246 secondary school students with gestational age information were recruited.</p> <p>Parental questionnaire specifying physician-diagnosed hay fever and eczema when children/adolescents were aged 11-16 yo.</p>	<p>Hay fever OR at 11-16 yo was 1.76 (95% CI 1.22-1.54, $P < 0.01$) for highest vs. lowest birth weight quintile, but this was not significant after adjustment for early life factors.</p> <p>Birth weight quintile not associated with eczema at 11-16 yo.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age considered in statistical analyses by testing in multivariate analyses for those with data on gestational age - Allergy based on medical diagnosis/criteria - Fetal size/birth data obtained from medical records - Only 21% loss to follow-up - Representative population sample <p>Weaknesses:</p> <ul style="list-style-type: none"> - Gestational age was only recorded for 30% of participants - was not significant in multivariate analyses - Parent-report of medical diagnosis, potential misunderstanding or recall failure <p>Note: weight recorded at home interview (24-37 days post-birth), then corrected by a sex-specific growth rate constant per day post-birth prior to analyses to estimate birth weight</p>
<p>Dunedin, New Zealand (Dunedin Multidisciplinary Child Development Study) Leadbitter <i>et al.</i> 1999 (102)</p>	<p>Prospective study</p> <p>Singleton infants ($n=1,037$) born at Queen Mary Maternity Hospital in Dunedin over a one-year period and still living in the Otago Province were recruited at 3 yo for a longitudinal cohort study.</p> <p>Parental questionnaire for eczema and hay fever symptoms and distribution when adolescents were 13 yo.</p>	<p>Birth weight category (analysed as 3 categories and as 5 categories) was not associated with risk of eczema or hay fever at 13 yo.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age adjusted for within statistical analyses - Fetal size/birth data obtained from medical records - Only 29% loss to follow-up from original cohort - Representative population sample <p>Weaknesses:</p> <ul style="list-style-type: none"> - Allergy not based on medical diagnosis or symptoms but based on name of medical condition; potential misunderstanding - Parent-report of medical condition, potential recall failure

Study region (cohort name) and reference	Study design, participants and outcome measures	Effect of birth weight/fetal growth on allergic outcomes	Study strengths & limitations
Taiwan (National survey by the Taiwan Environmental Protection Administration) Lin <i>et al.</i> 2015 (109)	<p>Prospective study</p> <p>Stratified systematic sampling of junior high students were for a national survey conducted by the Taiwan Environmental Protection Administration. 74,688 singleton-born junior high school students (8.9% <13 y, 67.7% 13-14 y, 23.5% ≥15 yo) returned questionnaires and were individually interviewed.</p> <p>Parental questionnaire specifying physician-diagnosed eczema and hay fever.</p>	Size for gestational age did not affect risk of eczema or hay fever.	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age included implicitly by use of size for gestational age - Allergy based on medical diagnosis - Fetal size/birth data obtained from medical records - Only 11% did not return questionnaire, and 13% of those who returned the questionnaire did not complete the personal interview, could not be linked to the birth registration database, had otherwise missing data or were not singletons - Stratified sampling of population - Representative population sample <p>Weaknesses:</p> <ul style="list-style-type: none"> - Parent-report of medical diagnosis, potential misunderstanding or recall failure - Upper and lower limits of participant ages not specified
Göteborg, Sweden Hesselmar <i>et al.</i> 2002 (75)	<p>Prospective study</p> <p>1,515 adolescents and adults participated. SGA participants (<i>n</i>=430) had previously participated in growth studies at the Paediatric Growth Research Centre. Controls (<i>n</i>=1,085) matched for variables including gestational age and gender were selected from the national birth register.</p> <p>Self-questionnaire specifying physician-diagnosed hay fever and eczema when participants were aged (15-25 yo, median 24 yo).</p>	<p>SGA (birth weight/length below -2 standard deviations) and term-born LBW individuals (born <2,500 g at 38-42 weeks gestational age) at 15-25 yo had no difference in rates of hay fever ever compared to matched controls of unspecified birth weight.</p> <p>Term-born LBW individuals had more current hay fever than those of normal birth weight (OR 1.65, 95% CI 1.05-2.59) but it was not associated with SGA.</p> <p>Current or ever eczema was not associated with SGA or LBW in term-born individuals.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age corrected for: SGA participants matched with AGA controls; LBW analyses included only full-term individuals (38-42 weeks) - Allergy based on medical diagnosis/criteria - Fetal size/birth data obtained from medical records - Only 37% loss to follow-up <p>Weaknesses:</p> <ul style="list-style-type: none"> - Parent-report of medical diagnosis, potential misunderstanding or recall failure
Finland (FinnTwin16 Study) Räsänen <i>et al.</i> 2001 (170)	<p>Prospective study</p> <p>4,646 twins born in Finland between 1975 and 1979 were identified from the Finnish Central Population Registry with birth weight and hay fever data.</p> <p>Parental questionnaire specifying physician-diagnosed hay fever completed when adolescents were 16 yo.</p>	Within twin pairs discordant for hay fever at 16 yo, affected twins had greater birth weights than their unaffected co-twins (monozygotic twins, <i>n</i> =68 pairs, median of 15 g heavier, 95% CI -136-116 g; dizygotic twins, <i>n</i> =253 pairs, median of 30 g heavier, 95% CI -20.0-82.1 g).	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age: accounted for by co-twin analyses - Allergy based on medical diagnosis - Fetal size/birth data obtained from medical records - Only 17% did not return the family questionnaire - Twin cohort study: corrects for some genetic and early postnatal environmental factors <p>Weaknesses:</p> <ul style="list-style-type: none"> - Parent-report of medical diagnosis, potential misunderstanding or recall failure - Population sample not representative (twins)

<p>Sweden (Swedish Medical Birth and Military Service Enrolment Registers) Bråbäck <i>et al.</i> 1998 (27)</p>	<p>Retrospective study</p> <p>Data from Military Service Enrolment Register and conscript examination used for current (12 mo prevalence) hay fever ($n=149,398$ male conscripts).</p> <p>Men were examined by a physician at age 17-20 yo.</p>	<p>Decreasing birth weight category (7 categories) was associated with decreasing risk of hay fever at 17-20 yo ($P<0.0001$). Using a birth weight of $\geq 4,000$ g as a reference, birth weight categories of $<1,500$ g (OR 0.69, 95% CI 0.49-0.97), 1,500-1,599 g (OR 0.72, 95% CI 0.60-0.88), 2,000-2,499 (OR 0.83, 95% CI 0.75-0.92) and 2,500-2,999 (OR 0.94, 95% CI 0.88-0.99) were associated with a lower risk of hay fever at 17-20 yo.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age included implicitly by use of size for gestational age (growth retardation defined as a birth weight below two standard deviations of the expected weight by Swedish sex/gestational age-specific standards) - Allergy status from enrolment form confirmed by clinical examination - Fetal size/birth data obtained from medical records - Study includes almost all of the male population at 18-19 yo <p>Weaknesses:</p> <ul style="list-style-type: none"> - Questionnaire validation found that 20% of hay fever cases were not diagnosed by routine conscript examinations - Of boys born in Sweden between 1973-1975, 10% were not examined as conscripts and thus did not participate in this study - Males only included in study
<p>Fifth Conscription District, Denmark Steffenson <i>et al.</i> 2000 (186)</p>	<p>Retrospective study</p> <p>Data from 4,795 men was retrieved from draft board for national service.</p> <p>Self-questionnaire for health issues likely to affect service at 18 yo during national service registration, followed by physician confirmation of eczema.</p>	<p>Rates of eczema did not differ between birth weight categories overall or after adjustment for gestational age in whole cohort, but LBW ($\leq 2,500$ g) was associated with greater risk of eczema at 18 yo (OR 4.9, 95% CI 1.1-22.7) within term-born individuals only.</p>	<p>Strengths:</p> <ul style="list-style-type: none"> - Gestational age included in statistical model - Allergy based on medical diagnosis - Fetal size/birth data obtained from medical records <p>Weaknesses:</p> <ul style="list-style-type: none"> - Male participants only

Abbreviations: AGA, adequate size for gestational age; CI, confidence interval; LBW, low birth weight; mo, months old; yo, years old; NBW, normal birth weight; OR, odds ratio; RR, risk ratio; SGA, small size for gestational age. "Hay fever ever" refers to hay fever at any age until reported for the study.

The following two sections (1.4.2 and 1.4.3; not including subheadings) are reproduced exactly as submitted for publication with the exception of formatting, which has been changed to maintain consistency throughout the thesis. It has been submitted for publication as:

Gatford KL, **Wooldridge AL**, Bischof RJ, Clifton VL, Kind KL. Pre-birth origins of allergy and asthma, accepted by J Reprod Immunol since thesis submission (Appendix 4)

1.4.2 Evidence from human cohorts for maternal asthma and allergy during pregnancy as allergy risk factors

Maternal asthma is a common gestational exposure, affecting ~12% of singleton pregnancies in an Australian cohort (37). Maternal asthma worsens during pregnancy in ~50% of women, and 20% of asthmatic women undergo exacerbations requiring medical intervention (140, 141). Asthma during pregnancy substantially increases risks of adverse pregnancy outcomes (143), including preeclampsia (↑54%), preterm birth (↑41%), SGA (↑22%), and LBW (↑46%). Risks of adverse neonatal outcomes including admission to neonatal intensive care (↑12%), respiratory distress syndrome (↑9%) and transient tachypnoea of the newborn (↑10%) are also increased when the mother has asthma, even after correction for prematurity as a comorbidity (129).

In addition to these short-term adverse outcomes, there is good epidemiological evidence suggesting that exposure to maternal asthma or allergy before birth increase risks of the same conditions in children. Maternal asthma is consistently a stronger risk factor for childhood asthma than paternal asthma, implying that the maternal contribution is not only genetic, but that the *in utero* and possibly lactational environment also contribute to risk (108). Exposure to active maternal allergy is associated with increased risks of multiple childhood allergies, although interestingly, not with childhood asthma. In the PAULA study cohort of 526 children born in greater Munich in Germany, atopic symptoms in the mother during pregnancy were associated with >175% greater odds of food sensitisation in children within the first year of life, 60% greater odds of eczema (atopic

dermatitis) in the first two years of life, and ~100% greater odds of hay fever (allergic rhinitis) at 4-5 years of age (82). Increased odds ratios for eczema in the first two years of life and of hay fever at 4-5 years of age were also observed in a sub-analysis of children from atopic mothers, also supporting the hypothesis that these relationships reflect programming by environmental factors in addition to genetic susceptibility (82). Although maternal atopic symptoms during pregnancy were not associated with altered odds of asthma before 4-5 years of age, nor with current wheeze at 4-5 years of age in the children, increased frequency of maternal infection with common colds during pregnancy was associated with more than double the odds for childhood asthma (82). Together, this evidence implicates *in utero* exposure to maternal inflammation - induced by maternal allergy, asthma or infection - as a factor that increases susceptibility of progeny to allergic disease postnatally. Altered T cell development is implicated in programming of allergic susceptibility by exposure to maternal allergy *in utero*. Compared to neonates born from non-allergic women, neonates from allergic women have a higher proportion of type 2 T helper (Th2) cells and lower ratio of Treg to Th2 cells in cord blood (57). In the same study, low type 1 T helper (Th1):Th2 and Treg:Th2 cell ratios in cord blood predicted increased risk of eczema development in the infants by two years of age (57). DNA in peripheral blood is also differentially methylated in peripheral blood of 1 year-old infants born to mothers with asthma, compared to infants of non-asthmatic mothers, and some of the changes in DNA methylation correlate with characteristics of asthma and allergy severity in the mother or with infant circulating immune cell abundance (70). Whether these methylation changes at birth predict subsequent allergic outcomes in children is yet to be determined. The effects of maternal asthma and exacerbations in pregnancy on pregnancy outcomes and fetal and placental responses differ depending on whether the fetus is male or female (37). Intriguingly, within the Isle of Wight Birth cohort, associations between allergy in parents and children were parent-of-origin specific and differed according to the sex of the child, such that maternal allergy was associated with increased risk in girls, and paternal allergy was associated with increased risk in boys (6). Whether effects of maternal asthma and allergy are sex-specific requires confirmation in other cohorts, and if

confirmed, further study is required to determine the extent to which this reflects effects of imprinted genes or sex-specific effects of the *in utero* environment on fetal immune development and allergic susceptibility.

1.4.3 Evidence from human cohorts for methyl donor abundance as an asthma and allergy risk factor

Adequate maternal folate (vitamin B₉) status before conception and in the first few weeks of pregnancy is critical for proper development of the embryonic neural tube. Periconceptional folic acid supplementation is an extremely effective preventative measure, reducing the risk of neural tube defects (NTDs) by at least 40% (21), and health authorities in most countries and the World Health Organisation therefore recommend intakes of folic acid supplementation of 0.4-0.5 mg/d from at least a month before conception and during the first trimester (66). Many pregnancies are unplanned, however, and these women are unlikely to know they are pregnant until after development of the neural tube during the 3rd and 4th weeks after conception. Voluntary and mandatory food fortification has therefore been implemented in many countries over the past 15 years to increase folate status in all women of reproductive age, and has further reduced rates of NTDs (26). Women at high risk of delivering a baby with an NTD, including those whose previous children have had NTDs, are recommended to consume 10-fold higher doses of 4-5 mg/d folic acid periconceptionally (66). Randomised clinical trials are also evaluating efficacy of high folic acid doses (comparing 0 and 4 mg/d from before pregnancy to 12 weeks post-conception, followed by 0.2 or 0.8 mg/d for the remainder of pregnancy) in prevention of all congenital malformations, not just NTDs (24).

The evidence collated in several recent systematic reviews is that maternal folic acid supplementation at the usual doses of 0.4-0.5 mg/d during the periconceptional period before conception and during the first trimester of pregnancy is not associated with increased rates of

childhood asthma (19, 28, 41). There is some evidence that higher doses of folic acid during pregnancy are associated with asthma, based on linkage of maternal and children pharmacy dispensing data for >39 000 pregnancies in the Netherlands (209). Similar associations are evident in those dispensed high-dose folic acid in either the first or third trimester alone (209). There is also some evidence from study of a prospective birth cohort to support the original suggestion, that maternal consumption of folic acid supplements specifically in late pregnancy may increase risks of childhood asthma (199). Maternal consumption of folic acid supplements in late gestation is associated with 6-26% greater risk of childhood asthma/wheeze in progeny (28). Effects of folic acid supplementation on incidence of allergic sensitisation and eczema in childhood vary between studies, with some finding increased risk and others no effect (28), and more data is needed to characterise effects of supplement at specific periods of pregnancy and at different doses. Tuokkola (191) and colleagues recently reported that in a cohort of 2,327 children in the Finnish Type 1 Diabetes Prediction and Prevention study, maternal folic acid supplement use but not dietary folate intake in the 8th month of pregnancy was associated with 40% greater risk of cow's milk allergy in 5 year-old children. This suggests that maternal folic acid supplementation in late gestation is likely to predispose progeny to later allergic disease in general, and not specifically asthma. Any changes to dietary recommendations about folic acid supplementation in pregnancy need to be made with care, in order not to confuse women about the benefits of periconceptional supplementation in reducing NTDs. Additional information is therefore required, including childhood allergic outcomes in trials of high-dose maternal folic acid, to clearly define the impact of high and late pregnancy consumption of folic acid on allergic outcomes, potentially providing the opportunity to intervene at a population level to decrease allergic disease incidence.

1.4.4 Strengths and limitations of human studies

1.4.4.1 Strengths

Human studies investigating the effect of maternal methyl donor status on offspring allergy examine allergy rates in offspring that have been subjected to different early postnatal environments. For example, if a mother has many allergies herself, her children may be less exposed to allergens within the home due to environmental avoidance, which prevents or reduces the intensity of allergic symptoms (30). Exposure to increased allergens is associated with increased allergic sensitisation (100), hence this environmental exposure may affect the reported allergic risk of factors tested in human studies.

Human studies have found varying associations between size at birth and allergic outcomes at different postnatal ages, and have identified that patterns of fetal growth may be at least as important as absolute size at birth for prenatal programming of allergy. Twin studies and large, standardised multi-national studies comprise some of the most robust studies within this field of research and more variables such as gestational age and parental atopy have been controlled or corrected for within recent studies compared with those conducted during the field's early stages. However, a thorough systematic review of the literature is still required to determine the potentially varying associations between size at birth and allergic outcomes as differences between ages and sexes and mechanistic pathways remain unclear. The link between maternal allergic states and subsequent offspring allergy has also been established in human studies, and has indicated a stronger link between maternal compared to paternal allergy, which implicates an inflammatory *in utero* environment as a likely causal factor. Neonatal T cell phenotypes, differential DNA methylation of relevant genes and some evidence for sex-differences in causal factors have been all investigated in human studies and have provided some key mechanistic information to support the concept of *in utero* programming of allergy. However, it is unknown how the fetus responds in real-time to maternal inflammatory stimuli and how the timing of the inflammatory stimuli affects the extent of allergic susceptibility, with these gaps in knowledge largely the result of ethical limitations. The role of maternal methyl donor intake during later stages of pregnancy has been

examined to some extent in humans, and suggests that high folic acid intake during late pregnancy is associated with increased risk of allergy, although findings are varied and require confirmation in further cohorts. However, randomised trials in normal pregnancies are ethically prohibited due to the suggestion that high folic acid intake during late gestation has adverse allergic outcomes during late pregnancy (209). Studies of maternal folic acid supplementation as a preventative treatment for conditions such as preeclampsia continue to be undertaken (177), however, follow-up assessments of offspring immune outcomes have unfortunately not been conducted. Nevertheless, experimental animal models are likely to provide the strongest evidence for or against the role of methyl donor intake in the prenatal programming of allergic susceptibility and to elucidate mechanisms of action.

The primary strength of studies within the field of prenatal programming of allergy is that most studies investigating the effects of poor fetal growth on allergic outcomes are of humans. These studies have been increasingly standardised and global – multi-centre studies like the ISAAC have allowed for the investigation of associations between poor fetal growth and allergic outcomes in offspring under very different environmental exposures. This is highlighted by the finding that this association differs between affluent and non-affluent countries (Table 1.1, 132). Twin studies enable the opposite – the investigation of associations under very similar early postnatal environmental exposures; the simplicity of this study design (heavier versus lower birth weight twin comparison) has yielded results similar to non-twin studies (Table 1.1, 76, 115, 132).

1.4.4.2 Limitations

Despite multiple studies of developmental programming of allergy, human studies alone are unable to fully characterise the effects of poor fetal growth on allergic outcomes, partially due to ethical limitations. In accepting that developmental programming can cause long-term adverse outcomes, we must also accept that imposing such challenges for research purposes is likely to cause these outcomes. Investigation of underlying mechanisms and responses to novel therapeutics in the fetus are limited as

due to risks for the pregnancy; monitoring of fetal health is generally restricted to imaging without direct sampling of tissues *in utero*. As such, most systemic diagnostic tests are conducted on cord blood samples, with studies unable to measure fetal systemic characteristics throughout pregnancy. Together with the need to establish safety and efficacy before clinical testing, minimising impact on human subjects means that the capacity to define mechanisms and test interventions in humans is limited. While much useful information has been gained from the analysis of cord blood samples, it is not sufficient to understand immediate fetal responses to adverse events during early-mid gestation. One such example is the fetal response to maternal asthma exacerbations, particularly because asthma exacerbations cannot ethically be deliberately induced in pregnant women and spontaneous asthma exacerbations usually occur away from diagnostic equipment.

Data availability also limits confirmation of direct causality, as it can be difficult to gather data on large numbers of potentially confounding factors with human participants. This is particularly a problem with retrospective studies or studies that limit their collection of data on perinatal environmental factors due to feasibility. Additionally, there can be wide variations in early perinatal life exposures within human populations, further complicating interpretation. Most human studies have not categorised SGA infants into symmetric and asymmetric SGA, thus studies recruiting SGA infants without taking newborn measurements may be limited as they cannot distinguish between these groups. This is important to note, as fetal organs develop at different stages during gestation, thus the timing of an insult to the fetus may affect what organ systems are adversely affected during postnatal life (12). Fetuses growth-restricted from early pregnancy grow symmetrically, whereas fetuses growth-restricted from late pregnancy grow asymmetrically, indicative of organ sparing (51); hence these groups may reflect acute and chronic fetal growth restriction respectively, which may have different effects on long-term postnatal outcomes. Not all studies in IUGR humans have differentiated between IUGR due to maternal under nutrition and that due to placental dysfunction. Nulliparous women are more likely to have IUGR infants compared to multiparous women (123), but

parity is not always recorded and may interact with effects of IUGR. Poor fetal growth is also caused by factors other than placental dysfunction and often occurs with comorbidities. One example of such a comorbidity is preterm birth, which itself reduces birth weight and is associated with deficiencies in adaptive immune function (reviewed in 128). Socioeconomic status is another example of an early life factor that is associated with both SGA and total plasma IgE concentrations and may confound studies in human cohorts (123). Within the human literature, the range of exposures corrected for varies greatly. The most common exposures that are corrected for in statistical analyses include gestational age, familial atopy, and having been breastfed for at least six months. Many more exposures remain uncorrected in statistical analyses, with one example being maternal dietary intake during pregnancy. Certain maternal dietary patterns are associated with decreased or increased risk of allergy in their children. For example, a Mediterranean diet during pregnancy is protective against allergy in children (35, 43) whereas a diet high in n-6 polyunsaturated fatty acids may increase the risk of allergy in children (176). However, collection of data on maternal diet is laborious and often not feasible and hence not considered in many studies. Other than twin studies, there is no way to correct for variation in early postnatal environment in human studies. Twinning restricts both placental and fetal growth (reviewed in 63), and recruitment for a study of placental insufficiency in twin pregnancies would not be feasible, hence twin studies alone are not ideal for investigating the effect of placental restriction of fetal growth on allergic susceptibility. Parity also affects birth weight, with first pregnancies resulting in smaller infants (reviewed in 63). Therefore, ethical limitations prevent thorough investigation of the mechanisms by which poor fetal growth affects allergic outcomes in human studies, and hence, both human and animal studies are required to fully characterise the effects of poor fetal growth on allergic outcomes. These limitations of human studies related to measurement capacity, confounding and highly variable environments and the need for more detailed information about perinatal environments can be minimised by use of animal studies.

1.5 Developmental programming of allergic disease in animal models

1.5.1 IUGR

The following section (1.5.1.1; not including subheading) is reproduced exactly as submitted for publication with the exception of formatting, which has been changed to maintain consistency throughout the thesis. It has been submitted for publication as:

Gatford KL, **Wooldridge AL**, Bischof RJ, Clifton VL, Kind KL. Pre-birth origins of allergy and asthma, accepted by J Reprod Immunol since thesis submission (Appendix 4)

1.5.1.1 Chronic experimental IUGR reduces allergic sensitisation

Allergic sensitisation has been reported in only a few experimental models of IUGR to date, with variable effects possibly reflecting the cause of IUGR (and hence different fetal exposures) as well as different developmental timings of restriction. In Wistar rats, maternal nutrient restriction to 50% of *ad libitum* intake from mating until delivery induces a severe IUGR phenotype, reducing birth weight of pups by 32-34%. Allergic responses of young adult progeny to airway allergen challenge, including ovalbumin (OVA)-specific IgE production, inflammatory cell airway infiltration, mucus secretion and collagen deposition were attenuated in progeny of feed-restricted mothers compared to control progeny (98, 99). Lung cytokine and transcription factor gene expression patterns in allergen-challenged progeny were also altered, suggesting a shift from Th1 to Th2 immune responses following *in utero* exposure to maternal undernutrition (99). In contrast, allergic responses to OVA sensitisation and a 2-week OVA inhalation exposure were increased rather than decreased in IUGR rat progeny (birth weight <10th centile of control progeny) when induced by a similar maternal undernutrition protocol throughout pregnancy in Sprague-Dawley rats (207). This accentuated allergic response after OVA challenge occurred in conjunction with increased lung endothelin-1 (*Et1*) gene and protein expression, together with increased histone acetylation but unchanged methylation of the *Et1* promoter, in IUGR compared to control progeny (207). Causality of the epigenetic changes and increased *Et1* expression in enhanced allergic responses of these IUGR

progeny has not yet been demonstrated. Why effects of maternal undernutrition on allergic susceptibility of progeny differ between these two sets of studies is not clear, but might relate to rat strain, progeny sex or differences in sensitisation dose or continuous vs intermittent OVA challenge protocols. A milder reduction of 17% in neonatal weight induced using a maternal pregnancy stress protocol in mice (24 h sound stress at d 12 and d 14 of pregnancy) was associated with increased allergic responses in adult progeny (164). Conversely, maternal noise-induced stress protocols (hourly exposure each day from d 15 to 21 of pregnancy) that did not alter pup size at birth reduced delayed hypersensitivity reaction to bovine serum albumin in sensitised male and female progeny (185). Further studies appear needed to clarify the effects of IUGR on allergic susceptibility in rodents and to determine which aspects of the *in utero* environment alter immune development and predispose to allergy.

In humans, IUGR is often associated with impaired placental function, and this can be mimicked experimentally by pre-mating removal of the majority of placental attachment sites before mating in sheep (placental restriction, PR), which reduces placental size and function (4, 173). We have applied established protocols for systematic sensitisation to allergens and cutaneous allergen challenges in this species to evaluate effects of PR on susceptibility to allergy (18). In our recent studies, PR reduced birth weight by 20%, and decreased delayed cutaneous hypersensitivity reactions to OVA despite increased IgE responses to allergens after sensitisation to OVA and house dust mite (Appendix 1, 203). Acute cutaneous inflammatory responses to histamine correlated positively with birth weight in singleton progeny of this cohort (Appendix 1, 203). We have since found that mast cell density in skin is not reduced in the adult PR progeny (Wooldridge *et al.*, unpublished, Chapter 3). We therefore hypothesise that loss of mast cell function explains the suppressed cutaneous delayed hyper-sensitivity inflammatory responses in the presence of normal or exaggerated IgE responses to allergens in PR sheep, but this requires direct testing. Overall, the balance of evidence from experimental models suggests that chronic IUGR induced by reduced nutrient supply to the

fetus is protective against allergy, consistent with the associations between low birth weight and reduced incidence of allergy reported in children.

