

Maternal allergic asthma during pregnancy alters fetal lung and immune development in sheep: potential mechanisms for programming asthma and allergy.

Amy L Wooldridge¹, Vicki L Clifton^{1, 2}, Timothy JM Moss^{3, 4}, Hui Lu³, Monerih Jamali⁶, Stefanie Agostino⁶, Beverly S Muhlhausler⁷, Janna L Morrison⁸, Robert De Matteo⁵, Megan J Wallace^{3, 4}, Robert J Bischof^{3, 6}, Kathryn L Gatford¹

Biography Dr Amy Wooldridge

Amy Wooldridge received her Bachelor's Degree in Animal Science at the University of Adelaide, where she then completed her Honours and doctoral training in developmental programming of allergic susceptibility. Thereafter, Amy moved to Perth to receive postdoctoral training on inflammatory conditions of preterm infants. She is now continuing her postdoctoral training at the University of Alberta in Canada, studying the effects of advanced maternal age on vascular adaptations to pregnancy.

¹ Robinson Research Institute and Adelaide Medical School, University of Adelaide, Adelaide SA 5005, Australia

²Mater Medical Research Institute, University of Queensland, Brisbane QLD 4101, Australia

³The Ritchie Centre, Hudson Institute of Medical Research, Clayton VIC 3168, Australia

⁴Department of Obstetrics and Gynaecology, Monash University, Clayton VIC 3168, Australia

⁵Department of Anatomy and Developmental Biology, Monash University, Clayton VIC 3800, Australia

⁶Department of Physiology, Monash University, Clayton VIC 3800, Australia

⁷Food and Nutrition Research Centre, Department of Food and Wine Sciences, 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, University of South Australia, Adelaide SA 5001, Australia.

Short title: Developmental programming by maternal asthma

Corresponding author:

This is an Accepted Article that has been peer-reviewed and approved for publication in the The Journal of Physiology, but has yet to undergo copy-editing and proof correction. Please cite this article as an 'Accepted Article'; [doi: 10.1113/JP277952](https://doi.org/10.1113/JP277952).

This article is protected by copyright. All rights reserved.

Dr. Kathryn L Gatford

Robinson Research Institute

University of Adelaide SA 5005 Australia

Phone: +61 8 8313 4158

Email: Kathy.gatford@adelaide.edu.au

Key points

- Experimental maternal allergic asthma in sheep provides an experimental model in which to test impacts on progeny
- Fetuses from allergic asthmatic ewes had fewer surfactant-producing cells in lungs
- A greater proportion of lymphocytes from thymus were CD44 positive in fetuses from allergic asthmatic ewes than controls
- These changes to fetal development might contribute to poor neonatal lung function and increased risk of allergy seen in offspring of pregnancies complicated by asthma

Abstract

Asthma is prevalent in pregnancy and increases the risk of disease in offspring, including neonatal respiratory distress and childhood asthma and allergy, but the mechanisms are not understood. We hypothesized that fetal lung structure and immune phenotype in late gestation fetal sheep would be impaired in our sheep model of maternal allergic asthma during pregnancy. Singleton-bearing ewes were either sensitized before pregnancy to house dust mite (HDM, allergic, n = 7) or non-allergic (control, n = 5), and subsequently subjected to repeated airway challenges with HDM (allergic group) or saline (control group) throughout gestation. Tissues were collected at 140 ± 1 days gestational age (term, ~147 days). The density of type II alveolar epithelial cells (surfactant protein C-immunostained) in the lungs was 30% lower in fetuses from allergic ewes than controls (P

< 0.001), but tissue-to-airspace ratio and numbers of leukocytes and macrophages were not different between groups. The proportion of CD44⁺ lymphocytes in the fetal thymus was 3.5-fold higher in fetuses from allergic ewes than control ewes (P = 0.043). Fewer surfactant-producing type II alveolar epithelial cells may contribute to the increased risk of neonatal respiratory distress in infants of asthmatic mothers, suggesting that interventions to promote lung maturation could improve their neonatal outcomes. If the elevated lymphocyte expression of CD44 persists postnatally, this would confer greater susceptibility to allergic diseases in progeny of asthmatic mothers, consistent with observations in humans. Further experiments are needed to evaluate postnatal phenotypes of progeny and investigate potential interventions.