1.5.1.2 Limitations in existing studies of experimental IUGR and allergic outcomes

The animal/mechanistic literature still has many gaps in knowledge. Before the studies described in this thesis, the field of prenatal programming of allergic susceptibility lacked studies using a placental dysfunction model that is developmentally similar to humans. The existing literature describes rat models of placental dysfunction, and these studies have been useful for establishing epigenetic mechanisms as the pathway by which these changes may occur. Rats give birth to multiple (usually >6) pups, however, whereas humans usually have singletons or twins. Large litter size may itself restrict fetal growth, which may have different mechanisms to placental dysfunction. Additionally, the immune system of the rat is still very underdeveloped at birth relative to humans (94). Hence, placental restriction will impact on different in stages of immune development these species, and early postnatal environmental factors such as lactation will have a greater influence on immune development during its critical window of development in rodents compared to more altricial species including humans.

1.5.2 Maternal allergy and asthma

The following section (1.5.2.1; not including subheading) is reproduced exactly as submitted for publication with the exception of formatting, which has been changed to maintain consistency throughout the thesis. It has been submitted for publication as:

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1.5.2.1 Experimental allergy and asthma in the mother pre-dispose progeny to allergy

To date, the hypothesis that susceptibility to allergic disease is programmed by *in utero* exposure to maternal atopic states has been tested primarily in mice. We have recently developed an ovine model of maternal allergic asthma which will enable this question to be evaluated in a large animal model and allow direct studies of fetal responses and longitudinal studies of individual progeny. This model will also be described below.

In the mouse, maternal allergic asthma before and during pregnancy increases susceptibility of pups to allergy, predisposing them to allergic responses to sensitisation (72). This is a systemic response, since pups are more likely to develop allergic responses to novel antigens as well as after sensitisation with ovalbumin, the allergen used to induce maternal allergy (72). At least in this mouse model, exposure during gestation or lactation was sufficient to induce allergic susceptibility in progeny, suggesting circulating inflammatory cells or signals in the mother can be transmitted to progeny across the placenta or in breastmilk (105). Transfer of allergen-specific T cells from donor mice to non-sensitised dams followed by airway allergen exposure during pregnancy also increased risk of allergic asthma in mouse progeny without causing overt maternal asthma, showing that the presence and stimulation of allergen-specific T cells during pregnancy are sufficient to program allergic susceptibility in progeny (79). Only induction of an allergic (Th2-biased) immune response to OVA increases progeny susceptibility to allergic sensitisation. If dams are sensitised to OVA using a protocol that induces a Th1-biased immune response, then pups are actually protected against allergic sensitisation to OVA, although protection by the maternal Th1 response is allergen-specific, in contrast to induction of susceptibility (121). Consistent with this protective effect of non-allergic maternal allergen exposures, maternal airway OVA exposure from early pregnancy until delivery, which did not induce maternal allergy, induced IL-10 and Treg-mediated immune tolerance to OVA in progeny that inhibited their allergic responses to OVA-sensitisation into adulthood (58). In a single study in dogs, maternal and paternal sensitisation to ragweed before mating was associated with

increased circulating antibody responses and asthmatic-type lung responses to inhaled ragweed in progeny. This study is limited, however, by use of pups from only two litters in each group and potential effects of maternal ragweed exposure during lactation (15). Together with the human data, these results in experimental models are consistent with the hypothesis that exposure to maternal allergy *in utero*, but not allergen exposure in the absence of allergy, increases the allergic susceptibility of progeny, and that this is not specific to the *in utero*-exposed allergen/s.

In order to directly evaluate the acute fetal and long-term progeny effects of maternal allergic asthma, and to enable evaluation of the effects of clinical and experimental interventions on these, we have recently developed an ovine model of maternal allergic asthma in pregnancy (Appendix 2, 38). Sheep are sensitised systematically by repeated immunisation with allergen, followed by repeated airway challenges with aerosolised allergen, utilising a protocol that induces an allergic asthmatic phenotype in non-pregnant sheep (17, 18). We mated ewes that had been sensitised and commenced airway challenges to house dust mite prior to pregnancy, and continued airway challenges with house dust mite throughout pregnancy (Appendix 2, 38). These pregnant ewes developed characteristics of allergic asthma including increased lung resistance, progressive increases in the eosinophil influx induced by airway allergen challenges, and increased deposition of smooth muscle around lung airways (Appendix 2, 38). The 12% reduction in relative fetal weight in late pregnancy in this model is consistent with effects of maternal asthma in human pregnancy, although additional studies are needed to determine whether fetal responses to maternal allergic asthma in sheep are sex-dependent as occurs in humans (37, Appendix 2, 38). We have begun to study the effects of maternal allergic asthma on fetal immune phenotype in this model. To date, the main effect we have observed is that fetuses from allergic ewes had a greater proportion of cluster of differentiation (CD)44-positive lymphocytes in thymus than control fetuses, with a similar trend in the lymphocyte population isolated from spleen (Wooldridge *et al.*, unpublished, Chapter 4). This cell adhesion molecule marker is involved in lymphocyte adhesion to endothelial cells via hyaluronic

acid and this interaction is essential for migration of activated T cells into sites of inflammation (44, 45). Blocking CD44 action in a mouse model of airway allergic inflammation prevented or attenuated many of the inflammatory responses to airway allergen challenge including eosinophil and lymphocyte accumulation in lung, antigen-induced increases in Th2 cytokines and chemokines in lung liquid and antigen-induced airway hyper-responsiveness (86). Anti-CD44 antibody treatment also inhibits the cutaneous delayed-type hypersensitivity in a murine model of contact allergy (32), consistent with the importance of CD44 in allergic inflammation. If the elevated CD44 expression in lymphocytes we see in late gestation fetuses in our ovine model of maternal allergic asthma persists, it may therefore predispose the progeny to allergic inflammation postnatally. The availability of this large animal model of maternal allergic asthma, where allergic sensitisation and tissue and molecular responses can be investigated in the same animals over time, will allow us to investigate these potential mechanisms for developmental programming of allergy.

1.5.2.2 Limitations in existing studies of experimental maternal allergy and asthma

Small rodent models have proven useful in elucidating some of the cellular and DNA methylation pathways behind the effects of maternal allergy and asthma on offspring allergic susceptibility. This has confirmed and expanded on findings from human studies that maternal allergy and not just allergen exposure is required for offspring to have increased allergic susceptibility. Experimental models have also confirmed that allergic susceptibility is not allergen-specific, which is not easy to determine from human studies due to the wide range of allergens and vastly different environments to which humans are exposed *in utero* and during early postnatal life. The primary limitation of current studies within this field are that most have used small animal models that do not allow the direct investigation of fetal responses to challenge *in utero* due to the use of animals that are too small for fetal instrumentation and that also undergo a greater proportion of development of the immune system postnatally compared to humans (94). Previously used rat and dog models are also in litter bearing species, in which intra-litter competition affects fetal growth. The establishment of a large animal model to directly

investigate fetal responses to maternal allergic asthma, in a species with singleton and twin pregnancies and with a relatively mature immune system at birth, will allow studies of mechanisms and interventions that are more likely translatable to humans.

1.5.3 One-carbon pathways

The following section (1.5.3.1; not including subheading) is reproduced exactly as submitted for publication with the exception of formatting, which has been changed to maintain consistency throughout the thesis. It has been submitted for publication as:

Gatford KL, **Wooldridge AL**, Bischof RJ, Clifton VL, Kind KL. Pre-birth origins of allergy and asthma, accepted by J Reprod Immunol since thesis submission (Appendix 4)

1.5.3.1 *Experimental manipulation of one-carbon pathways and progeny allergy*

The strongest experimental evidence for a role of methyl donor exposure *in utero* in allergic susceptibility comes from a study where female mice were fed diets containing high (HMD) or low (LMD) levels of methyl donors and cofactors important in one-carbon metabolism (folic acid, vitamin B₁₂, choline, L-methionine, zinc, and betaine) from 2 weeks before mating until weaning of the progeny (78). Compared to the LMD group, feeding HMD throughout pregnancy increased the severity of allergic airway disease (Th2-type immune responses) not only in the progeny exposed to this diet *in utero* (F1 generation), but also in the F2 generation (78). DNA methylation at multiple gene loci differed between HMD and LMD progeny, including greater methylation of runt-related transcription factor (*Runx3*) and decreased *Runx3* gene and protein expression in HMD progeny. These changes in *Runx3* may be partially responsible for greater allergic susceptibility as this gene negatively regulates allergic airway disease (78). A number of methylated genes are also important determinants of T cell lineage, providing another pathway for effects of methyl donor metabolism on immune phenotype. For example, demethylation of *FOXP3* correlates with greater expression of *FOXP3* in whole cord blood, as well as with circulating Treg cell numbers and suppressive activity of

Tregs in culture of mononuclear cells isolated from cord blood and challenged with common allergens (113).

Our findings that PR protects progeny against allergic sensitisation (Appendix 1, 203), discussed above, are also consistent with the hypothesis that decreased methyl donor abundance *in utero* may alter methylation of key genes to initiate a trajectory of immune system development that is subsequently less susceptible to developing allergy. In rodent models of PR, fetal one-carbon donor abundance is decreased, one-carbon pathway enzyme expression is altered and this is associated with hypomethylation of DNA and increased histone acetylation in multiple tissues (88, 118, 160). Consistent with the hypothesis that reduced placental methyl donor transport to the fetus protects against allergy in the PR sheep, when we supplemented PR ewes with methyl donors and cofactors in the last month of their five month gestation, the protective effects of PR against cutaneous delayed-type hypersensitivity after allergen sensitisation were partially lost (Wooldridge *et al.*, unpublished, Chapter 3). Effects of PR on antibody responses to allergen sensitisation were not altered by maternal methyl donor supplementation, however (Wooldridge *et al.*, unpublished, Chapter 3). We are currently investigating effects of our PR and maternal methyl donor supplementation on one-carbon metabolism in our ovine models to further evaluate the potential role of methyl donors in programming of allergy.

1.5.3.2 Limitations in existing studies of one-carbon pathways and progeny allergy

Experimental studies of maternal one-carbon intake have demonstrated that intakes of these nutrients can affect allergy and immune function in multiple generations, although it is unknown whether these are direct or indirect exposure effects. These studies have also confirmed the importance of methylation as a mechanism by which programming of allergy occurs. Whether increased maternal methyl donor supplementation can prevent the protective effects of PR, which is associated with decreased methyl donor supply to the fetus at least in rats, is currently unknown.

1.6 Thesis hypotheses and aims

Based on the literature and current gaps in knowledge describe above, the aims and hypotheses for my thesis are as follows:

Hypothesis One:

Intrauterine growth restriction, due to placental restriction (PR), will be protective against allergic susceptibility in the adolescent sheep.

Aim One:

To determine the effects of intrauterine growth restriction, due to PR, on allergic susceptibility in the adolescent sheep. Outcomes of this aim are described in experimental chapter 2.

Hypothesis Two:

Protective effects of PR against allergic susceptibility will be ameliorated by maternal dietary methyl donor and cofactor supplementation during late PR pregnancy in the adolescent sheep.

Aim Two:

To determine the effects of maternal dietary methyl donor and cofactor supplementation during late pregnancy on allergic susceptibility of PR progeny. Outcomes of this aim are described in experimental chapter 3.

Hypothesis Three:

In utero exposure to maternal systemic allergy and maternal asthma exacerbations will induce an allergic phenotype in thymic and splenic immune cells of the fetal sheep in late gestation.

Aim Three:

To investigate the effects of maternal allergic asthma on the fetal immune system in an ovine model. Outcomes of this aim are described in experimental chapter 4.

Chapter 2: Effect of placental restriction on susceptibility to allergy

2.1 Overview

Chapters 2 and 3 describe outcomes from a progeny cohort from control and placentally-restricted pregnancies, including those whose mothers were fed a methyl donor and cofactor dietary supplement as described in Chapter 3. Within the broader project, I assisted in animal management and measures of maternal and progeny size throughout the study, surgical implantation of catheters and metabolic testing in progeny. More specifically, my contributions to generating the outcomes described in Chapters 2 and 3 were as follows: I performed the majority of the allergic sensitisations and blood sampling to measure immune cell populations and antibody abundance before and after sensitisation, prepared animals for skin prick testing, assisted with measurement of skin wheal responses, conducted all antibody assays, assisted with all post-mortems, performed all histological processing, staining and mast cell counting in skin sections, analysed all data, and prepared all figures. The information in Chapter 2 has been published as a manuscript, of which I am first author and wrote the first draft (Appendix 1, 203). This was also the first publication describing this experimental cohort. Figures in Chapter 2 have been reproduced with permission from the American Journal of Physiology. Material in Chapter 3 has not yet been published. Metabolic outcomes have also been published from this cohort of control and placentally-restricted sheep plus additional treatment groups, and I am a co-author on that publication (Appendix 5, 112).

2.2 Statement of authorship – Placental restriction of fetal growth reduces cutaneous responses to antigen after sensitization in sheep

Statement of Authorship

Title of Paper	Placental restriction of fetal growth reduces cutaneous responses to antigen after sensitization in sheep
Publication Status	<input checked="" type="checkbox"/> Published <input type="checkbox"/> Accepted for Publication <input type="checkbox"/> Submitted for Publication <input type="checkbox"/> Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Amy Wooldridge		
Contribution to the Paper	Performed experiments; analysed data; interpreted results of experiments; prepared figures; drafted manuscript; edited and revised manuscript; approved final version of manuscript		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/10/15

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Robert Bischof		
Contribution to the Paper	Conception and design of research; performed experiments; analysed data; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript		
Signature		Date	20/10/2015

Name of Co-Author	Els Meeusen		
Contribution to the Paper	Conception and design of research; edited and revised manuscript; approved final version of manuscript		
Signature		Date	21/10/2015

Name of Co-Author	Hong Liu		
Contribution to the Paper	Performed experiments edited and revised manuscript; approved final version of manuscript		
Signature		Date	9/1/2016

Name of Co-Author	Gary Heinemann		
Contribution to the Paper	Performed experiments edited and revised manuscript; approved final version of manuscript		
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Name of Co-Author	Damien Hunter		
Contribution to the Paper	Performed experiments edited and revised manuscript; approved final version of manuscript		
Signature		Date	8/2/16

Name of Co-Author	Lynne Giles		
Contribution to the Paper	Analysed data; edited and revised manuscript; approved final version of manuscript		
Signature		Date	12/10/2015

Name of Co-Author	Karen Kind		
Contribution to the Paper	Analysed data; edited and revised manuscript; approved final version of manuscript		
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Name of Co-Author	Julie Owens		
Contribution to the Paper	Conception and design of research; edited and revised manuscript; approved final version of manuscript		
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Name of Co-Author	Vicki Clifton		
Contribution to the Paper	Conception and design of research; interpreted results of experiments; drafted manuscript; edited and revised manuscript; approved final version of manuscript		
Signature		Date	12/10/15

Name of Co-Author	Kathryn Gatford		
Contribution to the Paper	Conception and design of research; performed experiments; analysed data; interpreted results of experiments; prepared figures; drafted manuscript; edited and revised manuscript; approved final version of manuscript		
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2.3 Abstract

Prenatal and early childhood exposures are implicated as causes of allergy, but the effects of intrauterine growth restriction on immune function and allergy are poorly defined. We therefore evaluated effects of experimental restriction of fetal growth on immune function and allergic sensitisation in adolescent sheep. Immune function (circulating total red and white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, and basophils, and the antibody response to Clostridial vaccination) and responses to house dust mite (HDM) allergen and ovalbumin (OVA) antigen sensitisation (specific total Ig, IgG₁, and IgE antibodies, and cutaneous hypersensitivity) were investigated in adolescent sheep from placentally restricted (PR, $n=23$) and control ($n=40$) pregnancies. Increases in circulating HDM-specific IgE ($P=0.007$) and OVA-specific IgE ($P=0.038$) were greater in PR than control progeny. PR did not alter total Ig, IgG₁, or IgM responses to either antigen. PR increased OVA-specific but not HDM-specific IgA responses in females only ($P=0.023$). Multiple birth increased Ig responses to OVA in a sex-specific manner. PR decreased the proportion of positive cutaneous hypersensitivity responders to OVA at 24 h ($P=0.030$) but had no effect on cutaneous responses to HDM. Acute wheal responses to intradermal histamine correlated positively with birth weight in singletons ($P=0.023$). Intrauterine growth restriction may suppress inflammatory responses in skin downstream of IgE induction, without impairment in antibody responses to a non-

polysaccharide vaccine. Discord between cutaneous and IgE responses following sensitisation suggests new mechanisms for prenatal allergy programming.

2.4 Introduction

Increasing evidence suggests that environmental factors, including prenatal or early childhood exposures, modulate later allergy susceptibility (167). One such prenatal event is intrauterine growth restriction (IUGR), which affects 6–12% of babies in developed countries (92) and usually results in small size at birth for gestational age where the birth weight of babies falls below the tenth percentile for sex at a given gestational age (SGA, 206). IUGR and SGA babies have increased risks of perinatal mortality and morbidity and of adult metabolic and cardiovascular disease (62). Less is known about the effects of IUGR on postnatal immune function, including allergy. Low birth weight individuals are at greater risk of pneumococcal infection as infants (181) and may have impaired B-cell function as adults (135, 136), but these studies did not separate low birth weight due to IUGR and prematurity. Risks of allergic sensitisation and atopic diseases including eczema and hay fever in children and adults are positively related to size at birth, independent of gestational age, in some (23, 65, 87), but not all studies (75, 161). Similarly, in a twin study, the heavier birth weight twin had a higher risk of childhood atopic eczema (115). Although SGA is associated with increased, rather than decreased, risks of childhood and adult asthma (152, 196), these studies did not differentiate allergic and non-allergic asthma and possibly reflect impaired lung development following IUGR (188). Together, these findings suggest IUGR suppresses immune function and decreases the risk of postnatal allergy, although these human studies may be confounded by environmental factors that affect both fetal growth and immune function (148, 171).

Poor placental function is a major cause of IUGR due to reduced fetal substrate supply (175).

Restricted placental growth and function (PR) can be induced surgically in sheep, with similar prenatal and postnatal consequences as human IUGR (4, 173). Similar to humans, sheep sensitised

to allergens develop IgE responses, cutaneous hypersensitivity, and airway hyperresponsiveness following allergen challenge (125). This provides a repeatable, well-characterised protocol for induction of allergy in this species (17, 18, 125), allowing susceptibility to allergic challenge to be tested independent of prenatal environment. We therefore used the sheep to directly test the hypothesis that restricted fetal growth impairs immune function and reduces allergy susceptibility in adolescence.

2.5 Methods

All procedures were approved by the University of Adelaide Animal Ethics Committee (M-2010-139) and conducted in accordance with Australian guidelines (147).

2.5.1 Animal model

Placental growth of primiparous Merino × Border Leicester ewes was restricted by surgical removal of all but four visible endometrial placental attachment sites (caruncles) from each uterine horn (4, 173) at least 10 wk before timed mating (156). No maternal surgery occurred during pregnancy, and control ewes were unoperated. Pregnant control (CON, unoperated) and PR ewes were housed indoors from *day 110* of gestation until their spontaneously born lambs were weaned at 13 wk of age. Ewes were fed 1 kg Rumevite pellets daily (Ridley AgriProducts, Melbourne, Australia), with *ad libitum* access to lucerne chaff and water. Gestational ages, birth weights, and litter sizes were recorded. After being weaned, progeny were housed in outside paddocks in same sex groups of similar ages and fed 0.5 kg Rumevite pellets per sheep daily, with *ad libitum* access to oaten hay, pasture, and water. Sheep were housed indoors in individual pens for ≥6 days before and 3 days during cutaneous hypersensitivity testing, with 0.5 kg pellets/day and *ad libitum* access to lucerne chaff and water. Immune function was studied in 17 CON males (2 singletons, 13 twins, 2 triplets), 23 CON females (5 singletons, 18 twins), 10 PR males (5 singletons, 5 twins), and 13 PR females (9 singletons, 3 twins, 1 triplet).

2.5.2 Immunisation, sensitisation, and cutaneous hypersensitivity testing

Sheep were immunised with an anti-Clostridial vaccine (Ultravac 5-in-1; Pfizer Animal Health, West Ryde, Australia) at 5 and 9 wk of age (Figure 2.1). Sheep were then sensitised to house dust mite allergen (HDM; CSL, Parkville, Australia) and ovalbumin (OVA; A2512, Sigma, MO), each administered mixed with aluminium hydroxide as adjuvant (1:1) by subcutaneous injections (17, 194) at 20, 22, 24, and 26 wk of age. Immediate and delayed cutaneous responses (cutaneous hypersensitivity) to intradermal injections of 50 µl saline (negative control), histamine (10 µg/ml, H7375, Sigma), HDM (100 µg/ml), and OVA (10 µg/ml) were assessed at 28 wk of age (18). No adjuvants were given with intradermal injections. Skin wheal responses were measured with calipers at 0.5, 4, 2, and 48 h, and an average diameter across two perpendicular readings of ≥ 3 mm was classified as a positive reaction.

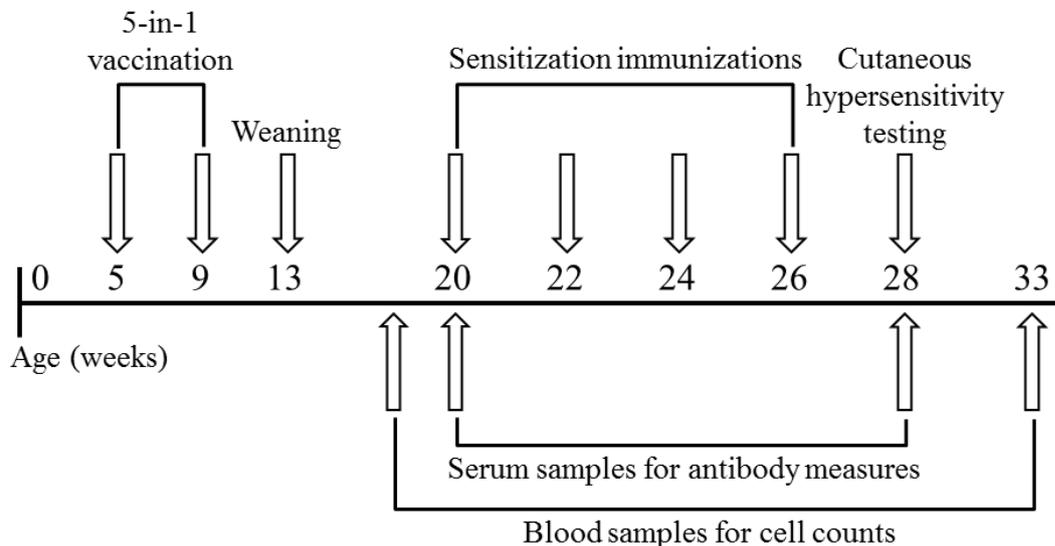


Figure 2.1 *In vivo* study timeline.

2.5.3 Serum antibody concentrations

Peripheral blood was collected at 20 wk of age and immediately before cutaneous hypersensitivity tests at 28 wk of age (Figure 2.1), and serum was stored at -80°C . Serum clostridial-specific total Ig

was assayed on ELISA plates precoated with 10 µg/ml Chauvoei antigen (Pfizer Animal Health, West Ryde, Australia), with samples taken at 28 wk diluted 1/500 in Blue Diluent (AsureQuality, Tullamarine, Australia). Sheep serum was used for standards (serially diluted to 1/32,000) and positive controls. Horseradish peroxidase (HRP)-conjugated rabbit anti-sheep IgG was diluted 1/2,000 in Blue Diluent and used as the detection antibody. Plates were developed with 3',3',5',5'-tetramethyl-benzidine dihydrochloride hydrate (TMB, Sigma, Castle Hill, Australia), and optical density was read at 450 nm.

HDM- and OVA-specific total Ig, IgG₁, IgE (17, 18, 180, 194), IgM, and IgA antibodies pre- (20 wk) and post- (28 wk) immunisation were determined in duplicate by ELISA, with optical density read at 450nm. IgM and IgA were assayed by ELISA as for total antigen-specific Ig (18, 194), but with rabbit anti-ovine IgA (Bio-Rad AbD Serotec, Kidlington, UK), or rabbit anti-ovine IgM (diluted 1/5,000, Bio-Rad AbD Serotec, Kidlington, UK) as primary antibody, and HRP-conjugated swine anti-rabbit Ig (diluted 1/1,000, Dako, Glostrup, Denmark) as secondary antibody. Antibody responses to sensitisation were classified as positive when they increased by greater than two fold relative to basal concentrations.

2.5.4 Cell counts

Peripheral blood was collected into EDTA-coated tubes at 18 (subset of 75% of cohort) and 33 wk of age. Samples were stained with Wright's Giemsa stain (Siemens, Munich, Germany). Total red blood cells (RBC) and white blood cells (WBC) were quantified using an automated cell counter (Cell Dyn 3700, Abbott Diagnostics, IL), then 100 WBC per sample were classified manually under light microscopy to differentiate WBC subtypes (neutrophils, lymphocytes, monocytes, eosinophils, and basophils).

2.5.5 Statistical analysis

Continuous and binary outcomes were analysed using a Generalised Linear Mixed Models framework that examined the effects of PR, litter size (singleton vs. multiple birth), and sex, treating the dam as the experimental unit and data from siblings as repeated measures on each dam. The distributions of continuous variables were assessed for normality, and a log, square root or inverse transformation was applied as necessary. Binary outcomes were analysed within this framework, assuming a binomial distribution and logit link function. Interaction effects were non-significant for all binomial outcomes, and the final model used for these included main effects only. Relationships between continuous variables were examined through the calculation of Pearson's correlation coefficient, restricted to singletons to remove effects of clustering due to ewes. Data were analysed using SPSS software, version 20.0 (SPSS, Chicago, IL) and are shown as estimated means \pm SE. $P < 0.05$ was accepted as statistically significant. Interactions are not mentioned unless significant.

2.6 Results

2.6.1 Birth weight and gestational age

PR reduced birth weight by 20% (CON: 5.70 ± 0.22 kg, PR: 4.55 ± 0.21 kg, $P < 0.01$), and multiple birth reduced birth weight by 14% (singleton birth: 5.52 ± 0.23 , multiple birth: 4.73 ± 0.20 , $P = 0.010$). Sex did not affect birth weight. Gestational age at birth was 139–150 days and was reduced by 2.2 days in PR pregnancies (CON: 147.1 ± 0.5 days, PR: 144.9 ± 0.5 days, $P = 0.004$). Neither litter size nor sex affected gestational age. Inclusion of gestational age as a covariate did not change effects of PR on continuous outcomes; therefore, it was not included as a factor in final analyses.

2.6.2 Circulating immune cells

At 18 wk of age, PR, litter size and sex did not affect concentrations of RBC, WBC, and WBC subtypes. At 33 wk of age, PR and litter size did not affect concentrations of RBC, WBC, and WBC subtypes except eosinophils. Effects of PR on eosinophil concentrations at 33 wk differed between sexes (PR

× sex interaction, $P = 0.019$) but did not differ between PR and CON in either males or females and were unaffected by litter size. Neutrophil concentrations at 33 wk were higher in males than females (males: $3.70 \pm 0.29 \times 10^9$ cells/l, females: $2.79 \pm 0.22 \times 10^9$ cells/l, $P = 0.025$), whereas the reverse was true for lymphocyte concentrations (males: $3.30 \pm 0.37 \times 10^9$ cells/l, females: $4.55 \pm 0.29 \times 10^9$ cells/l, $P = 0.020$). Sex did not affect concentrations of RBC, WBC, monocytes, eosinophils, and basophils.

2.6.3 Antibody responses to house dust mite (HDM) allergen and ovalbumin (OVA) sensitisation

PR and sex did not affect levels of HDM-specific (Figure 2.2A) or OVA-specific (Figure 2.2D) total Ig. Litter size did not affect HDM-specific total Ig responses (Figure 2.2G), but the OVA-specific total Ig response was greater in multiple birth than singleton birth sheep (Figure 2.2J, $P = 0.047$). The increases in HDM-specific IgE (Figure 2.2B, $P = 0.007$) and OVA-specific IgE (Figure 2.2E, $P = 0.038$) postsensitisation were higher in PR than CON sheep. Litter size and sex did not affect HDM-specific IgE responses (Figure 2.2H). There were no main effects of litter size or sex on OVA-specific IgE responses (Figure 2.2K), although effects of litter size differed between sexes (litter size × sex interaction, $P = 0.015$). OVA-specific IgE responses were greater in multiple birth than singleton birth females ($P = 0.003$) but did not differ with litter size in males. PR and litter size did not affect HDM-specific IgG₁ (overall: 1.53 ± 0.07 -fold increase) or OVA-specific IgG₁ (overall: 3.97 ± 0.31 -fold increase) responses to sensitisation. The HDM-specific IgG₁ response to sensitisation was greater in females than males (males: 1.34 ± 0.15 -fold increase, females: 1.72 ± 0.12 -fold increase, $P = 0.017$), and sex did not affect OVA-specific IgG₁ responses. The HDM-specific IgM (overall: 1.38 ± 0.06 -fold increase), OVA-specific IgM (overall: 1.46 ± 0.12 -fold increase), and HDM-specific IgA (overall: 1.14 ± 0.06 -fold increase) responses were not affected by PR (Figure 2.2C), litter size (Figure 2.2I), or sex. Females (1.25 ± 0.07 -fold increase) had greater OVA-specific IgA responses than males (0.96 ± 0.05 -fold increase, $P = 0.038$). Effects of PR on the OVA-specific IgA response differed between sexes (PR × sex interaction, $P = 0.006$), with a greater OVA-specific IgA response (Figure 2.2F) in PR females than

CON females ($P=0.023$) and no PR effect on OVA-specific IgA response in males. Similarly, effects of litter size on OVA-specific IgA responses differed between sexes (litter size \times sex interaction, $P=0.014$), with a greater OVA-specific IgA response (Figure 2.2L) in multiple birth females than singleton birth females ($P=0.015$) and no litter size effect on OVA-specific IgA response in males.

More PR than CON animals had a positive HDM-specific IgE response (CON: $32.1 \pm 0.1\%$ positive, PR: $89.1 \pm 0.07\%$ positive, $P=0.003$). Similarly, more multiple birth than singleton birth animals had a positive HDM-specific IgE response (singleton birth: $44.2 \pm 14.3\%$ positive responders, multiple birth: $83.0 \pm 7.7\%$ positive responders, $P=0.038$). Proportions of HDM-specific IgE responders did not differ between sexes. PR, litter size, and sex did not affect the proportion of sheep that were positive responders in terms of HDM-specific total Ig (overall: $26.8 \pm 6.6\%$ positive), IgG₁ (overall: $10.1 \pm 4.8\%$ positive), IgM (overall: $6.3 \pm 3.5\%$ positive), and IgA (overall: no positive responders, IgA increased by less than 2-fold in all sheep). Similarly, PR, litter size, and sex did not affect the proportion of sheep that were positive responders in terms of OVA-specific total Ig (overall: $94.0 \pm 23.5\%$ positive), IgE (overall: $7.0 \pm 4.0\%$ positive), IgG₁ (overall: $69.5 \pm 8.4\%$ positive), IgM (overall: $5.0 \pm 2.9\%$ positive), and IgA (overall: $3.4 \pm 2.6\%$ positive).

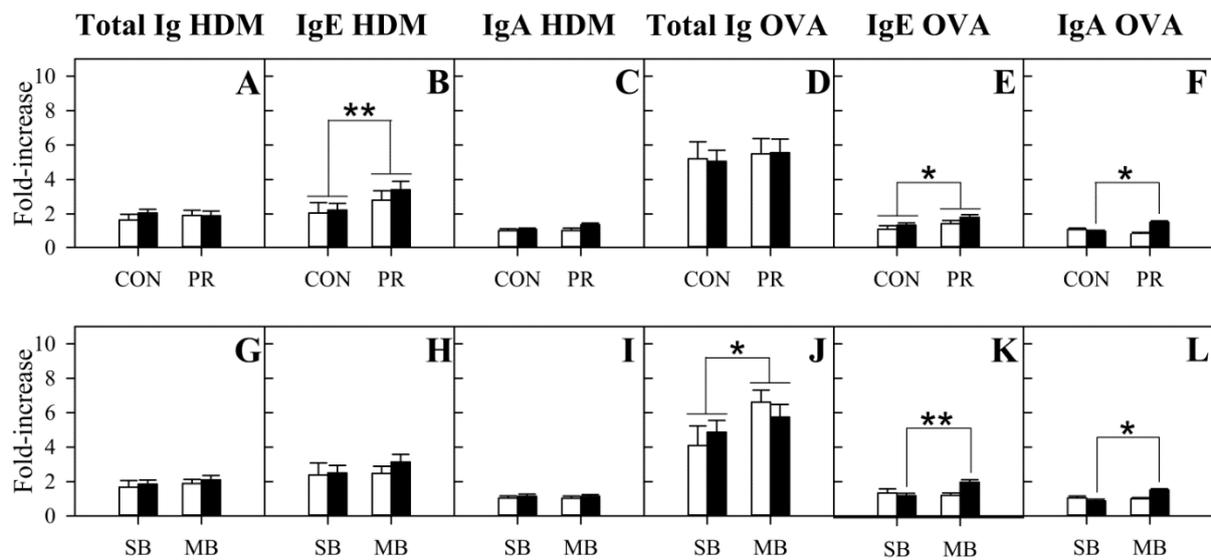


Figure 2.2 Serum antibody responses to house dust mite (HDM) allergen and ovalbumin (OVA)

sensitisation in control (CON) and placentally restricted (PR) progeny overall (A–F, CON: $n=17$ males and 23 females, PR: $n=9$ males and 13 females) and in singleton birth (SB) and multiple birth (MB) sheep (G–L, SB: $n=7$ males and 14 females, MB: $n=20$ males and 22 females). Data are shown as fold changes in HDM-specific total Ig (A, G), IgE (B, H), and IgA (C, I), and in OVA-specific total Ig (D, J), IgE (E, K), and IgA (F, L) from presensitisation to 2 wk postsensitisation. Open bars, males; solid bars, females. Values are estimated means \pm SE; * $P<0.05$; ** $P<0.01$.

2.6.4 Antibody responses to Clostridial vaccination

Antibody responses to vaccination against *Clostridium* spp. were highly variable, ranging from titers of 1.46 IU to 169.02 IU, with a mean of 11.75 ± 3.71 IU. Antibody responses to vaccination were not altered by PR, litter size, or sex (data not shown). *Cutaneous hypersensitivity responses.* All sheep had a positive cutaneous hypersensitivity response to HDM at 30 min, and this response was sustained to 4 h in most sheep (overall: $100.0 \pm 4.0\%$ positive). Similarly, most sheep had a positive acute response to OVA at 30 min (overall: $87.2 \pm 4.8\%$ positive) and 4 h (overall: $72.6 \pm 6.2\%$ positive responders). The proportions of HDM and OVA cutaneous hypersensitivity responders at 30 min and 4 h, and the proportion of HDM cutaneous hypersensitivity responders at 24 h (overall: $42.7 \pm 7.0\%$ positive) were not affected by PR, litter size, or sex. A lower proportion of PR than CON sheep had

positive cutaneous hypersensitivity responses to OVA at 24 h (CON: $49.8 \pm 10.0\%$ positive, PR: $15.7 \pm 7.8\%$ positive, $P=0.030$), and the proportion of responders was unaffected by litter size or sex. At 48 h after challenge, the proportion of HDM-positive responders was greater in singleton birth than multiple birth sheep (singleton birth: $53.0 \pm 11.5\%$ positive, multiple birth: $19.3 \pm 6.9\%$ positive, $P=0.027$) but was not affected by PR or sex. Males were more likely than females to have positive cutaneous hypersensitivity responses to OVA at 48 h (males: $36.7 \pm 10.5\%$ positive, females: $12.6 \pm 5.8\%$ positive), and the proportion of responders was unaffected by PR or litter size.

2.6.5 Correlation between birth weight and cutaneous histamine responses

Skin wheal diameter at 30 min after intradermal injection of histamine correlated positively with birth weight in singletons (Figure 2.3, $P=0.023$). There was no correlation, either overall or in singletons, between birth weight and skin wheal diameter at 4, 24, and 48 h after injection of histamine.

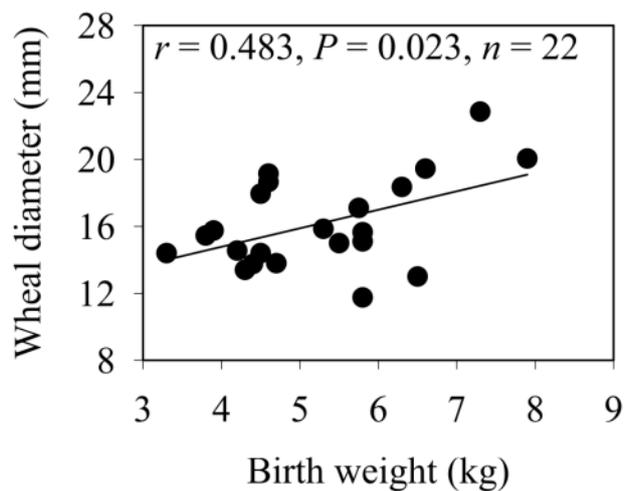


Figure 2.3 Relationship between birth weight and skin wheal response to histamine.

2.7 Discussion

Here we have shown directly that IUGR, induced by surgical restriction of placental attachment and function, alters later allergic responses in adolescent sheep, with fewer positive cutaneous

hypersensitivity responses than would be expected given changes in IgE. This is the first demonstration of altered allergy susceptibility after experimental IUGR, where IUGR and control progeny share a common postnatal environment. These outcomes reflect IUGR rather than prematurity, with >95% of PR lambs born within 7 days of normal term (147 days gestation in this breed). These results are consistent with reports from human epidemiological studies suggesting decreased susceptibility to allergy after SGA (23, 65, 87, 115). Furthermore, our results directly confirm an independent effect of the constrained prenatal environment on allergy.

The IgE responses to both HDM and OVA antigens were increased in PR compared with CON progeny, although the HDM-specific increases were of greater magnitude than those induced by OVA. This probably explains why PR increased the proportion of IgE responders to HDM but not OVA, because only 7% of sheep reached the threshold of a positive (>2-fold increase) IgE response to OVA. The different magnitudes of IgE responses may reflect different antigenic potential of the two preparations, since both antigens were given under the same conditions and timing to sheep in the present study. We have previously reported that the concentration of sensitising antigen influences IgE responses in sheep (17); however, testing effects of PR on responses to multiple antigen concentrations was beyond the scope of the present study. The approximately threefold overall increase in HDM-specific IgE is consistent with the increases we have reported previously in sensitised sheep (18). There is mixed evidence for effects of IUGR on IgE responses in humans, which may at least in part reflect confounding due to common pre- and postnatal exposures to an adverse environment. Studies of circulating antibody concentrations in SGA humans have largely focussed on IgE in response to environmental allergen exposure, with increased total IgE dependent on exposure levels in one study (123) but lower circulating IgE specific for common allergens in 5 to 7 year old children (23). Similar responses to vaccination (with bacterial antigens) in CON and PR lambs in the present study are consistent with previous findings that low birth weight and exposure to maternal seasonal undernutrition during gestation did not alter antibody production following vaccination

with nonpolysaccharide vaccines in humans (134-136). This evidence of enhanced or normal immunoglobulin responses to antigens and vaccination after SGA contrasts with the evidence that low-birth-weight infants have greater susceptibility to infectious diseases in early life (181) and exhibit markers of impaired B-cell function as adults (135, 136), although this evidence for greater susceptibility probably also reflects effects of prematurity. Together, these results suggest that some, but not all, immune responses are impaired by IUGR.

Effects of natural IUGR induced by twinning in the present study had similarities to effects of PR, with greater immunoglobulin responses to sensitisation in multiple birth (mostly twins) than in singleton birth progeny. These effects of multiple birth, however, were only evident for OVA-specific responses, whereas PR increased responses to both antigens. Twinning decreases placental function and reduces fetal growth in sheep (193) and in human twin pregnancies from 32 wk gestation (171). In the present study, multiple birth reduced birth weight to a lesser extent than PR, suggesting that this natural growth restriction was less severe than the surgically induced PR, possibly accounting for the smaller programming effect on immune function in later life. We also saw evidence of sex-specific programming of immune function, with enhanced IgA responses to OVA after PR or multiple birth and greater IgE responses to OVA in multiple birth than singleton birth progeny evident in females only. This contrasts with evidence that preimplantation methyl donor deficiency enhanced acute (haptoglobin) responses to antigens in male, but not female, young adult sheep (182). Different sex-specific susceptibility of immune function to perturbation during development between these two studies might reflect the different prenatal exposures or different interactions between sex and exposure for acute non-specific versus antigen-specific responses. Studies in humans and rats have shown that allergic disease rates and processes differ between sexes and are modulated by sex steroids, including potentiation of IgE responses to antigens by estradiol (reviewed in 36). Since IUGR decreases circulating estradiol in adolescent girls after puberty (81), however,

changes in estradiol seem unlikely to explain sex-specific differences in effects of litter size on IgE responses to antigens.

Cutaneous hypersensitivity responses were lower than might be expected given changes in circulating IgE. Despite increased IgE responses in PR progeny, cutaneous hypersensitivity responses to HDM were normal in PR progeny. More strikingly, cutaneous hypersensitivity responses to OVA were lower in PR progeny, despite elevated OVA-specific IgE responses in PR progeny. This suppressed cutaneous reactivity to antigens in the PR sheep is consistent with reduced cutaneous hypersensitivity reactions to phytohemagglutinin in SGA children born at ≥ 35 wk gestational age with known placental insufficiency or maternal hypertension compared with controls (55). Early life and adult environmental factors may interact in determining inflammatory responses to antigens. For example, perinatal exposure to short-day photoperiod in the Siberian hamster, which delays postnatal growth and reproductive development, programs increased adult hypersensitivity responses only when these animals were also housed in short-day photoperiod as adults (198). The contrasting effects of PR on antibody and inflammatory responses to sensitisation in the present study suggest an alteration in the inflammatory pathway downstream of IgE production, which may reduce inflammatory responses to antigens after IUGR. Consistent with this hypothesis, in the present cohort of sheep, acute cutaneous hypersensitivity responses to histamine correlated positively with birth weight. Acute responses to histamine include local inflammation, expression of eotaxin, and recruitment of eosinophils to the site of allergic skin reactions (131), with amplification by activation of the histamine H4 receptor on mast cells (208). Decreased acute responses to histamine in sheep with lower birth weights might therefore inhibit subsequent late phase reactions to antigens. Although circulating eosinophils were not measured concurrently with antibody abundance or acute reactions to sensitisation in the present study, eosinophil abundance was similar in CON and PR sheep 2 wk before the first sensitisation and 5 wk after cutaneous hypersensitivity testing, suggesting that a deficiency in peripheral blood eosinophils is not the primary mechanism

causing suppressed late phase reactions to HDM and OVA in PR sheep. Similarly, although elevated IgG concentrations can suppress IgE-mediated mast cell degranulation (189), increases in IgG after sensitisation were not altered by PR or litter size and are unlikely to explain differences in cutaneous responses between these groups. Further studies are needed to determine effects of IUGR on mast cell numbers and function, and the underlying mechanisms.

The mechanisms underlying the effects of IUGR on postnatal immune function are currently poorly understood. Reduced nutrient availability *in utero* might directly reduce cell proliferation in immune tissues. Thymus weight was reduced IUGR humans (42, 73) and newborn PR rats, and the spleen and thymus of PR rats had fewer lymphocytes at weaning (39). In adolescent humans, circulating concentrations of thymopoietin, a hormone produced by the thymus that regulates T cell differentiation and function, were lower in SGA than adequate size for gestational age individuals who had been exclusively breast-fed for at least 50 days after birth (122). Whether IUGR-induced changes in lymphocyte numbers in the thymus or spleen persist after weaning is unclear.

2.8 Perspectives and Significance

The incidence of allergy is increasing, and understanding the factors that determine individual susceptibility may help to identify potential preventative interventions. Studies of effects of prenatal environment on immune function in human populations are often confounded by use of birth weight as a marker, which reflects gestational age as well as prenatal environment, and by common prenatal and postnatal adverse exposures. The present study establishes an animal model in which to investigate effects of restricted growth *in utero* on postnatal immune function, independent of gestational age, and where all progeny share a common postnatal environment. Consistent with a lack of effect of birth weight in human studies, antibody responses to a protein-based vaccine were unaffected by PR in sheep, indicating that IUGR programs specific cell types and/or immune pathways without global suppression of immune function. Our finding of enhanced IgE responses,

but decreased cutaneous hypersensitivity responses to antigens after sensitisation, suggests a role for mast cells in programming of susceptibility to allergy. Which specific aspects of a restricted fetal environment induce changes in postnatal immune function, and the underlying mechanisms for this, require further investigation.

Chapter 3: Evidence for an epigenetic process for perinatal programming of allergy: maternal dietary methyl donor and cofactor supplementation through late gestation partially reverses protection against allergic sensitisation in an ovine model of IUGR

3.1 Overview

The work described in the following chapter utilised the same cohort as Chapter 2, with the addition of progeny from placentally-restricted (PR) ewes that were supplemented with methyl donors and cofactors for the last month of gestation, as well as additional control (CON) and PR progeny for whom studies were completed following the submission of my first paper (Appendix 1, 203). The methyl-supplemented group was added in order to assess the potential epigenetic mechanism for the developmental programming of allergy. The methods for progeny evaluations were already described in Chapter 2. The work described in this chapter has not yet been submitted for publication.

3.2 Introduction

Allergic diseases are one of the main causes of non-communicable disease in the world and are estimated to affect 30-40% of the world's population (205). Susceptibility to these diseases in postnatal life is programmed by an individual's early life environment (167). Intriguingly, the majority of epidemiological studies suggest that restricted growth before birth is protective against postnatal development of allergy. In contrast, risks of asthma are consistently increased in this group, likely reflecting poorer lung function rather than allergy (46, 137). Birth weight, independent of gestational age, is positively associated with risk of allergic sensitisation and atopic disease in many (23, 65, 87), although not all (75, 161, 186) studies of children and adults, whilst within twin pairs the risk of childhood atopic eczema is greater in the heavier birth weight twin. Consistent with small size for gestational age (SGA) being protective against later allergic disease, we have reported that surgical restriction of placental and fetal growth in sheep reduces cutaneous responses to challenge with allergen following a sensitisation protocol (Appendix 1, 203).

Epigenetic mechanisms such as altered DNA methylation have been postulated as explaining persistent effects of early life exposures on later life health, including for allergy (22, 120). In a recent meta-analysis of epidemiological data, maternal dietary supplementation with methyl donors such as folate was associated with increased risks of wheeze in young children (197). Although maternal supplementation was not associated with risk of allergic diseases including asthma, atopic dermatitis and eczema (197), the available data is variable and heavily influenced by other factors including variable timing and doses of supplements and evaluation of outcomes, as well as genetic and environmental factors. New evidence from a Finnish cohort, showed increased risk of food allergy in 5 year-old children when the mother consumed folic acid supplements in late pregnancy (191). Randomisation to folic acid supplements is not possible in human cohorts for ethical reasons given the protective effects against neural tube defects, as well as practically due to widespread use of food fortification and pregnancy supplements. Experimental evidence supports the hypothesis

that elevated maternal methyl donor abundance can increase risk of allergy development in progeny. Offspring from mice fed methyl donor and cofactor supplements from two weeks prior to mating and throughout gestation had greater specific IgE, allergic airway disease and cytokine responses to ovalbumin (OVA) following sensitisation (78). This shift to a more allergic phenotype was associated with increased methylation and a decrease in the expression of runt-related transcription factor 3 in the lung tissue of the offspring (78); a gene that down-regulates mechanisms that promote allergic airway disease by activating the induction of forkhead box P3 (FOXP3)-expressing functional regulatory T cells (Tregs, 89). Also consistent with a positive relationship between methyl status during pregnancy and later susceptibility to allergy, marginal maternal dietary restriction of methionine and choline during gestation and lactation in rat dams decreased the immune response to infection and decreased cutaneous hypersensitivity responses following sensitisation in progeny at five months of age (200). There is some evidence that these effects of maternal methyl donor abundance during pregnancy on allergy in progeny may be through changes in methylation of immune-regulating genes, induced before birth. DNA methylation of the *FOXP3* regulatory gene in T cells at birth is associated with reduced FOXP3 expression, decreased Treg function and Th2 cytokine production after *in vitro* stimulation (113), and is predictive of allergy risk in infants at one year of age (77).

Based on the above, we hypothesised that the apparently protective effects of restricted fetal growth (IUGR) against later susceptibility to allergy may be due to reduced supply of methyl donors *in utero*, particularly in late gestation. Restricted placental function reduces the abundance of hepatic one-carbon methyl donors and alters postnatal DNA methylation in day 0 rat liver (56, 118), while SGA in humans is associated with increased methionine in cord blood (52). Placentally-restricted (PR) sheep share many characteristics with IUGR humans (4, 173), including reduced immune responses to allergic sensitisation (Appendix 1, 203). If a reduced supply of methyl donors *in utero* is an underlying mechanism that programs the fetus for later protection against allergy, we

would predict that increased methyl donor abundance *in utero* would at least partially reverse the protective effects of PR against allergic susceptibility in the sheep. We therefore hypothesised that supplementing the maternal diet of PR dams with methyl donors and cofactors during the last month of pregnancy would increase the responses to allergic sensitisation in adolescent progeny compared to those of progeny from unsupplemented PR pregnancies. Because the changes in intradermal inflammatory response to antigen exposure did not correspond to IgE responses in our previous study of PR and control (CON) progeny, suggesting down-regulation of the cutaneous response to antigen-IgE complexes in PR progeny (Appendix 1, 203), we also tested the hypothesis that PR decreases intradermal abundance of inflammation-mediating mast cells in the present study.

3.3 Materials and Methods

All procedures were approved by the University of Adelaide Animal Ethics Committee (M-2009-145, M-2010-139 and M-2011-155) and conducted as per Australian guidelines (147).