Keywords: asthma, pregnancy, sheep, fetus, lung, immune

Introduction

Asthma is a chronic inflammatory disease of the airways, characterized by reversible airflow limitation or bronchial hyper-responsiveness, resulting in respiratory symptoms such as wheezing, shortness of breath and coughing (Bousquet *et al.*, 2010). The aetiology of asthma is variable, but allergic sensitisation is considered to be the most common factor triggering asthma development (Bousquet *et al.*, 2010). Asthma is common in pregnant women, affecting ~12% of pregnancies in Australia (Clifton *et al.*, 2009). Up to half of asthmatic women experience worsening of the disease during pregnancy (Schatz *et al.*, 1988; Murphy, 2015; Grzeskowiak *et al.*, 2016). As many as 20% of pregnant women with asthma require medical intervention for treatment of exacerbations, and approximately 6% require hospitalisation (Murphy *et al.*, 2006).

In addition to the impact on maternal health, maternal asthma increases risks of adverse pregnancy outcomes (Murphy *et al.*, 2006; Wang *et al.*, 2014), newborn respiratory problems (Murphy *et al.*, 2013), and allergy and asthma in childhood (Grieger *et al.*, 2016). In a large

retrospective study of >220,000 singleton births in the USA, including >17,000 mothers with asthma, the adjusted odds for neonatal hospitalisation and acute neonatal respiratory distress were increased by 10% and 9%, respectively, in infants of asthmatic mothers (Mendola *et al.*, 2014). Interestingly, maternal asthma increased the adjusted odds for acute neonatal respiratory distress to a greater extent (22%) in an analysis restricted to term-born infants within this cohort (Mendola *et al.*, 2014). It therefore appears that the effects of asthma on neonatal lung disease primarily reflect impaired fetal lung development, rather than prematurity. A recent meta-analysis suggests even stronger adverse effects of maternal asthma on the fetus, including a 49% increased risk of neonatal death and 50% increased risk of neonatal hospitalisation (Murphy *et al.*, 2013). The adverse effects of maternal asthma continue into childhood. Children of asthmatic mothers have a >3-fold increased risk of asthma compared to children of non-asthmatic mothers (Lim *et al.*, 2010). Similarly, children whose mothers had acute atopic symptoms during pregnancy have more than double the normal risk of food sensitisation and atopic dermatitis by 2 years of age, and of allergic rhinitis and inhalant sensitisation at 4-5 years (Illi *et al.*, 2014).

Increased risk of asthma and allergy in children of asthmatic and atopic mothers cannot be explained fully by genetics, since maternal asthma confers a greater risk than paternal asthma (Lim *et al.*, 2010). Furthermore, even within a population of mothers with a history of atopy (i.e. with genetic susceptibility), the risk of atopic dermatitis in infants was still increased if the mother had atopic symptoms during pregnancy. Experimental evidence further supports the concept that allergic susceptibility is programmed by exposure to maternal atopy, since maternal allergic asthma in mice sensitized to ovalbumin (OVA) increased airway inflammation and eosinophilia in offspring (Hamada *et al.*, 2003). Risks of pregnancy complications affecting the mother or newborn complications are predominantly associated with asthma exacerbations (Murphy *et al.*, 2005), and do not appear to be increased by maternal asthma that is well-controlled (Schatz *et al.*, 1995). Improved

maternal asthma control, which more than halved the incidence of exacerbations, reduced risks of neonatal hospitalisation and doctor-diagnosed asthma in children of asthmatic women (Powell *et al.*, 2011; Morten *et al.*, 2018).