3.3.1 Animal model

Placental growth of ewes was restricted (PR) by surgically removing all but four visible endometrial placental attachment sites (caruncles) from each uterine horn (4, 173). CON ewes were unoperated. PR surgery and animal management were as described previously, with ewes housed in individual pens throughout late gestation and the first month of lactation, then group-housed in pens until weaning at 13 weeks post-delivery, and progeny housed in same-sex groups in small paddocks except when pen-housed for a week for subcutaneous hypersensitivity testing (Appendix 1, 203). Lambs were weighed and measured (crown-rump length, shoulder height, abdominal and thoracic circumferences, skull width and length) within 24 h of birth. The present study included the sheep described in Chapter 2, with more sheep within each experimental group, and with an added experimental group consisting of progeny from a randomly allocated subset of PR ewes who were

fed a dietary methyl supplement from day 110 of gestation until delivery (PR+METHYL). These treatment groups were studied concurrently. The methyl supplementation consisted of 2 g rumen-protected methionine (Mepron, Evonik Degussa GmbH, Hanau, Germany), 1.2 g dustless sulphur, 0.7 mg of cobalt as 5% dustless cobalt and 300 mg folic acid (F7876, Sigma, St Louis, USA), and was given in the diet by top-dressing a small amount of lucerne chaff (~200 g) with the daily supplement dose when ewes received their morning feed at 0800 to 0900 hours. The dose of folic acid in the methyl supplementation reflects a relatively low flux through the rumen and was based on doses previously fed to dairy cattle, which resulted in a respective ~doubling of maternal plasma and liver folate concentrations (67). Methionine was given as Mepron, in a form that provides rumen by-pass protection of >70% (16), and at doses per body weight which in cattle increases by 20% the flux of methionine through the transmethylation pathway to produce the methyl donor S-adenosylmethionine (168). Sulphur and cobalt doses were selected to ensure that availability of these minerals would not limit ruminal bacterial production of sulphated amino acids such as methionine or production of vitamin B₁₂, each of which can be limited by feeding diets deficient in these minerals in sheep (182). The remainder of the morning ration was offered once the lucerne chaff and supplement were consumed. Immune function in progeny was studied in 24 CON males (2 singletons, 20 twins, 2 triplets), 25 CON females (7 singletons, 18 twins), 11 PR males (6 singletons, 5 twins), 17 PR females (12 singletons, 4 twins, 1 triplet), 10 PR+METHYL males (5 singletons, 5 twins) and 15 PR+METHYL females (8 singletons, 7 twins).

3.3.2 Immunisation, sensitisation and cutaneous hypersensitivity testing

The sheep were immunised at 5 and 9 weeks of age with anti-Clostridial vaccine (Ultravac 5-in-1; Pfizer Animal Health, West Ryde, Australia), and immunologically sensitised with 50 µg house dust mite allergen (HDM; CSL Ltd, Parkville, Australia) and 100 µg OVA (A2512, Sigma, Missouri, USA) with aluminium hydroxide as adjuvant (1:1) by subcutaneous injections at 20, 22, 24 and 26 weeks of age (Appendix 1, 17, 18, 203). Immediate and delayed cutaneous responses (cutaneous hypersensitivity,

skin prick test, SPT) to intradermal injections of 50 μ L saline (negative control), histamine (10 μ g/mL; histamine diphosphate, Sigma), HDM (100 μ g/mL) and OVA (10 μ g/mL) were assessed at 28 weeks of age (Appendix 1, 203). Skin wheal responses were measured with digital callipers at 0.5, 4, 24 and 48 h after injections, and an average diameter across two perpendicular readings of ≥ 3 mm was classified as a positive skin reaction (Appendix 1, 203).

3.3.3 Circulating blood cell counts and serum antibody concentrations

Peripheral blood was collected by jugular venepuncture prior to sensitisations at 20 weeks of age and immediately prior to cutaneous hypersensitivity tests at 28 weeks of age. Serum was stored at -80 °C until analysis of HDM- and OVA-specific total Ig, IgG₁, IgE, IgA and IgM at both time points, and Clostridial-specific total Ig at 28 weeks of age, in duplicate by antigen-specific ELISA (Appendix 1, 17, 18, 203). Antibody responses to sensitisation were classified as positive when they increased at least 2-fold relative to basal concentrations (17, 18). Peripheral blood was collected into EDTA-coated tubes at 18 weeks of age (two weeks prior to sensitisations, in 64% of the cohort) and 33 weeks of age (~7 weeks after final sensitisation, in all animals) for quantification of total red and white blood cells, and of white blood cell subtypes (Appendix 1, 203).

3.3.4 Quantitation of intradermal mast cells

Post-mortems were conducted at 52 weeks of age. A full-thickness section of skin (~1 cm²) was dissected from mid-way down the left side of the sheep over the third rib and fixed in 10% buffered formalin. Skin samples were subsequently embedded in paraffin and 5 μ m-thick cross-sections of skin tissue were stained with toluidine blue (0.5% toluidine blue in 0.5 M HCl, pH 0.8) for 30 min before light counterstaining with fresh 2% eosin in distilled water (modified from 201). The slides were scanned (NanoZoomer 2.0-HT (C9600-13) digital slide scanner, Hamamatsu Photonics, Japan) then fields spanning the depth of the upper dermis and perpendicular to the skin surface were digitally captured by random systematic sampling (0.299 mm²/field, 22-47 fields per sample,

NDP.view NanoZoomer Digital Pathology software version 1.2.36, Hamamatsu Photonics, Japan).

Numbers of cells with metachromatic purple staining and mast cell morphology such as the presence of small purple granules (Figure 3.2A) were counted in all fields (ImageJ, version 1.48, National Institutes of Health, USA).

3.3.5 Statistical analysis

Continuous and binary outcomes were analysed using a Generalised Linear Mixed Models framework that examined the effects of treatment (CON, PR, PR+METHYL), litter size (singleton birth vs. multiple birth), and sex, treating the dam as the experimental unit and sibling data as repeated measures on each dam. Where treatment effects or trends were apparent ($P < 0.1$), we compared means for each treatment by the least significant difference method based on *a priori* questions to 1) determine the effects of placental restriction (CON cf. PR treatments), 2) determine effects of maternal methyl donor and cofactor supplementation in progeny from PR pregnancies (PR cf. PR+METHYL treatments), and 3) assess whether maternal methyl donor and cofactor supplementation restored values to those of controls (CON cf. PR+METHYL treatments). The distributions of continuous variables were assessed for normality, and a square root, log or inverse transformation was applied as required. Binary outcomes (responders or non-responders in antibody fold-changes and SPT) were also analysed within this framework, assuming a binomial distribution and utilizing a logit link function. Interaction effects were non-significant for all binomial outcomes, and the final models used for these outcomes included only main effects. Relationships between continuous variables were analysed through the calculation of Pearson's correlation coefficient, restricted to singleton birth sheep to remove effects of clustering due to ewes. Data were analysed using SPSS version 20.0 (SPSS Inc., Chicago, USA) and are presented as estimated means \pm SE. $P < 0.05$ was accepted as significant. Interactions are not described unless significant.

3.4 Results

3.4.1 Birth phenotype and gestation length

Gestation length ranged between 139-151 d, and differed between treatments (overall: $P < 0.002$, singleton birth: $P=0.035$, Table 3.1). Within singleton birth sheep, PR pregnancies (145.9 ± 0.4 d) had gestation lengths that were 1.8 d shorter than CON pregnancies (147.7 ± 0.6 d, $P=0.014$), and PR+METHYL pregnancies (145.7 ± 0.4 d) were 2.0 d shorter than CON pregnancies ($P=0.019$). Gestation lengths were similar between PR and PR+METHYL pregnancies. Overall, pregnancies with male offspring (146.7 ± 0.4 d) were 1.0 d longer than pregnancies with female offspring (145.7 ± 0.3 d, $P=0.014$). In singleton birth sheep, pregnancies with male offspring (147.1 ± 0.5 d) were 1.1 d longer than pregnancies with female offspring (146.0 ± 0.3 d, $P=0.014$). Gestation length was not affected by litter size, either overall or in singleton birth sheep.

Birth weight differed between treatment groups (overall: $P < 0.001$, singleton birth: $P = 0.001$), such that birth weight was reduced by PR (overall: by 17% $P < 0.002$, singleton birth: by 24%, $P = 0.004$) and PR+METHYL (overall: by 26%, $P < 0.001$, singleton birth: by 35%, $P < 0.001$), in comparison to CON lambs (overall outcomes in Table 3.1; singleton birth CON: 6.37 ± 0.43 kg, PR: 4.83 ± 0.27 kg, PR+METHYL: 4.12 ± 0.30 kg). Birth weight was similar in PR and PR+METHYL sheep, both overall and in singleton birth sheep. Overall, birth weight of males was greater than that of females (males: 5.16 ± 0.19 kg; females: 4.46 ± 0.14 kg, $P < 0.001$), although birth weight did not differ between sexes within singleton birth sheep. Birth weight of singleton birth sheep was greater than that of multiple birth sheep (singleton birth: 5.10 ± 0.18 kg; multiple birth sheep: 4.52 ± 0.16 kg, $P=0.017$).

The crown-rump length of PR+METHYL offspring did not differ from PR offspring either overall or in singleton birth sheep, but crown-rump length was shorter in PR+METHYL than CON offspring both overall and in singleton birth sheep (overall outcomes: Table 3.1; singleton birth CON: 54.9 ± 1.9 cm, PR: 51.7 ± 1.3 cm, PR+METHYL: 48.5 ± 1.3 cm; overall: $P=0.002$, singleton birth: $P=0.026$). Crown-

rump length was greater in singleton birth sheep than in multiple birth sheep (singleton birth: 51.7 ± 0.8 cm; multiple birth sheep: 48.1 ± 0.8 cm, $P=0.002$), and did not differ between sexes overall or in singleton birth sheep.

Shoulder height at birth differed with treatments (overall: $P=0.006$, singleton birth: $P=0.017$) such that overall, shoulder height was lower in PR ($P=0.047$) and PR+METHYL ($P=0.047$) offspring compared to CON offspring, with no difference between PR and PR+METHYL offspring (overall outcomes Table 3.1). In singleton birth offspring, PR+METHYL offspring had lower shoulder heights than either CON ($P=0.012$) or PR ($P=0.029$) offspring, but PR offspring did not differ from CON offspring (singleton birth CON: 41.3 ± 1.7 cm, PR: 39.1 ± 0.9 cm, PR+METHYL: 36.3 ± 0.9 cm). Shoulder height did not differ between litter sizes or sexes overall or in singleton birth sheep.

Abdominal circumference at birth differed with treatments (overall: $P=0.009$, singleton birth: $P=0.042$) such that overall and in singleton birth sheep, PR+METHYL offspring had a lower abdominal circumference than CON offspring (overall: $P=0.002$, singleton birth: $P=0.013$), and PR was not significantly different from either CON or PR+METHYL offspring (overall outcomes: Table 3.1; singleton birth CON: 39.0 ± 1.7 cm, PR: 35.6 ± 1.1 cm, PR+METHYL: 33.5 ± 1.2 cm). Abdominal circumference did not differ between litter sizes or sexes overall or in singleton birth sheep.

Thoracic circumference at birth differed with treatments (overall: $P=0.001$, singleton birth: $P=0.023$) such that overall and in singleton birth sheep, PR+METHYL offspring had a lower thoracic circumference than CON offspring (overall: $P<0.001$, singleton birth: $P=0.003$, Table 3.1). Thoracic circumference was greater in singleton birth sheep than in multiple birth sheep (singleton birth: 36.9 ± 0.6 cm; multiple birth sheep: 35.1 ± 0.5 cm, $P=0.018$), and did not differ between sexes overall or in singleton birth sheep.

Effects of treatment on skull width at birth differed with litter size (treatment \times litter size $P < 0.001$; overall outcomes: Table 3.1). Within singleton birth sheep, skull width differed with treatment ($P = 0.001$) such that PR+METHYL singleton birth sheep had smaller skull widths compared to CON ($P < 0.001$) or PR ($P = 0.005$) singleton birth sheep. CON singleton birth sheep and PR singleton birth sheep did not differ in skull width (singleton birth CON: 6.7 ± 0.1 cm, PR: 6.4 ± 0.1 cm, PR+METHYL: 6.1 ± 0.1 cm). Within sheep from multiple birth litters, effects of treatment on skull width differed with sex (treatment \times sex: $P < 0.038$). There was no treatment effect on skull width within males from multiple birth litters, but there was a treatment effect within females from multiple birth litters ($P = 0.045$). Within these females from multiple birth litters, PR offspring had smaller skull widths than CON offspring ($P = 0.034$) and PR+METHYL offspring did not differ from either CON or PR offspring (multiple birth female CON: 6.2 ± 0.0 cm, PR: 6.0 ± 0.1 cm, PR+METHYL: 6.1 ± 0.1 cm). Overall skull width was greater in males than in females (males: 6.4 ± 0.1 cm; females: 6.2 ± 0.0 cm, $P < 0.001$), but this was not significant when analysed in singleton birth offspring.

Skull length at birth differed with treatments (overall: $P < 0.001$, singleton birth: $P = 0.004$) such that overall and in singleton birth sheep, PR+METHYL offspring had shorter skulls than both CON (overall: $P < 0.001$, singleton birth: $P = 0.001$) and PR (overall: $P = 0.011$, singleton birth: $P = 0.043$) offspring. PR offspring had shorter skulls than CON offspring in overall ($P = 0.012$) but not singleton analyses (overall outcomes: Table 3.1; singleton birth CON: 13.6 ± 0.3 cm, PR: 13.0 ± 0.2 cm, PR+METHYL: 12.4 ± 0.2 cm). Skull length was greater in singleton birth sheep than in multiple birth sheep (singleton birth: 13.0 ± 0.1 cm; multiple birth sheep: 12.6 ± 0.1 cm, $P = 0.013$), and did not differ between sexes overall or in singleton birth sheep.

Table 3.1 Effect of PR and late pregnancy maternal dietary methyl donor and cofactor supplementation on body size at birth

	CON (n=49)	PR (n=28)	PR+METHYL (n=25)	Treatment significance
Sex, M:F	24:25	11:17	10:15	
Birth, singleton:multiple	9:40	18:10	13:12	
Gestational length (days)	147.0 ± 0.5	145.0 ± 0.4	146.6 ± 0.5	0.035*†
Birth weight, kg	5.62 ± 0.22	4.64 ± 0.20	4.17 ± 0.20	<0.001*‡
Crown-rump length, cm	52.6 ± 1.1	49.7 ± 1.0	47.4 ± 1.0	0.002‡
Shoulder height, cm	40.4 ± 0.8	38.3 ± 0.6	37.0 ± 0.6	0.006*‡
Abdominal circumference, cm	37.3 ± 0.8	35.2 ± 0.8	33.7 ± 0.8	0.009‡
Thoracic circumference, cm	37.9 ± 0.7	35.9 ± 0.7	34.1 ± 0.7	0.001*‡
Skull width, cm	6.4 ± 0.1	6.3 ± 0.2	6.2 ± 0.1	0.003+^
Skull length, cm	13.3 ± 0.2	12.8 ± 0.1	12.3 ± 0.1	<0.001*†‡

Data are estimated means ± SE, from a Generalised Linear Mixed Models framework including treatment, litter size and sex as fixed factors and with the dam as the experimental unit. CON, control; PR, placental restriction; PR+METHYL, placental restriction and maternal methyl donor and cofactor supplementation; M, male; F, female. Where treatment effects or trends were apparent ($P < 0.1$), we compared means for each treatment by the least significant difference method based on *a priori* questions to 1) determine the effects of placental restriction (CON cf. PR treatments), 2) determine effects of maternal methyl donor and cofactor supplementation in progeny from PR pregnancies (PR cf. PR+METHYL treatments), and 3) assess whether maternal methyl donor and cofactor supplementation restored values to those of controls (CON cf. PR+METHYL treatments). Between-group differences for specific contrasts are shown as follows: * $P < 0.05$ CON cf. PR; † $P < 0.05$ PR cf. PR+METHYL, ‡ $P < 0.05$ CON cf. PR+METHYL. ^There was an interaction between treatment and other factors included in analysis, which has been described in the text.

3.4.2 Circulating red and white blood cell counts

At 18 weeks of age, treatment and sex did not affect circulating concentrations of red blood cells (overall mean: $12.1 \pm 0.2 \times 10^9/L$), white blood cells (overall mean: $10.2 \pm 0.4 \times 10^9/L$) and white blood cell subtypes (overall mean for neutrophils: $4.1 \pm 0.3 \times 10^9/L$; lymphocytes: $4.6 \pm 0.3 \times 10^9/L$; monocytes: $1.3 \pm 0.1 \times 10^9/L$; eosinophils: $0.1 \pm 0.0 \times 10^9/L$; basophils: $0.2 \pm 0.0 \times 10^9/L$) either overall or within singleton birth sheep, and litter size did not affect these concentrations in overall analyses.

At 33 weeks of age, treatment and sex did not affect levels of circulating red blood cells, total white blood cells, monocytes, eosinophils or basophils either overall or within singleton birth sheep and did not affect concentrations of circulating neutrophils or eosinophils in singleton birth sheep (Table 3.2). Treatment and litter size did not affect circulating concentrations of lymphocytes or neutrophils either overall or within singleton birth sheep. Overall, males had greater circulating concentrations of neutrophils than females ($P=0.005$, Table 3.2), but within singleton birth sheep this was not statistically significant. The circulating concentration of lymphocytes at 33 weeks of age was greater in females than in males, both overall and in singleton birth sheep (overall: $P=0.005$; singleton birth: $P=0.043$, Table 3.2).

Table 3.2 Effect of sex on circulating white blood cell subsets at 33 weeks of age

	Males	Females	Sex significance
<i>Overall</i>			
Red blood cells, x 10 ⁹ /L	10.2 ± 0.2	10.6 ± 0.1	0.060
White blood cells, x 10 ⁹ /L	8.8 ± 0.4	8.6 ± 0.3	>0.6
Neutrophils, x 10 ⁹ /L	3.7 ± 0.2	2.9 ± 0.2	0.005
Lymphocytes, x 10 ⁹ /L	3.2 ± 0.3	4.3 ± 0.2	0.005
Monocytes, x 10 ⁹ /L	1.4 ± 0.1	1.2 ± 0.1	>0.2
Eosinophils, x 10 ⁹ /L	0.1 ± 0.0	0.1 ± 0.0	>0.6
Basophils, x 10 ⁹ /L	0.1 ± 0.0	0.1 ± 0.0	>0.5
<i>Singleton birth</i>			
Red blood cells, x 10 ⁹ /L	10.2 ± 0.3	10.8 ± 0.2	0.100
White blood cells, x 10 ⁹ /L	8.6 ± 0.6	8.6 ± 0.4	>0.8
Neutrophils, x 10 ⁹ /L	3.3 ± 0.3	3.0 ± 0.2	>0.4
Lymphocytes, x 10 ⁹ /L	3.0 ± 0.5	4.3 ± 0.3	0.043
Monocytes, x 10 ⁹ /L	1.6 ± 0.2	1.1 ± 0.2	0.068
Eosinophils, x 10 ⁹ /L	0.1 ± 0.0	0.1 ± 0.0	>0.7
Basophils, x 10 ⁹ /L	0.1 ± 0.0	0.1 ± 0.1	>0.1

Data are estimated means ± SE, from a Generalised Linear Mixed Models framework including treatment, litter size and sex as fixed factors and with the dam as the experimental unit. Overall: male CON n=19, female CON n=23, male PR n=10, female PR n=15, male PR+METHYL n=10, female PR+METHYL n=12. Singleton birth: male CON n=2, female CON n=6, male PR n=5, female PR n=10, male PR+METHYL n=5, female PR+METHYL n=6.

3.4.3 Antibody responses

Antibody responses to Clostridial vaccination: Within the entire cohort and within singleton birth sheep alone, antibody responses to vaccination against *Clostridium* spp. were not affected by treatment or sex, nor by litter size overall, and varied greatly, ranging from 1.5 IU to 169.0 IU with a mean of 10 ± 3 IU.

HDM-specific total Ig, IgG₁, IgA and IgM antibodies: The proportion of animals that showed positive HDM-specific antibody responses (≥ 2 -fold increase after sensitisation), and the fold-increases, for total Ig, IgG₁, IgA and IgM did not differ between treatments overall or within singleton birth sheep. No singleton birth sheep exhibited positive HDM-specific IgA responses.

HDM-specific IgE antibodies: In the entire cohort, the proportion of animals with positive HDM-specific IgE responses was greater in PR sheep than CON sheep ($P=0.001$, Table 3.3) or PR+METHYL sheep ($P=0.001$, Table 3.3) and did not differ between CON and PR+METHYL sheep, nor with sex or litter size. Similarly in singleton birth sheep, the proportion of animals with positive HDM-specific IgE responses was greater in PR sheep than CON sheep ($P=0.001$, Table 3.3) with a similar trend for PR+METHYL sheep ($P=0.050$, Table 3.3) and did not differ between PR and PR+METHYL sheep. In singleton birth sheep, the proportion of HDM-specific IgE responders was greater in females than in males (females: $51.4 \pm 13.1\%$, males: $13.1 \pm 9.5\%$, $P=0.042$). In analyses of the entire cohort, effects of treatment on the fold increase in HDM-specific IgE differed depending on levels of other factors (treatment \times sex \times litter size $P=0.059$). In males alone, the fold-increase in HDM-specific IgE did not differ between treatments or litter sizes. In females, effects of treatment on the fold-increase in HDM-specific IgE differed with litter size (treatment \times litter size: $P=0.005$). In singleton birth females, the fold-increase in HDM-specific IgE differed between treatments ($P=0.015$) such that both PR ($P=0.027$) and PR+METHYL ($P=0.005$) sheep had greater fold-increases in HDM-specific IgE than CON sheep (CON: 1.39 ± 0.76 -fold, PR: 3.04 ± 0.59 -fold, PR+METHYL: 3.93 ± 0.67 -fold). Fold-increases in

HDM-specific IgE did not differ between singleton birth female PR sheep and singleton birth female PR+METHYL sheep.

Table 3.3 Effect of PR on the proportion of antibody responses to house dust mite

	CON	PR	PR+METHYL	Treatment significance
Multiple birth sheep (M:F)	22:18	5:5	5:12	
Singleton birth sheep (M:F)	2:7	5:11	5:8	
Overall HDM-specific IgE positive responders (%)	37.7 ± 8.0	78.4 ± 8.3	33.8 ± 9.8	0.005*†
Singleton HDM-specific IgE positive responders (%)	5.4 ± 6.1	63.5 ± 14.3	39.0 ± 16.0	0.005*
Singleton OVA-specific IgG ₁ response (fold-increase)	2.74 ± 0.88	2.74 ± 0.50	4.10 ± 0.54	0.030†

Data are estimated means ± SE, from a Generalised Linear Mixed Models framework including treatment, litter size and sex as fixed factors and with the dam as the experimental unit. CON, control; PR, placental restriction; PR+METHYL, placental restriction and maternal methyl donor and cofactor supplementation; M, male; F, female. Where treatment effects or trends were apparent ($P < 0.1$), we compared means for each treatment by the least significant difference method based on *a priori* questions to 1) determine the effects of placental restriction (CON cf. PR treatments), 2) determine effects of maternal methyl donor and cofactor supplementation in progeny from PR pregnancies (PR cf. PR+METHYL treatments), and 3) assess whether maternal methyl donor and cofactor supplementation restored values to those of controls (CON cf. PR+METHYL treatments). Between-group differences for specific contrasts are shown as follows: * $P < 0.05$ CON cf. PR; † $P < 0.05$ PR cf. PR+METHYL.

OVA-specific total Ig and IgM: The proportion of animals with positive OVA-specific total Ig or IgM antibody responses (≥ 2 -fold increase after sensitisation) did not differ between treatments, overall or within singleton birth sheep (data not shown).

OVA-specific IgE: The proportion of animals with positive OVA-specific IgE antibody responses (≥ 2 -fold increase after sensitisation) did not differ between treatments, overall or within singleton birth sheep (data not shown). In multiple birth sheep, the fold increase in OVA-specific IgE differed between treatments ($P=0.046$), such that PR+METHYL sheep had lower fold-increases in OVA-specific IgE than PR sheep (PR: 1.77 ± 0.19 -fold, PR+METHYL: 1.13 ± 0.18 -fold, $P=0.015$).

OVA-specific IgG₁: The proportion of animals with positive OVA-specific IgG₁ antibody responses (≥ 2 -fold increase after sensitisation) did not differ between treatments, overall or within singleton birth sheep (data not shown). Fold-increases in OVA-specific IgG₁ did not differ between treatments, sexes or litter size overall. Within singleton birth sheep, there was a treatment effect ($P=0.030$) such that PR+METHYL singleton birth sheep had greater fold-increases in OVA-specific IgG₁ than PR singleton birth sheep ($P=0.010$, Table 3.3). Fold-increases in OVA-specific IgG₁ in CON singleton birth sheep did not differ from either PR or PR+METHYL singleton birth sheep (Table 3.3). There was no effect of sex.

OVA-specific IgA: The proportion of animals with positive OVA-specific IgA antibody responses (≥ 2 -fold increase after sensitisation) did not differ between treatments, overall or within singleton birth sheep (data not shown). The effect of treatment on the fold increase in OVA-specific IgA differed between sexes (treatment \times sex: $P=0.018$). In females, treatment affected fold-increases in OVA-specific IgA ($P=0.024$), such that PR females had greater fold-increases in OVA-specific IgA than CON females (CON: 0.96 ± 0.09 -fold, PR: 1.37 ± 0.10 -fold, $P=0.009$). PR+METHYL females (1.21 ± 0.09 -fold) also tended to have greater fold-increases in OVA-specific IgA when compared with CON females ($P=0.053$), but did not differ from PR females; similar treatment differences were observed in

females from multiple birth litters when analysed separately. Also within females, litter size affected this antibody response, with multiple birth females having greater fold-increases in OVA-specific IgA compared with singleton females (multiple birth females: 1.33 ± 0.08 -fold, singleton birth females: 1.03 ± 0.07 -fold, $P=0.019$). There were no effects in males. Females had greater fold increases in OVA-specific IgA than males in multiple birth sheep (males: 0.97 ± 0.13 , females: 1.33 ± 0.09 , $P=0.027$).

3.4.4 Cutaneous hypersensitivity responses

Histamine: All sheep responded positively to histamine at 30 min after intradermal injection. Within the entire cohort, treatment, sex and litter size did not affect the proportion of positive cutaneous responses to histamine at 4 h (overall mean: $71 \pm 5\%$), 24 h ($14 \pm 4\%$) and 48 h ($8 \pm 3\%$) after injection.

HDM: All sheep responded positively to HDM at 30 min after intradermal injection. Within the entire cohort, treatment, sex and litter size did not affect the proportion of positive cutaneous responses to HDM at 4 h ($92 \pm 4\%$), 24 h ($47 \pm 6\%$) and 48 h ($33 \pm 5\%$) after intradermal injection ($P>0.091$ for all). Similarly, within singleton birth sheep, treatment and sex did not affect the proportion of positive cutaneous responses to HDM at 4, 24 or 48 h after injection.

OVA: Within the entire cohort, treatment, sex and litter size did not affect the proportion of positive cutaneous responses to OVA at 30 min ($91 \pm 3\%$), 4 h ($73 \pm 5\%$), 24 h ($39 \pm 5\%$, Figure 3.1A) or 48 h ($27 \pm 5\%$). Within singleton birth sheep, treatment and sex did not affect the proportion of positive cutaneous responses to OVA at 30 min ($92 \pm 6\%$), 4 ($76 \pm 9\%$) and 48 h ($29 \pm 8\%$) after intradermal injection, and sex did not affect responses to OVA at 24 h, but treatment did change responses at 24 h after injection of OVA (Figure 3.1B, $P=0.049$). The proportion of singleton birth PR sheep that responded positively to OVA was lower than that of CON singleton birth sheep ($P=0.002$, Figure

3.1B). The proportion of positive cutaneous responders to OVA at 24 h was intermediate in PR+METHYL singleton birth sheep and not different from either CON or PR singleton birth sheep (Figure 3.1B).

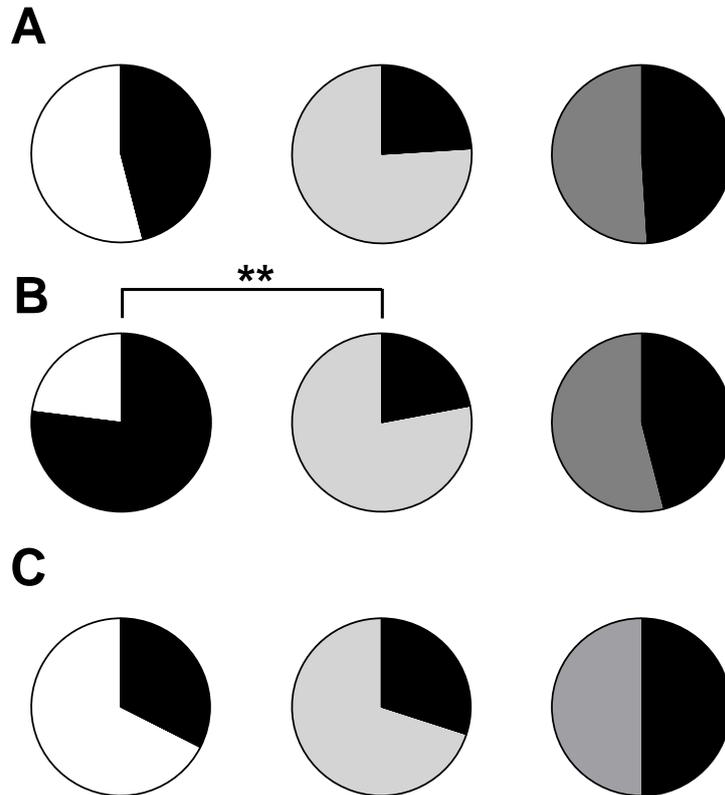


Figure 3.1 Proportion of positive (black shaded, skin wheal diameter ≥ 3 mm) and negative (not black shaded, skin wheal diameter < 3 mm) responders at 24 h after intradermal challenge with ovalbumin in control (CON; white), placentally-restricted (PR; light grey) and PR with maternal dietary methyl donor and cofactor supplementation (PR+METHYL; dark grey) after a sensitisation protocol. A. Overall cohort (CON: n=24 males, n=25 females; PR: n=11 males, n=17 females; PR+METHYL: n=10 males, n=15 females). B. Singleton birth sheep only (CON: n=2 males, n=7 females; PR: n=6 males, n=12 females; PR+METHYL: n=5 males, n=8 females). C. Multiple birth sheep only (CON: n=22 males, n=18 females; PR: n=5 males, n=5 females; PR+METHYL: n=5 males, n=7 females).

3.4.5 Mast cell density

Upper dermis mast cell densities (Figure 3.2A) were higher in sheep from multiple birth pregnancies than in those from singleton birth pregnancies (overall means, singleton birth: 43.5 ± 2.2 mast cells/mm², multiple birth: 53.7 ± 2.4 mast cells/mm², $P=0.002$, Figure 3.2B) and did not differ between sexes, either overall or in singleton birth sheep. Effects of treatment differed between litter size groups (treatment \times litter size $P=0.092$, Figure 3.2B). Within singleton birth sheep, there was a treatment effect ($P=0.040$, Figure 3.2B), such that PR+METHYL sheep had greater upper dermis mast cell densities than CON ($P=0.040$) and PR ($P=0.027$) sheep. Upper mast cell densities did not differ between CON and PR singleton birth sheep. Within multiple birth sheep, effects of treatment differed between sexes ($P=0.020$, Figure 2B), with no treatment effects on mast cell densities evident in females. In males from multiple birth litters, mast cell densities were >50% higher in PR than CON sheep ($P<0.001$). Wheal diameter responses during acute response to histamine (30 min and 4 h) and during delayed phase response to OVA and HDM allergens (24 and 48 h) in SPT were not correlated with mast cell densities in adult skin, overall or within singletons.

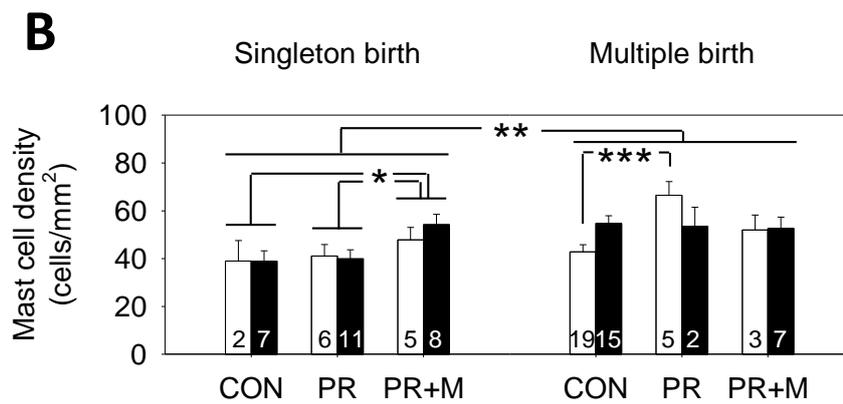
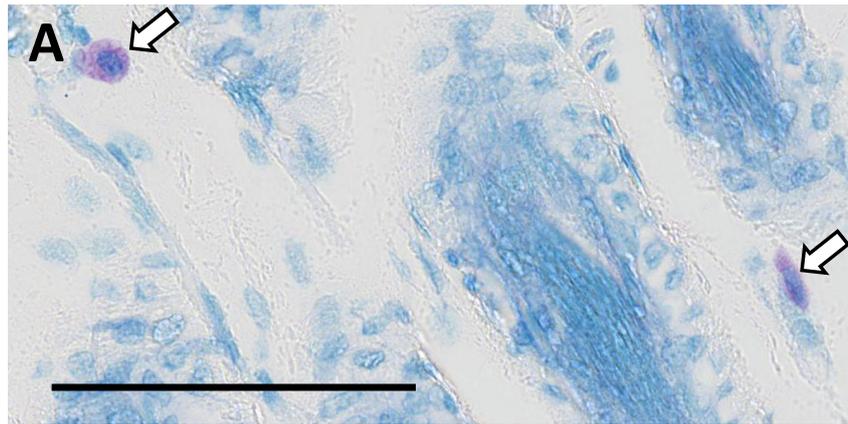


Figure 3.2 A. Upper dermis of skin sections from adult sheep were stained with toluidine blue; mast cells (indicated by arrows) stain metachromatically purple. Scale bar is 100 μ m in length. B. Upper dermis mast cell density in singleton birth and multiple birth male (open bars) and female (closed bars) sheep in control (CON), placentally-restricted (PR) and PR with maternal dietary methyl donor and co-factor supplementation (PR+M). Values are estimated means \pm SE; * P <0.05; ** P <0.01, *** P <0.005.

3.5 Discussion

Consistent with our hypothesis, we have shown in the present study that maternal dietary methyl donor and cofactor supplementation during late gestation in PR sheep pregnancies partially blocks the protection against adolescent susceptibility to allergy afforded by restricted growth before birth. Cutaneous hypersensitivity responses to allergens after prior sensitisation were intermediate in PR progeny whose mothers had received methyl donor and cofactor supplementation in late

pregnancy, compared to progeny of un-supplemented PR and control pregnancies. Altered antibody responses to allergic sensitisation did not explain the effect of maternal methyl donor supplementation on changes in cutaneous hypersensitivity. Methyl supplementation also did not rescue the restricted birth phenotype induced by PR in this model of IUGR. Our results therefore suggest that the one-carbon pathway plays a specific role in developmental programming of the immune system and allergy.

The maternal supplementation strategy chosen for the present study was designed to increase maternal circulating abundance of both one-carbon donors and cofactors important for the one-carbon pathway. The rationale for this approach was based on previous observations of reduced one-carbon metabolite abundance and altered methylation in day 0 rat IUGR pups (56, 118), and on epidemiological and experimental findings suggesting that increased maternal methyl donor consumption in late gestation predisposes progeny to allergy (68, 78). Ewes in the present study received not only folic acid and rumen-protected methionine, but also cobalt and sulphur to maximise rumen bacterial synthesis of vitamin B₁₂ and sulphated amino acids such as methionine, respectively (182). Pilot data from a previous experiment demonstrated that this supplement increased maternal plasma folate by ~10-fold during late gestation without altering maternal plasma homocysteine concentrations (Gatford *et al.* unpublished data). Nevertheless, additional evidence that PR perturbs the one-carbon pathway is required to support the hypothesis of an epigenetic mechanism underlying allergic protection in this sheep model. To confirm the degree to which the provision of methyl donors prevented the effects of PR, and by which mechanism, will require a combination of analyses. One of these analyses is direct measurement of plasma one-carbon metabolites from ewes and newborn lambs to confirm methyl donor transfer and identify treatment group differences in abundance. Another required analysis is direct measurement to confirm a change in the epigenetic state of genes relevant to allergic disease, discussed later.

Maternal dietary supplementation with methyl donors and cofactors did not normalise the decrease in birth weight of PR sheep reported previously in this cohort (Appendix 1, 203). In fact, birth size of PR+METHYL neonates was generally slightly smaller than the PR group, and this was significant for skull size measures. This is despite the fact that the gestation lengths were normalised in supplemented PR ewes and were similar to CON ewes, and is consistent with the relatively small effect of PR on gestation length (~ 2 d shorter compared to 147 d at term in CON) not being a major cause for the reduction in size at birth in PR offspring. These responses to maternal methyl-donor supplementation in PR ewes differ from those reported in a meta-analysis of human studies, where maternal supplementation with folic acid or 5-methyltetrahydrofolate from the second trimester of pregnancy until term increased birth weight and did not change gestation age at delivery (54). Interestingly, maternal folic acid supplementation protects mice from LPS-induced preterm birth, suggesting that maternal folic acid supplementation down-regulates inflammation-associated processes that promote parturition (210). The lack of effect of the supplement on birth weight is consistent with methyl-donor abundance not being the limiting factor for fetal growth in this experimental model of placental restriction where supply of nutrients such as oxygen and glucose are limited by reduced placental blood flow and nutrient transfer (153-155).

Consistent with our previous report comparing CON and PR offspring, circulating immune cells (proportions and concentrations) and antibody responses to Clostridial vaccination were not affected by prenatal exposure to PR or PR+METHYL. We have previously reported that antigen-specific IgE responses to HDM but not OVA were upregulated in PR compared to CON sheep. Overall, maternal methyl donor supplementation reduced the proportion of IgE responders to HDM in PR progeny to similar response rates as occurred in CON progeny, although the magnitude of increases in HDM-specific IgE after sensitisation were similar in PR and PR+METHYL progeny. There was a reduced magnitude of OVA-specific IgE response in PR+METHYL compared with PR sheep, but only in progeny from multiple birth litters. Thus, changes in OVA-specific IgE responses do not appear to

underlie the treatment effects in cutaneous hypersensitivity responses, which we observed only within singleton birth progeny. Overall, this suggests that methyl donor supply *in utero* probably had limited effects on the processes involved in antigen-induced antibody production in our experimental model. In contrast, Hollingsworth reported that *in utero* exposure to a methyl-supplemented diet containing folic acid, vitamin B₁₂, choline, L-methionine, zinc and betaine in mice increased antigen (OVA)-induced increases in IgE and IgG₁ as well as allergic responses to airway challenge with OVA including eosinophil influx and the Th2 cytokine IL-13 (78). Interestingly, we observed that the effects of prenatal environment on response rates and size differed between antigens, with no effects of treatment on the proportion of sheep with a positive OVA-specific IgE response. In mice, OVA-sensitisation of dams before pregnancy increases susceptibility of progeny to asthma development in response to systemic and airway challenge with either OVA or casein, suggesting that the programming effect of an allergic mother in this case is not antigen-specific (72). Nevertheless, our results suggest that assessing responses to multiple antigens may be needed to fully characterise effects of the prenatal environment on allergic susceptibility.

As we have reported previously, PR progeny had reduced rates of cutaneous hypersensitivity responses to OVA compared to CON progeny, and this was antigen-specific, with no change in this response to HDM challenge (Appendix 1, 203). This is consistent with the majority of epidemiological studies suggesting a protective effect of IUGR, SGA or LBW against development of allergic disease such as eczema in later life (68). Our new findings of intermediate rates of cutaneous hypersensitivity responses to OVA in the PR+METHYL progeny suggests that *in utero* supply of methyl donors may be important determinants of the inflammatory response to allergens. Others have suggested that epigenetic mechanisms underlie the persistent effects of early life exposures on allergic disease (167), and our findings suggest a plausible role for altered methylation in these associations. Also consistent with the hypothesis that increased methyl donor abundance promotes allergic susceptibility and may involve altered methylation of gene networks important for immune

function, maternal supplementation with methyl donors throughout pregnancy in mice was associated with hypermethylation of runt-related transcription factor 3 which is a negative regulator of allergic airway disease (78). Furthermore, altered methylation patterns of immunoregulatory genes such as increased methylation of *FOXP3* in human cord blood correlate positively with a neonate's risk of developing allergic disease in early life (77). We suggest that effects of PR and PR+METHYL treatments on epigenetic state of immune-regulating genes should be the subject of further studies.

Unexpectedly, maternal methyl donor and cofactor supplementation increased mast cell density in the upper dermis of adult singleton-birth PR progeny. This increased mast cell abundance in PR+METHYL progeny might contribute to their partial loss of protection against cutaneous sensitisation to OVA. Consistent with this hypothesis, increased cutaneous mast cell densities persisted for several months after experimentally-induced allergic dermatitis in mice and were associated with increased allergic airway hyper-reactivity following sensitisation and challenge with an independent allergen (74). Whether methyl donor supplementation and metabolism alter mast cell proliferation, differentiation or lifespan, and potential epigenetic mechanisms for such changes, requires further investigation. The similar mast cell densities in PR and CON progeny do not, however, support our hypothesis that restricted growth *in utero* decreases mast cell density in the upper dermis, based on evidence for decreased mast cell activation in PR progeny (Appendix 1, 203). In contrast to our hypothesis, within multiple birth progeny, PR males had greater cutaneous mast cell density compared with CON males. It is possible that mast cell chemoattractant release such as c-Kit ligand (126) may be upregulated as a result of PR of the multiple birth male sheep compared with CON multiple birth male sheep, however, this our finding of increased mast cell density in PR compared with CON multiple birth males requires confirmation with a larger study size. Perhaps the discord between the singleton and multiple birth responses is due to the combined effect of twinning and placental restriction. One possible reason that a combined effect was seen in males

only is that male fetuses may be greater affected by secondary insults (such as the combination of PR and twinning) during gestation, as male fetuses tend to have worse pregnancy outcomes than female fetuses (reviewed in 48). It remains unclear why fetal growth restriction may increase mast cell density in the skin, as we were unable to confirm in our study what the surrounding cytokine milieu was in the skin that may have caused more mast cell progenitors to localise and terminally differentiate within the skin. How mast cell density determinants (reviewed in 59) may be affected by either fetal stress or birth weight-related factors has not yet been explored and therefore remains a topic for further research. Decreased cutaneous hypersensitivity responses in PR compared to CON sheep unexplained by mast cell density might therefore reflect either decreases in mast cell function, or changes in other regulatory pathways such as altered basophil function (138). A further possibility is that mast cell density may have changed between cutaneous hypersensitivity testing at 28 weeks of age and post-mortem skin sample collection at 52 weeks of age. To our knowledge, this is the first report that maternal methyl donor supplementation during pregnancy affects mast cell abundance in progeny.

In the present study, maternal methyl donor and cofactor supplementation in late gestation reduced the protective effect of PR on cutaneous hypersensitivity responses after OVA sensitisation in singleton-birth sheep, consistent with a possible epigenetic mechanism contributing in part to protective effects of IUGR against allergic susceptibility. Studies investigating the effects of PR and methyl donor supplementation on one-carbon metabolism and methylation of genes important for immune function are required to define the underlying pathways. Maternal methyl donor supplementation also decreased antibody responses to allergen/antigen in some subgroups of progeny, but not in those in which cutaneous responses were decreased, implying that other mechanisms are also involved in programming of allergy by the prenatal environment. This also adds to evidence that late gestation methyl donor supplementation may increase allergy risk in progeny,

and suggest that supplementation strategies should focus on the peri-conception period rather than the entirety of pregnancy.

Chapter 4: Effects of maternal asthma on the fetal immune system

4.1 Overview

Development of the experimental model of maternal asthma in pregnancy used in this chapter has been published (Appendix 2, 38), the manuscript of which I am a co-author (©2015 The Authors. The Journal of Physiology ©2015 The Physiological Society). Within this broader pilot study, my roles included assisting with management of the cohort in late pregnancy, including body weight measurements, endoscopic airway challenges, catheterisation surgeries, catheter maintenance, lung function tests (response to a bronchoconstrictor), bronchoalveolar lavage fluid sampling, blood sampling, post-mortem tissue collection and antibody assays. Specifically relating to this chapter, I assisted with all post-mortem collections of fetal tissues, performed all immune cell isolations from fetal spleen and thymus, antibody labelling and flow cytometry of immune cells, IgE assays, analysis of data and preparation of all results figures. The first section of this chapter consists of an overview of the experimental model. Methods described in sections 4.4.1-4.4.4 are taken directly from the manuscript, as is Figure 4.1, which is reproduced in this thesis with permission from the Journal of Physiology. This published work is followed by chapter sections relating specifically to the hypotheses of this thesis, addressing the effects of maternal asthma on development of the fetal immune system, which have not yet been submitted for publication.

4.2 Statement of authorship form – Development of an experimental model of maternal allergic asthma during pregnancy

Statement of Authorship

Title of Paper	Development of an experimental model of maternal allergic asthma during pregnancy
Publication Status	<input checked="" type="checkbox"/> Published <input type="checkbox"/> Accepted for Publication <input type="checkbox"/> Submitted for Publication <input type="checkbox"/> Unpublished and Unsubmitted work written in manuscript style
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PhD Candidate's Contribution

Name of Candidate	Amy Wooldridge
Contribution to the Paper	Performed experiments; edited and revised manuscript; approved final version of manuscript
Overall percentage (%)	20%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.
Signature	Date 2/11/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- I. the candidate's stated contribution to the publication is accurate (as detailed above);
- II. permission is granted for the candidate to include the publication in the thesis; and
- III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Vicki Clifton
Contribution to the Paper	Conception and design of research; performed experiments; analysed data; interpreted results of experiments; drafted manuscript; edited and revised manuscript; approved final version of manuscript
Signature	Date 15/11/16

Name of Co-Author	Timothy Moss
Contribution to the Paper	Conception and design of research; performed experiments; analysed data; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript
Signature	Date 14 November 2017

Name of Co-Author	Kathryn Gattford		
Contribution to the Paper	Conception and design of research; performed experiments; analysed data; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript		
Signature		Date	17 November 2016

Name of Co-Author	Bahar Liravi		
Contribution to the Paper	Performed experiments; edited and revised manuscript; approved final version of manuscript		
Signature		Date	10/11/2016

Name of Co-Author	Dasom Kim		
Contribution to the Paper	Performed experiments; analysed data; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript		
Signature		Date	17/11/2016

Name of Co-Author	Beverly Mülhhauser		
Contribution to the Paper	Conception and design of research; performed experiments; analysed data; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript		
Signature		Date	15/11/2016

Name of Co-Author	Janna Morrison		
Contribution to the Paper	Conception and design of research; performed experiments; analysed data; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript		
Signature		Date	14/11/16

Name of Co-Author	Andrew Davies		
Contribution to the Paper	Conception and design of research; performed experiments; analysed data; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript		
Signature		Date	15/11/2016

Name of Co-Author	Rob De Matteo		
Contribution to the Paper	Conception and design of research; performed experiments; analysed data; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript		
Signature		Date	15/11/16

Name of Co-Author	Megan Wallace		
Contribution to the Paper	Conception and design of research; performed experiments; analysed data; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript		
Signature		Date	14/11/2016

Name of Co-Author	Robert Bischof		
Contribution to the Paper	Conception and design of research; performed experiments; analysed data; interpreted results of experiments; edited and revised manuscript; approved final version of manuscript		
Signature		Date	10/11/2016

4.3 Introduction and experimental paradigm

Maternal asthma affects up to 12% of human pregnancies (37, 95), and increases the risk of intrauterine growth restriction, pre-eclampsia, preterm birth (143) and offspring allergy (108). The mechanisms responsible for these observations are unknown, partially due to ethical limitations with human studies and the lack of a large animal model. A well-characterised model of asthma in non-pregnant sheep shows symptoms similar to allergic asthma, including hallmarks such as increased systemic allergen-specific IgE levels, increased circulating and airway eosinophils, airway wall remodelling and impaired lung function (17, 83, 125, 184). As such, the broader experiment was conducted with the aim of applying this approach to study allergic asthma during pregnancy, including fetal responses. Allergic asthma was induced in ewes prior to mating, using the established protocol that included sensitisation injections, followed by serial airway challenges with house dust mite (HDM), and collection of bronchoalveolar lavage (BAL) samples immediately before and 48 h after challenge (17). Ewes also received aerosolised HDM (allergic ewes) or saline (control ewes) challenges throughout pregnancy to simulate asthma exacerbations. Singleton-bearing ewes underwent surgery during late gestation for maternal and fetal catheterisation and tissues were collected near term as described in detail below (Section 4.4).

Our findings from the broader experiment have already been published (Appendix 2, 38). Briefly, fetal body weight (relative to maternal weight) was 12% lower in asthmatic sheep pregnancies compared to control sheep pregnancies (Appendix 2, 38), consistent with intrauterine growth restriction (IUGR) observed in humans (129, 143, 145). Asthmatic sheep had placentae with a more mature phenotype (Appendix 2, 38) suggesting compensation for impaired substrate and/or oxygen supply (4). Maternal asthma also resulted in decreased expression of fetal lung surfactant proteins (Appendix 2, 38), which would be expected to impair neonatal lung function consistent with effects of maternal asthma in human neonates from asthmatic women (129). The inflammatory response to HDM challenge in the lung of allergic sheep worsened throughout gestation, as assessed by

eosinophil migration in response to airway challenge and by measurement of airway resistance in response to a bronchoconstrictor (Appendix 2, 38). However, as non-pregnant ewes were not included in this pilot study, we were unable to determine if this worsening inflammatory response is due to accumulating effects of repeated allergen exposure (91, 184) or due to the effects of pregnancy itself, which worsens asthmatic phenotype in humans (141). Another limitation of this broader study is that sex differences were unable to be examined due to insufficient power. Overall, the allergic asthmatic phenotype induced changes in fetal growth and development that are consistent with the effects of maternal asthma in humans, establishing that this large animal model can be used to evaluate mechanisms and interventions in pregnancies complicated by maternal asthma.

In humans, maternal asthma is a risk factor not only for childhood asthma (33, 96, 97), but also increased risk of other allergic diseases in children (119), suggesting that maternal asthma induces persistent changes in the immune phenotype of progeny. These correlations between maternal and childhood allergy or asthma in humans may, however, be due in part to their common genetics. Allergic conditions such as asthma are highly heritable, with the heritability of asthma estimated to be between 50 and 90% (150). Nevertheless, maternal environment appears to contribute to this association, since maternal atopy is a stronger predictor of childhood atopy than is paternal atopy (111). Differentiating these genetic and environmental contributions, and exploring the underlying mechanisms, are challenging in human cohorts, and is further complicated by potential confounding between the prenatal and postnatal environment.

In humans, maternal allergy is associated with a more allergic phenotype of cord blood immune cells in neonates including decreased proportions and function of regulatory T lymphocytes, or regulatory T cells (Tregs, 57). This shift in immune phenotype at birth is likely to impact later allergy risk. For example, the presence of a Th2 (allergic) phenotype in cord blood mononuclear cells (lower Treg

cells, IL-10 production and forkhead box P3, *FOXP3*, expression) is associated with increased risk of childhood allergy by three years of age (130). In sheep, T cells begin to migrate from the thymus to the spleen from 75-80 days of the 147-150 day gestation in sheep (reviewed in 31). Similarly, in the human, mature functional T cells begin to leave the thymus and colonise the periphery from ~14 weeks of gestation (94). This prenatal pattern of T cell maturation and export contrasts with the later development in mice, in which thymic export begins shortly after birth and peripheral Tregs are only detectable from three days after birth (7). As in humans, maternal inflammatory challenges in sheep alter immune phenotype in the fetus and in early postnatal life. Repeated intra-amniotic lipopolysaccharide (LPS) exposure from 90-110 days gestational age increase cluster of differentiation (CD)4+ (127) and decrease CD8+ (103, 127) cells in the thymus of fetal and neonatal postnatal lambs, respectively. Effects of the LPS exposure in late gestation persisted into postnatal life, with an increase in the proportion of CD8+ lymphocytes in the ovine pulmonary lymph node at 7 weeks of age (103). These studies demonstrate that the fetal and early postnatal immune phenotype is programmed by *in utero* exposure to a maternal inflammatory state in sheep, consistent with associations seen in humans. The effects of maternal allergy on fetal immune phenotype have not been examined in this species, however.

The aims of the present study were therefore to determine the effect of maternal allergic asthma with exacerbations on immune cell populations in the spleen and thymus, and on circulating allergen-specific antibodies in the late gestation fetal sheep. We hypothesise that *in utero* exposure to maternal systemic allergy and maternal asthma exacerbations will induce an allergic phenotype in thymic and splenic immune cells of the fetal sheep in late gestation.

The following sections (4.4.1-4.4.3) are reproduced exactly as published with the exception of formatting, which has been changed to maintain consistency throughout the thesis, and the omission of paragraphs detailing methods for outcomes irrelevant to this thesis. Figure 4.1, which has been reproduced with permission from the Journal of Physiology, has been edited only to omit details for outcomes irrelevant to this thesis. However, some of these other irrelevant outcomes have been briefly mentioned below due to the inclusion of unchanged, published text, in line with university policy. It has been published as:

Clifton VL, Moss TJ, **Wooldridge AL**, Gatford KL, Liravi B, Kim D, Mühlhäusler BS, Morrison JL, Davies A, De Matteo R, Wallace MJ, Bischof RJ. (2016) Development of an experimental model of maternal allergic asthma during pregnancy, *J Physiol* 594(5):1311-1325. doi: 10.1113/JP270752 (Appendix 2)

4.4 Methods

4.4.1 Animals and experimental design

All experimental animal procedures were approved by the Animal Ethics Committees of Monash University (MARF/2013/133) and the University of Adelaide (M-2014-126), and were conducted in accordance with Australian guidelines (146).

Merino ewes (1-2 years of age) were allocated randomly to either non-immunised control ($n=9$) or sensitised ($n=31$) groups (Figure 4.1). Sheep in the sensitised group were immunised with HDM using four subcutaneous (s.c.) injections of 50 μg of solubilised HDM extract (*Dermatophagoides pteronyssinus*; CSL Ltd, VIC, Australia) in sterile 0.9% NaCl, and aluminium hydroxide as adjuvant (1:1), with injections given at 2 week intervals (17). Peripheral blood was collected by venepuncture prior to the commencement of HDM immunisations and again 7 days (d) after the final immunisation, to determine HDM-specific serum Immunoglobulin type E (IgE) levels and allergic status of immunised sheep; time-matched samples were collected from control sheep. HDM-specific

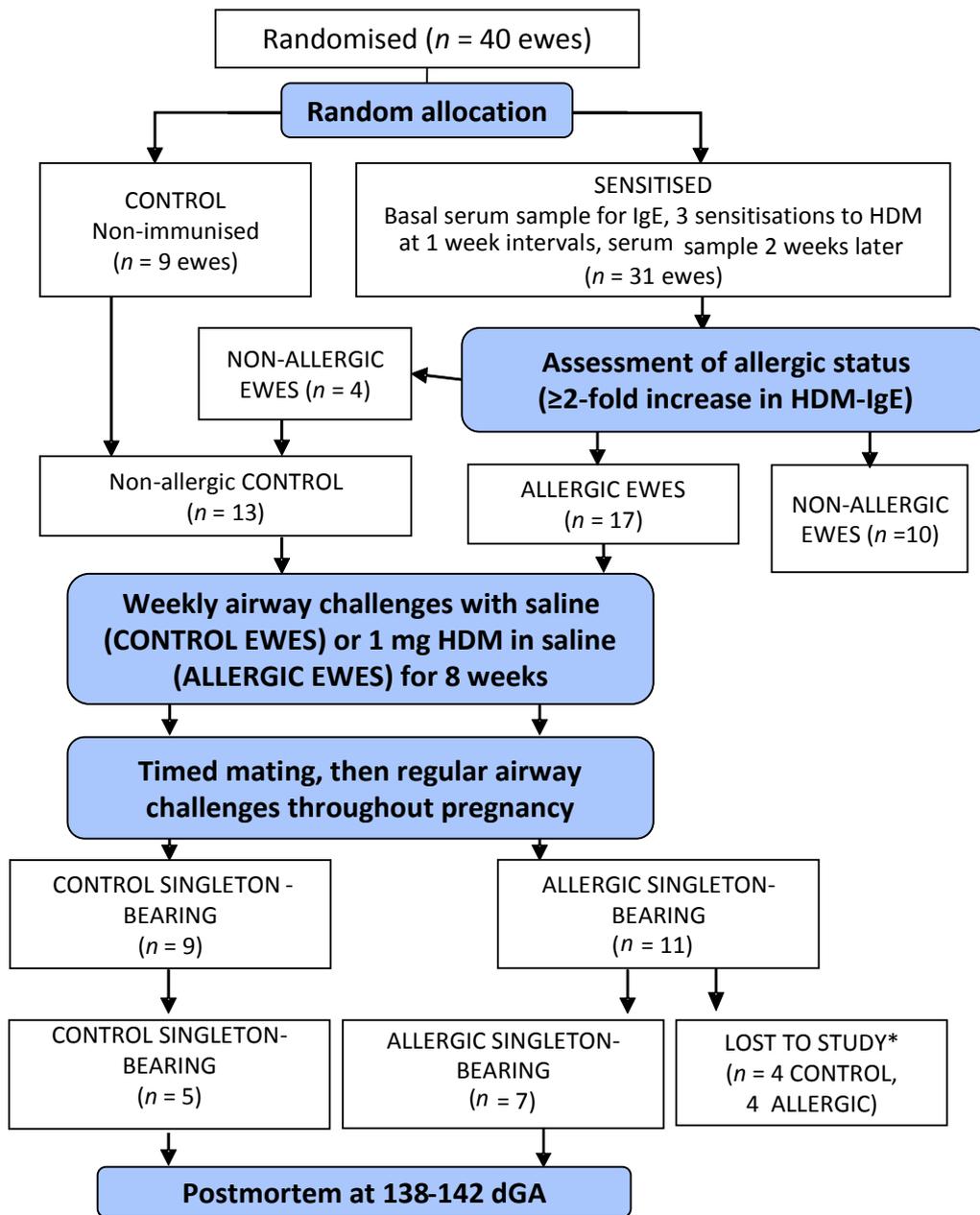
IgE levels were determined in duplicate samples by ELISA, with optical density read at 450 nm (A_{450}) (17, 18). Of the 31 sensitised sheep, there were 17 that showed a two-fold or greater increase in IgE levels after HDM immunisation, and these were defined as allergic (17).

Allergic and control sheep then received weekly endoscopic airway challenges with HDM or saline, respectively (described below), for eight weeks before timed mating with Merino rams (Figure 4.1). Oestrus was synchronised using intravaginal sponges containing 300 mg of the synthetic progestagen flugestone acetate (Eazi-breed CIDR device, Zoetis Australia Pty Ltd, NSW, Australia) for a 12-d period. Mating dates were recorded, and pregnancy status and fetal number were determined by ultrasound at 40-45 d gestational age (dGA; term ~147 dGA).

Throughout pregnancy, allergic sheep received endoscopic airway challenges with HDM every two weeks, and non-allergic sheep received endoscopic airway challenges with saline every four weeks (Figure 4.1). All animals were housed and handled as one group. Sheep were housed outdoors in small paddocks during allergen sensitisation and airway challenges until approximately 90-100 dGA. During this period, sheep grazed natural pastures and were supplemented with lucerne hay. Pregnant sheep were housed indoors in individual pens for the remainder of the experimental period, in a facility with a 12 h:12 h dark-light cycle, and were fed 0.8-1.0 kg lucerne chaff and 0.85 kg ewe and lamb pellets (Rumevite, Ridley AgriProducts, VIC, Australia) daily, with water available *ad libitum*.

Outcomes were studied in only singleton-bearing ewes from each group. Singleton-bearing non-allergic animals (control, $n=9$) included 5 sheep from the non-sensitised group plus 4 sheep from the sensitised group who did not show increases in IgE after the sensitisation protocol, and the allergic group consisted of 11 singleton-bearing sheep (Figure 4.1).

Figure 4.1 Study design. *Lost to study: 4 allergic and 4 control singleton-bearing ewes were lost to study due to non-pregnancy (detected at surgery; $n=1$ allergic ewe), sick on farm ($n=1$ control), failure to recover post-surgery ($n=0$ control, 1 allergic ewe), fetal death ($n=1$ control, 0 allergic ewes) or premature delivery ($n=2$ control, $n=2$ allergic ewes).



4.4.2 Endoscopic airway challenges

For endoscopic airway challenges, sheep were restrained unsedated in a custom-designed body harness, and a flexible fibre-optic endoscope (Model FG-16X; Pentax Ltd, VIC, Australia) was inserted into the lung via the nasal passage (17). In allergic ewes, 5 ml of 100 µg/ml HDM extract (in sterile saline) was delivered as a bolus infusion into each of the right and left caudal lobes of the lung, whilst control non-allergic ewes were similarly challenged with the same volume of saline (17, 125).

4.4.3 Post-mortem and tissue collection

Ewes were humanely killed at 140 ± 1 dGA by i.v. administration of an overdose of sodium thiopentone (Thiobarb, Jurox Pty Ltd, Rutherford, NSW, Australia). The uterus was removed by hysterectomy, amniotic fluid sampled, and the fetus removed and weighed. Maternal and fetal lung, heart, liver, kidneys, spleen, brain and fat depots (perirenal, retroperitoneal and omental), were dissected and weighed. Visceral fat weight was calculated as the sum of omental, retroperitoneal and perirenal fat depot weights. Maternal lungs and heart were processed as described below. All placentomes were removed from the endometrium, individually weighed and scored for phenotype (Type A, B, C or D; 195).

4.4.4 Flow cytometry

The fetal spleen, neck thymus and chest thymus were collected and weighed, then samples of fetal spleen and chest thymus were collected separately into cold Hank's buffered saline solution (HBSS 1470 1x, GIBCO) for cell staining and analysis by flow cytometry as described below. Immune cell populations were isolated from fetal spleen and/or thymus in 7 singleton-bearing allergic sheep and 5 singleton-bearing control sheep (Figure 4.1). Fetuses collected at post-mortem comprised two males and three females from control ewes, and three males and four females from allergic ewes.

To isolate cells, tissue samples of spleen and thymus were cut into small pieces and cells passed through a 70 µm filter cup (BD Bioscience) with several 5 ml washes of ice-cold HBSS. In spleen cell samples, red blood cells were lysed by the addition of Tris-buffered ammonium chloride (0.17 µM Tris, 0.16 µM ammonium chloride, pH 7.2; 17). Spleen and thymus cell samples were transferred into a 15 ml Falcon™ tube, washed twice by centrifugation for 5 min at 300 *g* and assessed by light microscopy to determine cell viability (trypan blue staining) and cell number (using a haemocytometer).

Cells aliquots were immunostained for expression of CD4, CD8, CD5, CD14, CD25, CD44, major histocompatibility complex class I (MHC I) or MHC Class II (MHC II), using fluorochrome-conjugated monoclonal antibodies (mAbs) as detailed in Table 4.1 (17) or isotype-matched control antibodies to validate cell-specific staining for each of the mAbs. For staining, cell samples were resuspended at 2×10^7 /ml in wash buffer (2% bovine serum albumin/0.05% sodium azide/phosphate-buffered saline) with the addition of normal sheep serum to block Fc and non-specific Ig binding sites. Cells were then plated into wells (50 µl/well) of a V-bottomed 96-well plate (Nunc), Ab mix (25 µl) added to each well and the plate incubated for 20 min on ice. Plates were then washed twice (centrifugation at 300 *g* for 2 min followed by resuspension of cells in 200 µl wash buffer), and fixed with the addition of 100 µl of 2% paraformaldehyde (Fluka) in PBS to the final cell pellet, and stored at 4°C prior to analysis by flow cytometry. Lymphocytes were gated based on forward-scatter (FSC) and side-scatter (SSC) characteristics (17), and 10,000 events were acquired using a BD LSR II flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA), with data analysed using FlowJo™ software (Figure 4.2, TreeStar, San Carlos, CA, USA). Lymphocytes were successfully isolated from all fetuses, and fluorescence-activated cell sorting (FACS) analyses was completed for a minimum of 4 fetuses per treatment for each antibody and tissue combination.

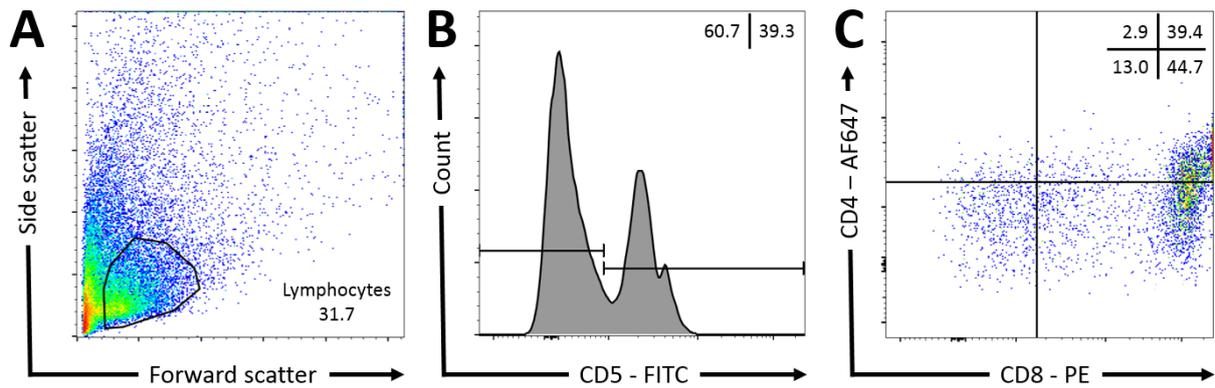


Figure 4.2 Gating strategies - representative FACS profiles from the spleen of an individual sheep showing A) lymphocyte gating, B) gating of CD5 positive lymphocytes and C) CD4/CD8 quadrant gating of CD5+ lymphocytes. In each plot the percentage of cells per gated region/quadrant is noted.

4.4.5 Statistical analyses

Outcomes in control and allergic sheep were compared by one-way ANOVA using SPSS version 20.0 (SPSS Inc., Chicago, USA). Data are presented as means \pm SE. $P < 0.05$ was accepted as significant.

Table 4.1 Antibodies used to stain cell receptors for flow cytometry

1° antibody	Label[#]	Raised in, type (clone, catalogue no.)	Dilution	Source
Anti-ovine CD4 ^{&}	AF647	Mouse, mAb (clone 44.97; in-house)	1:1000	In-house*
Anti-ovine CD4	FITC	Mouse, mAb (clone 4.38; MCA2213F)	1:500	AbD Serotec
Anti-ovine CD5 ^{&}	FITC	Mouse, mAb (clone 25.91; MCA2215F)	1:100	AbD Serotec
Anti-ovine CD8 ^{&}	PE	Mouse, mAb (clone 38.65; MCA2216PE)	1:200	AbD Serotec
Anti-human CD14	AF647	Mouse, mAb (clone TUK-4; MCA1568A647)	1:400	AbD Serotec
Anti-ovine CD25	FITC	Mouse, mAb (clone 9.14; MCA2218F)	1:100	AbD Serotec
Anti-ovine CD44	FITC	Mouse, mAb (clone 25.32; MCA2219F)	1:150	AbD Serotec
Anti-ovine MHCI	FITC	Mouse, mAb (clone 41.17; MCA2224F)	1:50	AbD Serotec
Anti-ovine MHCII (DQ/DR)	FITC	Mouse, mAb (clone 49.1; MCA2228F)	1:200	AbD Serotec

[#]FITC, fluorescein isothiocyanate; PE, phycoerythrin; AF647, Alexa Fluor[®] 647; mAb, monoclonal antibody. Unless specified, each preparation was only labelled with a single antibody. [&]Antibodies used concurrently in samples for 3-colour flow cytometry. *Antibody was conjugated in-house using AF647 antibody-labelling kit (Life Technologies); kindly provided by Dr Bahar Liravi (Biotechnology Research Laboratories, Dept Physiology, Monash University).

4.5 Results

4.5.1 Post-mortem organ weights

Aspects of maternal, placental and fetal phenotype have been reported previously (Appendix 2, 38). Maternal body weight, placental weight and average placentome weight did not differ between groups, nor did fetal body weight or the weights of the fetal spleen, chest thymus, neck thymus or total thymus (Table 4.2). Fetal body weight as a ratio of maternal body weight also did not differ between groups, nor did the weights of the fetal spleen, chest thymus, neck thymus or total thymus as a ratio of fetal body weight (Table 4.2). Although the total number of placentomes tended to be greater in control than allergic pregnancies, this difference was not significant ($P=0.070$, Table 4.2). The relative fetal body weight was 12% lower in allergic pregnancies compared to control pregnancies ($P=0.038$, Table 4.2).

Table 4.2 Placental phenotype and maternal and fetal weights at post-mortem.

		Control (n=5)	Allergic (n=7)	P-value
Maternal	Body weight (kg) [#]	38.9 ± 2.1	39.6 ± 1.4	0.766
Placental	Placental weight [#]	344 ± 51	323 ± 23	0.689
	Total placentomes (no.) [#]	76.4 ± 6.5	73.7 ± 4.7	0.070
	Average placentome weight (g) [#]	4.39 ± 0.37	4.40 ± 0.21	0.976
Fetal	Body weight (kg) [#]	4.11 ± 0.28	3.69 ± 0.14	0.167
	Body weight (% of maternal weight) [#]	10.6 ± 0.5	9.3 ± 0.3	0.038
	Spleen (g) [#]	5.93 ± 0.78	6.07 ± 0.48	0.880
	Spleen (% of fetal weight) [#]	0.14 ± 0.02	0.16 ± 0.01	0.322
	Chest thymus (g)	7.34 ± 0.42	7.18 ± 0.43	0.805
	Chest thymus (% of fetal weight)	1.8 ± 0.1	2.0 ± 0.1	0.257
	Neck thymus (g)	12.13 ± 0.95	11.88 ± 0.96	0.861
	Neck thymus (% of fetal weight)	3.0 ± 0.2	3.1 ± 0.2	0.640
	Total thymus (g)	19.88 ± 1.15	19.16 ± 1.15	0.685
	Total thymus (% of fetal weight)	4.7 ± 0.4	5.1 ± 0.2	0.433

Values are means ± SE. [#]Reported previously (Appendix 2, 38).

4.5.2 Fetal antibodies to HDM allergen

Levels of HDM-specific IgE were below the detectable limit in fetal plasma samples (data not shown).

4.5.3 Fetal immune cell phenotype

The percentage of lymphocytes positive for CD44 was greater in the thymus of fetuses from allergic compared to control ewes (Figure 4.3, $P=0.043$). The percentage of lymphocytes in spleen that were CD44+ did not differ between treatments (Figure , $P>0.1$). In both thymus and spleen, the percentage of lymphocytes that were CD5+, CD14+, CD25+, MHCII+ or MHCII+ did not differ between treatments. While there was no difference in the percentage of CD5+ lymphocytes, the intensity of CD5 expression (mean fluorescence intensity, MFI) tended to be decreased in fetal thymocytes from allergic ewes (control 2530 ± 158 ; allergic 1924 ± 212 ; $P=0.051$; data are geometric means \pm SE). The percentage of double-positive CD4+CD8+, single-positive CD8-CD4+ or CD8+CD4- and double-negative CD4-CD8- lymphocytes in spleen and thymus did not differ with treatment overall. Percentages of cells expressing each combination of CD4 and CD8, and the total percentage of CD4+ and CD8+ lymphocytes did not differ with treatment, within CD5+ and CD5- lymphocyte sub-populations.

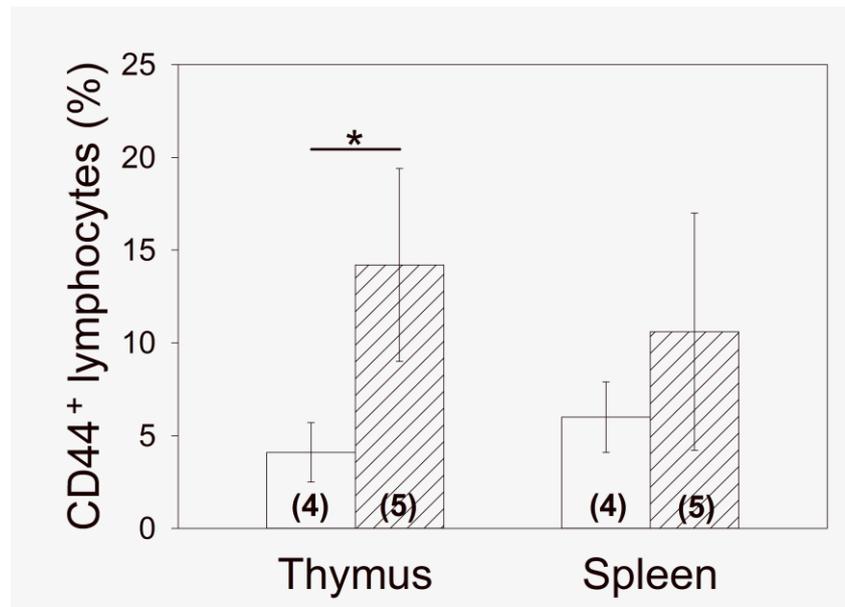


Figure 4.3 Percentage of lymphocytes positive for CD44 expression (% positive) from fetal thymus and fetal spleen taken from control (open bars) and allergic (striped bars) pregnancies. Data are means \pm SE; number of fetuses shown in brackets; * $P < 0.05$, control versus allergic.

4.6 Discussion

In contrast to our hypothesis, maternal asthma did not affect fetal weight of immune tissues or proportions of Th1 and Th2-expressing lymphocytes in the fetal thymus and spleen in late gestation. We did, however, observe an increased proportion of thymic lymphocytes positive for the cell adhesion marker, CD44. Increased CD44 expression, if it were to persist, might promote the traffic or circulation of lymphocytes to affected peripheral tissues during allergic (or other inflammatory) responses.

Fetal immune tissue weights: Maternal allergic asthma did not alter fetal thymus and spleen weights at late gestation in the present study. Whilst *in utero* inflammation is known to decrease thymus size (47), to our knowledge this is the first report of thymus and spleen size responses to maternal allergy. Fetal thymus size on ultrasound is decreased in human pregnancies complicated by both chorioamnionitis and funisitis (47). In sheep, intra-amniotic LPS during late gestation acutely

decreased chest thymus weights relative to body weight in one study (93), but not total thymus (chest + neck) weights in another (127). Overall, these provide some support for the suggestion that *in utero* exposure to an inflammatory environment may reduce thymus size. As the fetal sheep thymus is at its largest relative to fetal size at 125-130 dGA (reviewed in 31) and we examined fetal organs at 140 ± 1 dGA, it is possible that we missed the time point at which the greatest difference in fetal thymus weight between fetuses from control and asthmatic pregnancies may have occurred. It is also possible that maternal allergy alters the function of these immune tissues independent of effects on size. We suggest that future studies include measures of the micro-environment such as extracellular matrix proteins and cytokines, in addition to further phenotypic and functional analyses of immune cells isolated from thymus and spleen to tease out possible changes in the Th1 and Th2 cell populations.

Circulating antibodies: Allergic ewes were selected on the basis of elevated circulating (plasma) levels of HDM-specific IgE post-sensitisation, as previously reported (Appendix 2, 38). The lack of HDM antibodies in fetal plasma confirms previous reports that the ovine placenta provides a complete barrier to immunoglobulin transfer from mother to fetus, except where significant placental damage leads to intermingling of maternal and fetal circulations (165). It also suggests that the HDM antigen itself did not cross the placenta, since the fetal sheep can mount antibody responses to foreign allergens from mid-gestation (50). In contrast, antibodies cross from maternal to fetal circulation in human pregnancy, as do allergens including Der p 1, the main allergenic component of HDM (117). Induction of an altered fetal immune cell phenotype by maternal allergic asthma in sheep must therefore be due to exposure to an inflammatory *in utero* environment rather than being due to fetal exposure to the allergen itself. In humans, rats and mice, antibodies cross the placenta (85, 90), and so these potentially also contribute to development of fetal allergy. Nevertheless, the fact that increased allergic susceptibility in the offspring of sensitised mouse dams

is not specific to the allergens of maternal exposure (72) suggests that systemic responses are induced by the maternal allergic response itself.

Lack of differences in other cell surface markers: Unexpectedly, given the evidence for increased allergic susceptibility in progeny of asthmatic or allergic human mothers (82, 108) and progeny of allergic mice (72, 79), exposure to chronic maternal allergic asthma did not alter expression of the majority of immune markers we examined in immune tissues of the late gestation fetal sheep, including CD4, CD8 and MHC II. In mice, *in utero* exposure to chronic allergic asthma increased the number of splenic CD4+ lymphocyte that did not co-express either FOXP3 or CD25, although numbers of CD4+FOXP3+CD25+ (Treg) cells were unchanged (80). In the sheep, *in utero* exposure to intra-amniotic injection of 20 mg LPS in late gestation reduced the proportions of fetal thymocytes positive for CD8 and MHC II, and increased the ratio of CD4:CD8 seven days after exposure (127). This inflammatory exposure was initiated later in gestation than the present study, and in response to acute fetal rather than chronic maternal inflammation. Differences in immune programming between these models may also reflect impacts of repeated hypoxic exposure, secondary to asthmatic exacerbations.

Increased expression of CD44: Fetuses from allergic ewes had a greater proportion of lymphocytes expressing CD44 in thymus. The phenotype change in thymic but not splenic lymphocytes might suggest changes in the phenotype of immature T cells that remain in thymus, but additional studies are needed to identify which population of lymphocytes express CD44 in these fetal sheep. In particular, staining of lymphocytes for co-expression of CD44 and FOXP3 is likely to be informative. Unfortunately, a staining protocol for ovine FOXP3 was not sufficiently optimised by the time of post-mortems, and we were unable to include data for this marker. FOXP3 receptor expression is an important functional indicator of Treg cell function and is positively associated with Treg cell-mediated suppression of inflammation (113). Reduced FOXP3 expression and IL-10 production by

cord blood mononuclear cells in human neonates is associated with increased allergic diseases at three years of age (130), suggesting this marker may be a useful predictor of immune function. We acknowledge also the relatively small sample size available for this analysis, with FACS analysis able to be performed in only 4-6 fetuses per treatment for each antibody and tissue combination. The apparently tissue-specific effects therefore require validation in a larger cohort. Nevertheless, to the best of our knowledge, this is the first demonstration of developmental programming of CD44 by *in utero* exposure to maternal allergy. In contrast to the effects of chronic maternal allergic inflammation on CD44 expression in the present study, the proportion of CD44+ lymphocytes in the medulla of the fetal thymus was not changed in a different model of intrauterine inflammation in sheep, induced by intra-amniotic LPS administration (127). In that study, using an immunohistochemical approach, CD44+ cells were commonly found in the thymic medulla but were scarce in the thymic cortex (127). In the present study we isolated lymphocytes from a complete cross-section of the thymus, and were therefore unable to determine whether CD44 expression increased uniformly, was differentially increased in one region or whether the increase in expression reflects changes in the thymus medulla:cortex ratio. Expression of CD44 on the lymphocyte cell surface allows adhesion to endothelial cells and is essential for migration of activated T cells from the circulation into sites of inflammation (44, 45). This process is also critical for allergic responses, since loss or inhibition of CD44 impairs inflammatory responses, including those induced by airway and cutaneous allergen exposure in sensitised mice (32, 86). Furthermore, elevated CD44 expression has been reported on BAL lymphocytes following allergen challenge in sheep (17). Therefore, if the elevated CD44 expression in thymocytes from fetuses exposed to maternal allergic asthma persists postnatally, this may contribute to the increased susceptibility to allergy that has been reported in children of asthmatic and allergic mothers (82, 108).

Overall, these results support our hypothesis that *in utero* exposure to maternal systemic allergy and maternal asthma exacerbations would induce an allergic phenotype in thymic and splenic immune

cells of the fetal sheep in late gestation. In particular, increased CD44 expression on lymphocytes may reflect some level of maternal asthma-induced immune activation *in utero*. Clearly, further research is needed to determine if these changes in immune cell phenotype (as well as more specific changes) persist and result in an increased susceptibility to allergic disease in the affected offspring.

Chapter 5: General discussion

5.1 Introduction

Allergy affects 30-40% of the world's population (205), costing the economy thousands of dollars per affected child per year in the United States of America (71), and the incidence of allergy is increasing (139). In Chapter 1, the evidence that early life exposures program, or permanently alter, susceptibility to developing allergy in later life, was reviewed with a particular focus on effects of restricted fetal growth and maternal allergy and asthma. Human studies of size at birth and later allergic outcomes have reported inconsistent associations, possibly due to the potential for relationships differing with different allergic conditions, populations and ages, in addition to variable postnatal environments (see Table 1.1). Although a structured systematic review of human epidemiological studies is still required to clearly describe the relationship between small size at birth and later allergic disease (Appendix 3, 204), the most common general finding is that size at birth appears to be protective (190) whereas maternal asthma is a risk factor for allergy (157).

Prior to the work described in this thesis, it had been reported that maternal dietary methyl donor intake during at least late gestation was positively associated with allergic susceptibility in offspring (78, 209). Increased maternal dietary intake of methyl donors and cofactors during pregnancy has been suggested as a potential cause for increased offspring allergic susceptibility (78, 209). Similarly, reduced fetal one-carbon metabolite abundance and altered methylation caused by intrauterine growth restriction (IUGR) in rats (56, 118) had been identified as a potential pathway for prenatal programming of multiple postnatal outcomes, although allergic outcomes had not been reported in that model. It was therefore unknown whether the protective effects of IUGR on allergy could be explained by decreased fetal methyl donor supply, or could be reversed by increasing the supply of these nutrients. Meanwhile, the effects of maternal allergy on offspring immune function have to date only been adequately tested in mice. These murine studies had identified that the resulting greater allergic susceptibility of offspring was allergen-independent (53, 72, 79, 105), suggesting that

a change in immune cell populations related to recognition of allergens and allergen-specific antibody production was the cause. While exposure to maternal allergy during gestation or lactation was sufficient to induce offspring allergic susceptibility (105), and cellular phenotypic changes were identified as a mechanism for this change (79), these findings have yet to be tested in a species with a more mature immune system at birth.

In the studies described in this thesis, I therefore initially explored the effects of an induced model of placental restriction (PR) on immune phenotype and susceptibility to allergic sensitisation in the adolescent sheep (Chapter 2). Next, I explored the potential role of methyl donors in the protective effect of PR against allergic susceptibility by investigating the same outcomes in progeny of PR ewes fed a methyl donor and cofactor supplement in the last fifth of pregnancy (Chapter 3). Finally, I directly investigated the environmental contribution of maternal allergy to the programming of immune function by investigating the effects of experimentally-induced allergic maternal asthma on immune cells in the fetal sheep thymus and spleen in late gestation. Chapter 4 represents the first study to describe the effects of maternal allergic asthma on fetal immune phenotype in a large animal model.

5.2 Developmental programming of allergic susceptibility

Human epidemiological studies (see Table 1.1) and Chapter 2 of this thesis support previous speculation (166) that the stressed, growth-restricted fetus 'prioritises' development of organs such as the brain and liver over the immune system, potentially even when brain sparing is not immediately apparent from direct newborn measurements. Despite this resulting in short-term increased susceptibility to postnatal infection (1, 181), this has the long-term effect of 'protecting' against allergic sensitisation (Table 1.1, Chapter 2). Conversely, maternal asthma restricts fetal growth (129, 143, 145) yet is associated with increased risk of allergy in offspring (108). The decreased fetal/birth size resulting from both experimental prenatal exposures investigated within

this thesis is comparable to findings in humans that fetal stress is associated with reduced birth weight (14, 20, 145, 175). Integration of findings from both models is worthy of investigation as these are likely to provide insight into the main causal mechanisms for programming of allergic susceptibility.

Chapter 3 is the first study to describe the effects of combined exposures of IUGR and maternal methyl donor and cofactor supplementation on postnatal immune function. In line with directions of associations in human studies (Table 1.1), PR alone protected against allergic inflammatory responses in the skin after sensitisation (Chapter 2), whereas the maternal methyl donor supplementation prevented some of these protective effects (Chapter 3), consistent with a possible epigenetic mechanism. This demonstrates that whilst maternal dietary methyl donor supplementation can partially ameliorate the protective effect of PR on offspring allergic susceptibility, consistent with a possible epigenetic mechanism, it is not completely protective. These changes did not appear to be the result of dampened antibody responses to sensitisation (Chapters 2-3), suggesting that other pathways are responsible. Although mast cell density, hypothesised to be responsible for inflammation in the skin was investigated (Chapter 3), this was not altered by PR. Interestingly, however, the singleton placentally-restricted, maternal dietary methyl donor and cofactor-supplemented (PR+METHYL) sheep had greater upper dermis mast cell densities compared to singletons from both control and PR groups. Therefore, the results suggest that different regulatory mechanisms may underlie the effects of IUGR and methyl donor availability on immune programming, and that altered cutaneous structure and inflammatory responses are likely to be important pathways underlying reduced allergic inflammation after IUGR. Chapter 3 is the first study to report that maternal methyl donor supplementation during pregnancy affects mast cell abundance in progeny. The notion that cell-mediated immunity plays a role in the protective effects of IUGR on allergic responses is supported by an earlier study that found small for gestational age children ≥ 35 weeks gestational age born to mothers with known placental insufficiency or

hypertension had reduced cutaneous hypersensitivity reactions to phytohemagglutinin compared with “healthy” controls of variable gestational age (range 31-41 wk) and birth weight (mean 3,130 g; range 1,460-4,095 g; 55). Early postnatal malnourishment also reduces these responses to phytohemagglutinin (124), supporting a role for suppressed cell-mediated immunity as a mechanism underlying effects of perinatal growth on allergic susceptibility. A possible target to examine as a causal mechanism for reduced allergic inflammation in the skin after IUGR is mast cell function, as potentially the methyl donor supplementation offsets the effects of reduced mast cell function as a result of having increased mast cell density. To the best of my knowledge, however, mast cell function after IUGR or maternal methyl donor supplementation has not yet been investigated.

As reported in Chapter 4, experimentally-induced allergic maternal asthma increased levels of cluster of differentiation (CD)44+ cells as a proportion of lymphocytes in the fetal ovine thymus. CD44 is integrally involved in allergic responses (32, 44, 45, 86), therefore an increase in CD44+ cells represents a potential mechanistic pathway for increased allergy/asthma risk in offspring of maternal allergic asthmatic mothers. It remains unknown which immune cells express the increased CD44 in this model and unconfirmed whether the increased CD44 expression does in fact increase transfer of T cells from circulation to tissues (32). Potentially that would increase immune cell traffic in response to allergens, causing increased inflammation at the site of allergen contact. Whether this increase in CD44+ cells persists postnatally and confirmation of whether it is associated with allergic outcomes in offspring should be the study of further research.

It remains unknown whether the change in allergic phenotype due to maternal allergic asthma exposure is sex-specific between parent and offspring, as has been suggested by a study in humans (6). Whilst the study in Chapter 4 was a pilot study and as a result not sufficiently powered to detect sex differences in outcomes, the study of sex differences in a larger cohort would be an ideal target for future research. This is not only due to sex differences in allergy rates, which differ across ages

(reviewed in 36), but also because there are sex differences in how fetuses respond to stressors whilst *in utero* and in some cases the sex of the fetus also alters maternal physiology. For example, female fetuses of asthmatic women have reduced birth weights, whereas the birth weights of male fetuses are not affected, and these female fetuses are associated with increased maternal circulating monocytes and reduced placental 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) enzyme activity (142), probably the cause of the reduced fetal growth (179). Due to the range of potential causal mechanisms for prenatal programming outlined above (mast cell density, CD44+ lymphocytes, possible other unidentified causal mechanisms), careful analysis of sex differences caused by exposure to maternal allergic asthma may be very revealing of mechanisms behind prenatal programming. By subsequently comparing and contrasting multiple immune outcomes caused by various *in utero* exposures, we may identify consistent changes that appear in varying degrees with the different exposures known to program susceptibility to allergy. Examining these outcomes at different ages will assist in determining how robust certain changes are, whether some changes persist more than others and why that is the case.

5.3 Strengths and limitations of the studies in this thesis

The primary strength of the studies within this thesis lie in their novelty and use of large experimental (ovine) models of IUGR and maternal allergic asthma conditions, where the exposure is experimentally-induced and thus not confounded by genetics or other factors. The genetic heterogeneity of sheep is more similar to that of humans than in small rodents. In the case of maternal asthma, it is impossible to thoroughly measure the fetal response to maternal asthma exacerbations as it is ethically impossible to induce an asthma attack in a pregnant woman and these exacerbations would not otherwise be likely to occur whilst under medical observation. In comparison with human epidemiological studies, ovine models enable comparative groups to have a common postnatal environment and enable control of deliberate allergen exposure amounts and exposure timings. The ovine PR model has been well-characterised, whereas the ovine maternal

allergic asthma model is the first of its kind, heralding future advances in knowledge about the fetal response to maternal allergy and asthma exacerbations. This new ovine model will enable the *in vivo* fetal responses to allergic inflammation and exacerbations to be measured in real-time. Fetal immune outcomes resulting from exposure to maternal allergic asthma had previously relied on postnatal studies of small rodents, in which the immune system continues to develop during early postnatal life (94), meaning that early postnatal variables play a larger role in immune programming than they do in other species where a greater proportion of this development occurs before birth. Future studies in this model are expected to investigate a wider range of immune outcomes. This includes the characterisation of cytokine profiles, and analysis of *in vitro* responses to allergen challenge of fetal immune cells collected before and after maternal airway allergen challenge, and flow cytology to characterise regulatory T cells (Tregs) using the marker forkhead box P3 (FOXP3), which we were unable to do in the present study.

Limitations of the studies described in Chapters 2 and 3 not already mentioned above include that skin samples for mast cell analysis were collected at post-mortem instead of at a time point closer to skin prick testing; mast cell density may have changed during this time since this increases with age (163). Direct measurement of DNA methylation status of relevant genes (for example, *FOXP3*) could not be performed as part of this study. The lack of one-carbon metabolite data in the present study is partially offset by existing unpublished data, which has confirmed metabolite transfer from ewe to fetus after the same dietary supplement (Gatford *et al.* unpublished data). For logistical and resource reasons, it was not possible to include a CON+METHYL treatment group to directly test whether methyl donor and cofactor supplementation in normal ovine pregnancy increases adolescent allergic outcomes. Together with studies analysing the methylation status of relevant immune-regulatory genes, this extra treatment group would have enabled us to better clarify the contribution of methyl donors to the prenatal programming of allergic disease.

5.5 Conclusion

Overall, my findings have consistently shown programming of allergic susceptibility by environmental exposures before birth. In particular, I have used experimental ovine models in novel studies to directly show that restricted growth before birth and maternal methyl donor supply in late gestation each program allergic inflammatory responses in progeny, while maternal allergic inflammation changes fetal immune phenotype. The findings of this thesis highlight that different *in utero* exposures have varying effects on offspring immune function. In order to protect against the development of allergy via intervention during the prenatal period, a multi-target approach is therefore required, taking into account fetal nutrient and substrate supply in addition to potential inflammatory exposures.

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APPENDICES

Appendix 1 Placental restriction of fetal growth reduces cutaneous responses to antigen after sensitization in sheep

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Placental restriction of fetal growth reduces cutaneous responses to antigen after sensitization in sheep

Amy L. Wooldridge,^{1,2,3} Robert J. Bischof,⁵ Els N. Meeusen,⁵ Hong Liu,^{1,2} Gary K. Heinemann,^{1,2} Damien S. Hunter,^{1,2,3} Lynne C. Giles,⁴ Karen L. Kind,^{1,3} Julie A. Owens,^{1,2} Vicki L. Clifton,^{1,2} and Kathryn L. Gatford^{1,2}

¹Robinson Institute, ²School of Paediatrics and Reproductive Health, ³School of Animal and Veterinary Sciences, ⁴School of Population Health, University of Adelaide, and the ⁵Biotechnology Research Laboratories, Department of Physiology, Monash University, Australia

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Wooldridge AL, Bischof RJ, Meeusen EN, Liu H, Heinemann GK, Hunter DS, Giles LC, Kind KL, Owens JA, Clifton VL, Gatford KL. Placental restriction of fetal growth reduces cutaneous responses to antigen after sensitization in sheep. *Am J Physiol Regul Integr Comp Physiol* 306: R441–R446, 2014. First published February 5, 2014; doi:10.1152/ajpregu.00432.2013.—Prenatal and early childhood exposures are implicated as causes of allergy, but the effects of intrauterine growth restriction on immune function and allergy are poorly defined. We therefore evaluated effects of experimental restriction of fetal growth on immune function and allergic sensitization in adolescent sheep. Immune function (circulating total red and white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, and basophils, and the antibody response to Clostridial vaccination) and responses to house dust mite (HDM) allergen and ovalbumin (OVA) antigen sensitization (specific total Ig, IgG₁, and IgE antibodies, and cutaneous hypersensitivity) were investigated in adolescent sheep from placentally restricted (PR, $n = 23$) and control ($n = 40$) pregnancies. Increases in circulating HDM-specific IgE ($P = 0.007$) and OVA-specific IgE ($P = 0.038$) were greater in PR than control progeny. PR did not alter total Ig, IgG₁, or IgM responses to either antigen. PR increased OVA-specific but not HDM-specific IgA responses in females only ($P = 0.023$). Multiple birth increased Ig responses to OVA in a sex-specific manner. PR decreased the proportion of positive cutaneous hypersensitivity responders to OVA at 24 h ($P = 0.030$) but had no effect on cutaneous responses to HDM. Acute wheal responses to intradermal histamine correlated positively with birth weight in singletons ($P = 0.023$). Intrauterine growth restriction may suppress inflammatory responses in skin downstream of IgE induction, without impairment in antibody responses to a nonpolysaccharide vaccine. Discord between cutaneous and IgE responses following sensitization suggests new mechanisms for prenatal allergy programming.

allergy; animal model; intrauterine growth restriction; developmental programming; sheep

INCREASING EVIDENCE suggests that environmental factors, including prenatal or early childhood exposures, modulate later allergy susceptibility (29). One such prenatal event is intrauterine growth restriction (IUGR), which affects 6–12% of babies in developed countries (15) and usually results in small size at

birth for gestational age where the birth weight of babies falls below the tenth percentile for sex at a given gestational age (SGA, 42). IUGR and SGA babies have increased risks of perinatal mortality and morbidity and of adult metabolic and cardiovascular disease (9). Less is known about the effects of IUGR on postnatal immune function, including allergy. Low-birth-weight individuals are at greater risk of pneumococcal infection as infants (34) and may have impaired B-cell function as adults (22, 23), but these studies did not separate low birth weight due to IUGR and prematurity. Risks of allergic sensitization and atopic diseases including eczema and hay fever in children and adults are positively related to size at birth, independent of gestational age, in some (4, 10, 14), but not all studies (12, 28). Similarly, in a twin study, the heavier birth weight twin had a higher risk of childhood atopic eczema (16). Although SGA is associated with increased, rather than decreased, risks of childhood and adult asthma (26, 40), these studies did not differentiate allergic and nonallergic asthma and possibly reflect impaired lung development following IUGR (36). Together, these findings suggest IUGR suppresses immune function and decreases the risk of postnatal allergy, although these human studies may be confounded by environmental factors that affect both fetal growth and immune function (25, 30).

Poor placental function is a major cause of IUGR due to reduced fetal substrate supply (32). Restricted placental growth and function (PR) can be induced surgically in sheep, with similar prenatal and postnatal consequences as human IUGR (1, 31). Similar to humans, sheep sensitized to allergens develop IgE responses, cutaneous hypersensitivity, and airway hyperresponsiveness following allergen challenge (19). This provides a repeatable, well-characterized protocol for induction of allergy in this species (2, 3, 19), allowing susceptibility to allergic challenge to be tested independent of prenatal environment. We therefore used the sheep to directly test the hypothesis that restricted fetal growth impairs immune function and reduces allergy susceptibility in adolescence.

METHODS

All procedures were approved by the University of Adelaide Animal Ethics Committee (M-2010-139) and conducted in accordance with Australian guidelines (24).

Address for reprint requests and other correspondence: K. Gatford, School of Paediatrics and Reproductive Health, Univ. of Adelaide, South Australia 5005, Australia (e-mail: kathy.gatford@adelaide.edu.au).

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R441

Appendix 2 Development of an experimental model of maternal allergic asthma during pregnancy

Development of an experimental model of maternal allergic asthma during pregnancy

Vicki L. Clifton^{1,9}, Timothy J. M. Moss^{2,3}, Amy L. Wooldridge¹, Kathryn L. Gatford¹, Bahar Liravi⁵, Dasom Kim⁵, Beverly S. Muhlhausler⁷, Janna L. Morrison⁸, Andrew Davies^{5,6}, Robert De Matteo⁴, Megan J. Wallace^{2,3} and Robert J. Bischof^{2,5}

¹Robinson Research Institute and School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA 5005, Australia

²The Ritchie Centre, Hudson Institute of Medical Research, Monash University, Clayton, VIC 3168, Australia

³Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC 3168, Australia

⁴Department of Anatomy and Developmental Biology, Monash University, Clayton, VIC 3168, Australia

⁵Department of Physiology, Monash University, Clayton, VIC 3168, Australia

⁶School of Biomedical Sciences, Peninsula Campus, Monash University, Frankston, VIC 3199, Australia

⁷FOODplus Research Centre, School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, SA 5005, Australia

⁸Early Origins of Adult Health Research Group, School of Pharmacy and Medical Sciences, Sansom Institute for Health Research, University of South Australia, Adelaide, SA 5001, Australia

⁹Mater Medical Research Institute, University of Queensland, Brisbane, Qld 4101, Australia

Key points

- We studied the effects of preconceptional allergen sensitisation and repeated airway allergen challenges during pregnancy on maternal immune and airway functions during pregnancy, and maternal, fetal and placental phenotype in late pregnancy in sheep.
- This protocol induced maternal responses consistent with an allergic asthmatic phenotype. During pregnancy, lung resistance and the eosinophil influx induced by allergen challenges increased progressively in allergic sheep, and in late pregnancy airway smooth muscle content was greater in allergic than control ewes.
- Effects on fetal growth and development were consistent with those of maternal asthma in humans. Maternal allergic asthma decreased relative fetal weight by 12%, reduced fetal lung expression of surfactant protein B, and altered placental morphology.
- This provides an animal model in which to identify mechanisms underlying fetal effects of maternal asthma in pregnancy, including fetal physiological responses to exacerbations, and to evaluate responses to clinically used treatments and novel interventions.

Abstract Maternal asthma during pregnancy adversely affects pregnancy outcomes but identification of the cause/s, and the ability to evaluate interventions, is limited by the lack of an appropriate animal model. We therefore aimed to characterise maternal lung and cardiovascular responses and fetal–placental growth and lung surfactant levels in a sheep model of allergic asthma. Immune and airway functions were studied in singleton-bearing ewes, either sensitised before pregnancy to house dust mite (HDM, allergic, $n = 7$) or non-allergic (control, $n = 5$), and subjected to repeated airway challenges with HDM (allergic group) or saline (control group) throughout gestation. Maternal lung, fetal and placental phenotypes were characterised at 140 ± 1 days gestational age (term, ~ 147 days). The eosinophil influx into lungs was greater after HDM challenge in allergic ewes than after saline challenge in control ewes before mating and in late gestation. Airway resistance increased throughout pregnancy in allergic but not control ewes, consistent with increased airway smooth muscle in allergic ewes. Maternal allergic asthma decreased relative fetal weight (-12%) and altered placental phenotype to a more mature form. Expression of surfactant protein B mRNA was 48% lower in fetuses from allergic ewes than

Appendix 3 Systematic review protocol: relationship between fetal growth rate and postnatal allergy

Relationship between birth weight or fetal growth rate and postnatal allergy: a systematic review protocol

Amy L. Wooldridge¹ · Mark McMillan^{1,2} · Helen S. Marshall^{1,2} · Kathryn L. Gatford¹

¹Robinson Research Institute and School of Medicine, The University of Adelaide, Adelaide, Australia, ²Vaccinology and Immunology Research Trials Unit (VIRTU), Women's and Children's Hospital, North Adelaide, Australia

Review question/objective: The objective of this systematic review is to synthesize the best available evidence on the relationship between size at birth or fetal growth and postnatal allergy. Specifically, this review aims to assess evidence regarding relationships between absolute birth weight at term, birth weight corrected for gestational age, expressed as relative to population or customized growth data, or fetal growth measures and physician-diagnosed or parent- and self-reported postnatal clinical allergic disease (eczema/atopic dermatitis, hay fever/rhinitis, allergic asthma or anaphylaxis).

The specific review question is: what is the association between the absolute birth weight at full-term or birth weight relative to population or customized data and corrected for gestational age or direct measures of fetal growth, and physician-diagnosed or parent- and self-reported clinical allergic disease (eczema/atopic dermatitis, hay fever/rhinitis, allergic asthma or anaphylaxis)?

Keywords allergy; anaphylaxis; eczema; fetal growth; hay fever

Background

Description of the conditions of interest

Allergy is a common non-communicable disease worldwide that is estimated to affect 30-40% of the world's population¹ and is increasing in prevalence, particularly in young children.² Allergic conditions, including anaphylaxis and asthma, can become life-threatening if not well-managed. Common allergic conditions include eczema (atopic dermatitis), hay fever (allergic rhinitis), allergic asthma and anaphylaxis.

Eczema, also known as atopic dermatitis, is an inflammatory skin condition induced by sensitization and subsequent cutaneous exposure to allergen/s.¹ Hay fever, also known as allergic rhinitis, is an allergic condition resulting from IgE-mediated inflammation of the nasal mucosa and produces symptoms including: watery eyes, a runny nose and sneezing.¹ The term asthma is used to describe a complex chronic inflammatory disease syndrome affecting the airways, characterized by bronchial hyperresponsiveness and progressive airway remodeling and loss of function.³ Allergy is one of the

causes of asthma;⁴ this review addresses allergic asthma but not asthma or wheezing that are because of other causes such as exercise-induced asthma or non-specified causes. Anaphylaxis is a severe, potentially fatal, systemic allergic reaction, which is rapid in onset and occurs suddenly after contact with an allergy-causing substance.⁵

Description of the epidemiological relationship of interest

Recent evidence suggests that the risk of allergy in older life is highly influenced by exposures in early life, including prenatal growth and gestational age at delivery, mode of delivery, maternal pregnancy diet and family size.⁶ Epigenetic changes and altered microbiome development have been suggested as potential mechanisms for lasting effects of some perinatal exposures on susceptibility.⁷⁻⁹ Growth before birth and size at birth have been associated with risks of allergy in a number of human cohort studies,⁶ including in twin studies, where siblings share a common postnatal environment and genetic similarities.^{10,11} Experimental models of manipulated fetal growth have also induced altered allergy susceptibility in progeny. In rats, maternal under-nutrition throughout gestation reduced birth weight by approximately 30% and reduced antigen-specific circulating IgE and airway allergic responses to acute

Correspondence: Amy L. Wooldridge, amy.wooldridge@alumni.adelaide.edu.au

There is no conflict of interest in this project.

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Appendix 4 Pre-birth origins of allergy and asthma

Manuscript Details	
Manuscript number	JRI_2016_173
Title	Pre-birth origins of allergy and asthma
Article type	Review Article
Abstract	
<p>Allergy is a chronic disease that can develop as early as infancy, suggesting that early life factors are important in its aetiology. Variable associations between size at birth, a crude marker of the fetal environment, and allergy have been reported in humans and require comprehensive review. Associations between birth weight and allergy are however confounded in humans, and we and others have therefore begun exploring the effects of early life events on allergy in experimental models. In particular, we are using ovine models to investigate whether and how a restricted environment before birth protects against allergy, whether methyl donor availability contributes to allergic protection in IUGR, and why maternal asthma during pregnancy is associated with increased risks of allergic disease in children. We found that experimental intrauterine growth restriction (IUGR) in sheep reduced cutaneous responses to antigens in progeny, despite normal or elevated IgE responses. Furthermore, maternal methyl donor supplementation in late pregnancy partially reversed effects of experimental IUGR, consistent with the proposal that epigenetic pathways underlie some but not all effects of IUGR on allergic susceptibility. Ovine experimental allergic asthma with exacerbations reduces relative fetal size in late gestation, with some changes in immune populations in fetal thymus suggestive of increased activation. Maternal allergic asthma in mice also predisposes progeny to allergy development. In conclusion, these findings in experimental models provide direct evidence that a perturbed environment before birth alters immune system development and postnatal function, and provide opportunities to investigate underlying mechanisms and develop and evaluate interventions.</p>	
Keywords	Pregnancy; Developmental programming; Experimental models; IUGR; Folic acid
Corresponding Author	Kathryn Gatford
Order of Authors	Kathryn Gatford, Amy Wooldridge, Karen Kind, Robert Bischof, Vicki Clifton
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Appendix 5 Effect of placental restriction and neonatal exendin-4 treatment on postnatal growth, adult body composition and in vivo glucose metabolism in the sheep

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Effect of placental restriction and neonatal exendin-4 treatment on postnatal growth, adult body composition, and in vivo glucose metabolism in the sheep

Hong Liu,^{1,2} Christopher G. Schultz,⁵ Miles J. De Blasio,^{1,2} Anita M. Peura,^{1,2} Gary K. Heinemann,^{1,2} Harryanto H. Hunter,^{1,2} Damien S. Hunter,^{1,2,3} Amy L. Wooldridge,^{1,2} Karen L. Kind,^{1,3} Lynne C. Giles,⁴ Rebecca A. Simmons,⁶ Julie A. Owens,^{1,2} and Kathryn L. Gafford^{1,2}

¹Robinson Research Institute and ²School of Paediatrics and Reproductive Health, ³School of Animal and Veterinary Sciences, and ⁴School of Population Health, University of Adelaide, Adelaide, South Australia, Australia; ⁵Department of Nuclear Medicine, PET and Bone Densitometry, Royal Adelaide Hospital, Adelaide, South Australia, Australia; and ⁶University of Pennsylvania Medical School, Philadelphia, Pennsylvania

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Liu H, Schultz CG, De Blasio MJ, Peura AM, Heinemann GK, Harryanto H, Hunter DS, Wooldridge AL, Kind KL, Giles LC, Simmons RA, Owens JA, Gafford KL. Effect of placental restriction and neonatal exendin-4 treatment on postnatal growth, adult body composition, and in vivo glucose metabolism in the sheep. *Am J Physiol Endocrinol Metab* 309: E589–E600, 2015. First published July 28, 2015; doi:10.1152/ajpendo.00487.2014.—Intrauterine growth restriction (IUGR) increases the risk of adult type 2 diabetes (T2D) and obesity. Neonatal exendin-4 treatment can prevent diabetes in the IUGR rat, but whether this will be effective in a species where the pancreas is more mature at birth is unknown. Therefore, we evaluated the effects of neonatal exendin-4 administration after experimental restriction of placental and fetal growth on growth and adult metabolic outcomes in sheep. Body composition, glucose tolerance, and insulin secretion and sensitivity were assessed in singleton-born adult sheep from control (CON; $n = 6$ females and 4 males) and placentally restricted pregnancies (PR; $n = 13$ females and 7 males) and in sheep from PR pregnancies that were treated with exendin-4 as neonates (daily sc injections of 1 nmol/kg exendin-4; PR + exendin-4; $n = 11$ females and 7 males). Placental restriction reduced birth weight (by 29%) and impaired glucose tolerance in the adult but did not affect adult adiposity, insulin secretion, or insulin sensitivity. Neonatal exendin-4 suppressed growth during treatment, followed by delayed catchup growth and unchanged adult adiposity. Neonatal exendin-4 partially restored glucose tolerance in PR progeny but did not affect insulin secretion or sensitivity. Although the effects on glucose tolerance are promising, the lack of effects on adult body composition, insulin secretion, and insulin sensitivity suggest that the neonatal period may be too late to fully reprogram the metabolic consequences of IUGR in species that are more mature at birth than rodents.

intrauterine growth restriction; exendin-4; glucose tolerance; insulin action; body composition

INTRAUERINE GROWTH RESTRICTION (IUGR) in humans impairs adult insulin action in part via impaired insulin sensitivity and secretion and hence, increases the risk of type 2 diabetes (T2D) (23, 37). Poor growth before birth increases the risk of T2D, accounting for ~18% of the population lifetime risk of this disease (13). Even after correcting for gestational age, low-birth weight individuals remain at elevated risk of impaired glucose tolerance, inadequate insulin secretion, and risk of

T2D as adults (21, 24, 31, 34, 37, 38, 56). Prevention of T2D is a clinical challenge. Lifestyle interventions improve short-term glucose control (28, 55) but do not reduce long-term risk of cardiovascular morbidity or mortality (33). These poor outcomes of interventions initiated after disease onset lead us to suggest that the better approach to improve long-term outcomes in those at elevated risk of T2D would be via prevention of disease onset, including interventions that reverse the mechanisms that are initiated during IUGR and underlie the increased risk of T2D.

One intervention that may ameliorate the risk of T2D after IUGR is short-term neonatal exendin-4 administration. Exendin-4 is a long-acting analog of the hormone glucagon-like peptide 1 (GLP-1), which stimulates β -cell proliferation and function in rodents and human islets in vitro, with evidence of increased β -cell mass following short- and medium-term treatment (days to weeks) in rodents (reviewed in Ref. 29). Chronic treatment with exendin-4 and other GLP-1 receptor agonists improves insulin secretion in diabetic rats (61) and in human T2D patients, where current evidence from clinical trials suggests that GLP-1 receptor agonists increase β -cell function and may preserve existing β -cells but probably do not increase β -cell mass (reviewed in Ref. 29). Daily administration of exendin-4 for the first 6 days of neonatal life (1 nmol/kg body wt) successfully prevents development of diabetes in the IUGR rat without altering glucose metabolism in control progeny (52). Furthermore, neonatal exendin-4 treatment of IUGR rats reduced adult fasting plasma insulin concentrations, prevented oxidative stress in the liver, reversed defects in insulin signaling, and normalized hepatic insulin sensitivity (43). A well-defined mechanism by which neonatal exendin-4 normalizes insulin secretion in the IUGR rat is via increased expression of pancreatic and duodenal homeobox 1 (*Pdx1*), which is a regulator of β -cell function and mass. Neonatal exendin-4 administration reverses IUGR-induced deacetylation of histone tails in the promoter of *Pdx1*, preventing subsequent progression to DNA methylation of the *Pdx1* promoter and associated *Pdx1* gene silencing with age and normalizing β -cell mass in IUGR progeny (40). In rodents, β -cells develop late in gestation, with remodeling and functional maturation occurring ~10–17 days postnatal age, and the rat pancreas is still functionally immature at birth (7, 48). Therefore, both experimental restriction of the prenatal nutrient supply and neonatal exendin-4 treatment occur prior to pancreatic maturation in this species. Whether neonatal exendin-4 will also improve adult

Address for reprint requests and other correspondence: K. L. Gafford, Robinson Research Institute and School of Paediatrics & Reproductive Health, Univ. of Adelaide, Adelaide, SA 5005, Australia (e-mail: kathy.gafford@adelaide.edu.au).

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Appendix 6 In utero programming of allergic susceptibility

In utero Programming of Allergic Susceptibility

Jessica A. Grieger Vicki L. Clifton Astrud R. Tuck Amy L. Wooldridge
Sarah A. Robertson Kathryn L. Gatford

Robinson Research Institute and School of Medicine, University of Adelaide, Adelaide, S.A., Australia

Key Words

Allergy · Developmental programming · Diet · Epigenetics · Prenatal risk factors

Abstract

Background: Around 30–40% of the world's population will experience allergy, the most common and earliest-onset noncommunicable disease. With a steady rise in the incidence of allergic disease over recent decades, up to 18% of children will suffer a respiratory, food or skin allergy before their 18th birthday. There is compelling evidence that the risk of developing allergy is influenced by early life events and particularly in utero exposures. **Methods:** A comprehensive literature review was undertaken which outlines prenatal risk factors and potential mechanisms underlying the development of allergy in childhood. **Results:** Exposures including maternal cigarette smoking, preterm birth and Caesarean delivery are implicated in predisposing infants to the later development of allergy. In contrast, restricted growth in utero, a healthy maternal diet and a larger family size are protective, but the mechanisms here are unclear and require further investigation. **Conclusion:** To ameliorate the allergy pandemic in young children, we must define prenatal mechanisms that alter the programming of the fetal immune system and also identify specific targets for antenatal interventions.

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Introduction

Allergy is the most frequent and earliest-onset non-communicable disease, affecting 30–40% of the population worldwide [1]. A progressive rise in allergic diseases in the late 20th and early 21st centuries is evident, particularly in younger children, with a current estimate that 18% of children <18 years of age suffer from respiratory, food or skin allergy [2].

Extensive evidence implicates exposures and events during critical stages in pregnancy in altering offspring phenotype and disease predisposition in later life. This evidence forms the basis of the developmental origins of health and disease (DOHaD) hypothesis [3]. In recent years, the DOHaD hypothesis has expanded to propose that allergy susceptibility also originates prenatally. Allergic diseases frequently manifest within the first months after birth [4], the developing immune system is sensitive to a range of factors and events in utero [5] and neonatal immune responses differ between infants who later do or do not develop allergic disease [reviewed in 6, 7]. Recent studies on animals [8, 9] provide further evidence that perturbations during fetal life can change immune development and function to predispose individuals to postnatal atopy. Despite this, the nature of the relevant prenatal exposures and the pathways by which they impact fetal immune development and postnatal allergic susceptibil-

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E-Mail karger@karger.com
www.karger.com/iaa

Correspondence to: Dr. Kathryn L. Gatford
Robinson Research Institute and School of Medicine
University of Adelaide
Adelaide, SA 5005 (Australia)
E-Mail Kathryn.gatford@adelaide.edu.au

Appendix 7 Placental restriction in multi-fetal pregnancies increases spontaneous ambulatory activity during daylight hours in young adult female sheep

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ORIGINAL ARTICLE

Placental restriction in multi-fetal pregnancies increases spontaneous ambulatory activity during daylight hours in young adult female sheep

M. Kaur^{1,2}, A. L. Wooldridge^{1,2}, M. J. Wilkes³, W. S. Pitchford³, P. I. Hynd³, G. K. McConell⁴ and K. L. Gatford^{1,2*}

¹Robinson Research Institute, The University of Adelaide, SA, Australia

²Discipline of Obstetrics & Gynaecology, School of Medicine, The University of Adelaide, SA, Australia

³School of Animal and Veterinary Sciences, The University of Adelaide, SA, Australia

⁴Institute of Sport, Exercise and Active Living, Victoria University, Melbourne, VIC, Australia

Intrauterine growth restriction (IUGR) has adverse effects on metabolic health and early life, whereas physical activity is protective against later development of metabolic disease. Relationships between birth weight and physical activity in humans, and effects of IUGR on voluntary activity in rodents, are mixed and few studies have measured physical activity in a free-ranging environment. We hypothesized that induced restriction of placental growth and function (PR) in sheep would decrease spontaneous ambulatory activity (SAA) in free-ranging adolescent and young adult progeny from multi-fetal pregnancies. To test this hypothesis, we used Global Positioning System watches to continuously record SAA between 1800 and 1200 h the following day, twice during a 16-day recording period, in progeny of control (CON, $n = 5$ males, 9 females) and PR pregnancies ($n = 9$ males, 10 females) as adolescents (30 weeks) and as young adults (43 weeks). PR reduced size at birth overall, but not in survivors included in SAA studies. In adolescents, SAA did not differ between treatments and females were more active than males overall and during the day (each $P < 0.001$). In adults, daytime SAA was greater in PR than CON females ($P = 0.020$), with a similar trend in males ($P = 0.053$) and was greater in females than males ($P = 0.016$). Adult SAA was negatively correlated with birth weight in females only. Contrary to our hypothesis, restricted placental function and small size at birth did not reduce progeny SAA. The mechanisms for increased daytime SAA in adult female PR and low birth weight sheep require further investigation.

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Key words: behavior, physical activity, placental insufficiency, sex differences, sheep

Introduction

Intrauterine growth restriction (IUGR) arises from maternal, fetal and/or placental factors that prevent the fetus from achieving its genetic potential for growth.^{1,2} In developed countries IUGR, which is most commonly caused by placental insufficiency, affects 6–12% of births.^{3,4} Placental insufficiency progressively restricts transfer of nutrients and oxygen to the developing fetus, reducing growth particularly in late gestation.^{5,6} In human studies, low birth weight or small size at birth for gestational age (SGA) are often used as surrogate markers of IUGR.⁷ There is conflicting evidence from human cohorts that voluntary levels and intensity of physical activity are altered in low birth weight compared with normal birth weight adolescents and adults.^{8–12} In human cohorts, increased physical activity throughout life, and in childhood or adolescence is associated with decreased risk of developing metabolic disease in adult life.^{13–16} Decreased physical activity after IUGR may, therefore, contribute to the increased risk of metabolic disease in this population.^{17–19}

A meta-analysis categorizing adolescents and adults as active or inactive by self-report showed an inverse U-shaped relationship between birth weight and levels of leisure time physical activity (LTPA).⁸ Other studies confirm these findings with low and high birth weight individuals self-reporting lower levels of LTPA compared with those born of average birth weight.^{8,10,11} In contrast, LTPA measured objectively through accelerometer data during adolescence was either not related¹² or positively related⁹ to birth weight. Similarly, reduced birth weight due to maternal famine exposure in mid- or late-gestation did not significantly alter self-reported physical activity.²⁰ Variable gender differences in physical activity have also been reported in humans, with LTPA either not differing between genders in adolescents and adults^{8,12} or females being more sedentary than males in adolescence.⁹ In addition to the variable effects of birth weight and sex, it is difficult to infer causality from human studies due to confounding by environmental factors that affect growth before birth and activity during postnatal life. For example, the risk of an SGA birth increases with lower socio-economic status^{21,22} and socio-economic status is positively correlated with levels of physical activity in both adolescents and adults.^{23,24} Animal models of IUGR where progeny are delivered at term may be useful in separating out these effects of prenatal and postnatal

*Address for correspondence: Dr K. L. Gatford, Discipline of Obstetrics & Gynaecology, School of Medicine, University of Adelaide, SA 5005, Australia. (Email Kathy.gatford@adelaide.edu.au)

Appendix 8 A review of fundamental principles for animal models of DOHaD research: an Australian perspective



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REVIEW

A review of fundamental principles for animal models of DOHaD research: an Australian perspective

H. Dickinson^{1*}, T. J. Moss¹, K. L. Gatford², K. M. Moritz³, L. Akison³, T. Fullston², D. H. Hryciw⁴, C. A. Maloney⁵, M. J. Morris⁵, A. L. Wooldridge², J. E. Schjenken², S. A. Robertson², B. J. Waddell⁶, P. J. Mark⁶, C. S. Wyrwoll⁶, S. J. Ellery¹, K. L. Thornburg⁷, B. S. Muhlhauser⁸ and J. L. Morrison⁹, for the ANZ DOHaD Animals Models of DOHaD Research Working Group

¹ *The Ritchie Centre, Hudson Institute of Medical Research, Monash University, Clayton, VIC, Australia*
² *School of Medicine and Robinson Research Institute, University of Adelaide, Adelaide, SA, Australia*
³ *School of Biomedical Sciences, The University of Queensland, St Lucia, QLD, Australia*
⁴ *Department of Physiology, University of Melbourne, Parkville, VIC, Australia*
⁵ *School of Medical Sciences, UNSW Australia, Sydney, NSW, Australia*
⁶ *Faculty of Science, The University of Western Australia, Crawley, WA, Australia*
⁷ *Heart Research Center, Oregon Health & Science University, Portland, OR, USA*
⁸ *School of Agriculture, Food and Wine, FODplus Research Centre, The University of Adelaide, Adelaide, SA, Australia*
⁹ *School of Pharmacy and Medical Sciences, Sanson Institute for Health Research, University of South Australia, Adelaide, SA, Australia*

Epidemiology formed the basis of 'the Barker hypothesis', the concept of 'developmental programming' and today's discipline of the Developmental Origins of Health and Disease (DOHaD). Animal experimentation provided proof of the underlying concepts, and continues to generate knowledge of underlying mechanisms. Interventions in humans, based on DOHaD principles, will be informed by experiments in animals. As knowledge in this discipline has accumulated, from studies of humans and other animals, the complexity of interactions between genome, environment and epigenetics, has been revealed. The vast nature of programming stimuli and breadth of effects is becoming known. As a result of our accumulating knowledge we now appreciate the impact of many variables that contribute to programmed outcomes. To guide further animal research in this field, the Australia and New Zealand DOHaD society (ANZ DOHaD) Animals Models of DOHaD Research Working Group convened at the 2nd Annual ANZ DOHaD Congress in Melbourne, Australia in April 2015. This review summarizes the contributions of animal research to the understanding of DOHaD, and makes recommendations for the design and conduct of animal experiments to maximize relevance, reproducibility and translation of knowledge into improving health and well-being.

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Key words: developmental stage, outcome/system, developmental origins of health and disease, programming

From epidemiology to animal models to clinical intervention

To guide further animal research in the field of Developmental Origins of Health and Disease (DOHaD), the Australia and New Zealand DOHaD Society (ANZ DOHaD) Animals Models of DOHaD Research Working Group convened at the 2nd Annual ANZ DOHaD Congress in Melbourne, Australia in April 2015.¹ This review summarizes the contributions of animal research to the understanding of DOHaD, and makes recommendations for the design and conduct of animal experiments to maximize relevance, reproducibility and translation of knowledge into improving health and well-being.

The seminal epidemiological studies of leaders in the field of DOHaD in the 1980s and 1990s illustrated the link between early life and adult health and gave birth to a new scientific discipline. Epidemiological studies have taught us a great deal about DOHaD but they cannot explain mechanisms underlying the associations they observe. Hence, the development of animal models has been critical to our understanding of how early developmental events impact lifelong health.

The most valuable DOHaD animal models mimic complications of human pregnancies, cause similar changes in early life growth and development, and cause lasting alterations in phenotypes in the offspring. With these models, researchers have studied changes in specific organs at the physiological, structural and molecular level to discover likely mechanisms. With characterization of these underlying processes, it has been possible for scientists to identify potential interventions to prevent or ameliorate the negative impacts of early life events. Although it is tempting to test such interventions in a human population, without knowing the long-term effects of the intervention we may actually be causing harm. Thus, carefully designed studies in the same animal models are required to

*Address for correspondence: H. Dickinson, The Ritchie Centre, Hudson Institute of Medical Research, Monash University, 246 Clayton Rd, Clayton, VIC 3168, Australia.
(Email Hayley.dickinson@hudson.org.au)

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Appendix 9 Placental restriction in multi-fetal pregnancies and between-twin differences in size at birth alter neonatal feeding behaviour in the sheep

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ORIGINAL ARTICLE

Placental restriction in multi-fetal pregnancies and between-twin differences in size at birth alter neonatal feeding behaviour in the sheep

R. F. Peter¹, J. Gugusheff¹, A. L. Wooldridge^{2,3}, K. L. Gatford^{2,3,†} and B. S. Muhlhausler^{1,*†}

¹Department of Food and Wine Science, School of Agriculture, Food and Wine, FOODplus Research Centre, The University of Adelaide, Adelaide, SA, Australia

²Discipline of Obstetrics and Gynaecology, Adelaide Medical School, Adelaide, SA, Australia

³Robinson Research Institute, The University of Adelaide, Adelaide, SA, Australia

Most individuals whose growth was restricted before birth undergo accelerated or catch-up neonatal growth. This is an independent risk factor for later metabolic disease, but the underlying mechanisms are poorly understood. This study aimed to test the hypothesis that natural and experimentally induced *in utero* growth restriction increase neonatal appetite and milk intake. Control (CON) and placentally restricted (PR) ewes carrying multiple fetuses delivered naturally at term. Outcomes were compared between CON ($n = 14$) and PR ($n = 12$) progeny and within twin lamb pairs. Lamb milk intake and feeding behaviour and ewe milk composition were determined using a modified weigh-suckle-weigh procedure on days 15 and 23. PR lambs tended to have lower birth weights than CON (-15% , $P = 0.052$). Neonatal growth rates were similar in CON and PR, whilst heavier twins grew faster in absolute but not fractional terms than their co-twins. At day 23, milk protein content was higher in PR than CON ewes ($P = 0.038$). At day 15, PR lambs had fewer suckling bouts than CON lambs and in females light twins had more suckling attempts than their heavier co-twins. Birth weight differences between twins positively predicted differences in milk intakes. Lactational constraint and natural prenatal growth restriction in twins may explain the similar milk intakes in CON and PR. Within twin comparisons support the hypothesis that prenatal constraint increases lamb appetite, although this did not increase milk intake. We suggest that future mechanistic studies of catch-up growth be performed in singletons and be powered to assess effects in each sex.

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Key words: catch-up growth, fetal growth restriction, milk composition, milk intake, newborn

Introduction

Intrauterine growth restriction (IUGR), defined as a failure of the fetus to achieve its genetic growth potential,¹ affects 6–12% of births globally.² In developed countries, the major cause of IUGR is placental insufficiency, which limits the supply of nutrients and oxygen to the developing fetus and thus restricts fetal growth.³ The majority of IUGR individuals undergo a period of accelerated or catch-up growth in the early postnatal period, and ‘catch-up’ in body weight to normally grown infants by 6 months to 2 years of age.^{4,5} Markers of IUGR, such as small size at birth for gestational age (SGA), and accelerated neonatal growth are each independent predictors of poor cardiometabolic health outcomes, including diabetes, coronary heart disease, hypertension and obesity, in postnatal life.^{6–9}

While the importance of catch-up growth for determining future metabolic health outcomes of the child is well established, the mechanisms which underlie catch-up growth remain poorly understood. There are suggestions, however, that increased appetite and food intake in the early neonatal period

may play an important role. In a small clinical study, SGA infants consumed a greater volume of breast milk relative to their body weight in the early neonatal period, than infants who were of average or above average birth weight.¹⁰ Studies in rodents and sheep have also provided indirect evidence of higher milk consumption during the neonatal period in growth-restricted offspring in comparison with their normally grown counterparts, based on more rapid neonatal growth rates and higher milk intakes post-weaning.^{11,12} Studies in small ruminants (goats) by Laporte-Broux *et al.* also reported that daily feed intake between birth and 2 months *post-partum* of kids artificially reared on milk was higher in female kids born to goats who were feed-restricted in late pregnancy compared with progeny of *ad libitum*-fed mothers.¹³ Interestingly, however, they saw no effect of maternal feed restriction on suckling behaviour or milk intake in male kids reared by their mothers.¹⁴ In our established sheep model of placental and fetal growth restriction,^{15–17} we have previously demonstrated that during their active period of catch-up growth, 15-day-old lambs from placentally restricted (PR) ewes suckle for longer in the acute phase of weigh-suckle-weigh (WSW) tests than control (CON) lambs.¹⁸ However, milk intake was not quantified in this previous study.

In addition to the volume consumed, milk composition is also an important determinant of offspring growth rate and

*Address for correspondence: B. S. Muhlhausler, FOODplus Research Centre, The University of Adelaide, Adelaide, South Australia 5064, Australia. (Email beverly.muhlhausler@adelaide.edu.au)

[†]Joint senior authors.