The mechanisms by which maternal asthma increases the risks of lung disease and susceptibility to asthma in progeny are largely unknown. Understanding these mechanisms is critical for development of interventions to improve neonatal and lifelong health of children whose mothers do not achieve control of their asthma during pregnancy. To investigate mechanisms for effects of maternal asthma on the fetus and offspring, and to evaluate interventions, we established a sheep model of maternal allergic asthma induced by allergen sensitisation before mating and repeated airway challenges during pregnancy, which decreases fetal growth and alters fetal lung development (Clifton *et al.*, 2015). This protocol generates an asthmatic phenotype in pregnant ewes (Clifton *et al.*, 2015), as it does in non-pregnant sheep (Bischof *et al.*, 2003; Bischof *et al.*, 2008), including increases in systemic allergen-specific IgE levels, circulating and airway (BAL) eosinophils, and airway smooth muscle accumulation around the airways, together with decreased lung function. Similar immune responses and structural changes are typical of the human asthmatic lung (James *et al.*, 2012). We have reported previously that mRNA expression of surfactant protein B is decreased in lungs of fetuses from allergic compared to control ewes, with similar trends for other surfactant proteins (Clifton *et al.*, 2015), but the underlying mechanisms are unknown. In the current study, we used this established ovine model to determine the effects of maternal allergic asthma on lung and immune cell phenotypes in late gestation fetuses, two systems likely to impact risks of neonatal lung disease, and development of allergy and asthma in progeny.

Materials and Methods

Ethical approval

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), were conducted in accordance with Australian guidelines, and conform to the principles and regulations as described in the Editorial by Grundy (Grundy, 2015).

Animals and experimental design

The studies described in this paper were performed in samples collected in a cohort of allergic and control ewes described previously (Clifton *et al.*, 2015). Detailed information on the numbers of animals at each stage of the experiment, protocols for allergen sensitisation and airway challenges, and of collection of samples in these sheep have been described previously (Clifton *et al.*, 2015). Briefly, ewes were first sensitized to house dust mite (HDM) allergen (Bischof *et al.*, 2003; Wooldridge *et al.*, 2014), then exposed to repeated airway challenges with HDM, weekly for 8 weeks prior to mating, and fortnightly throughout gestation, with the final challenge conducted at a gestational age of 123 ± 1 days. (Clifton *et al.*, 2015). From an initial cohort of 31 ewes that underwent allergic sensitisation and 9 non-sensitised ewes, we obtained 17 allergic ewes and 10 control ewes that were mated, resulting in 9 control and 11 allergic ewes carrying singleton pregnancies (Clifton *et al.*, 2015). At 140 ± 1 days of gestation (range 138-144 days: term is 147 days), control (n=5) and asthmatic (n=7) ewes and their singleton fetuses that completed the study were humanely killed by i.v. administration of an overdose of sodium thiopentone (Thiobarb, Jurox Pty Ltd, Rutherford, NSW, Australia), immediately following collection of maternal blood. The uterus was removed by hysterectomy, amniotic fluid was sampled, the fetus was removed and weighed and a sample of fetal venous blood was collected by venepuncture of the fetal jugular vein. Maternal and fetal plasma samples, as well as amniotic fluid samples, were stored at -20°C for later analyses of antibody and cytokine levels. Tissues (including lung,

spleen, intra- and extrathoracic thymus) were collected from two male and three female control fetuses, and three male and four female fetuses from allergic ewes. Fetal tissues were dissected, weighed and processed as described below.

Antibody, cortisol and cytokine analyses

HDM-specific total immunoglobulin (Ig), IgG₁, IgE, IgA and IgM levels were determined in duplicate samples by enzyme-linked immunosorbent assay (ELISA) as described previously (Bischof *et al.*, 2003; Bischof *et al.*, 2008; Wooldridge *et al.*, 2014). Plasma cortisol concentrations in maternal and fetal plasma were measured using a commercially available ELISA kit (OB-EA65, Oxford Biomedical Research, Rochester Hills, USA), according to the manufacturer's guidelines. The standard curve for this assay ranges from 0.005 to 50 ng/ml (= 0.5 to 5000 ng/mL in plasma before extraction). Amniotic fluid cytokines, consisting of interleukin (IL)-6, IL-10, IL-13, and tumor necrosis factor (TNF)- α were also measured by ELISA (Liravi *et al.*, 2015; Melville *et al.*, 2017). The limits of detection for the cytokine ELISAs were 1.1 ng/ml (IL-6), 0.5 BU/ml (IL-10), 2.4 ng/ml (IL-13) and 1.6 ng/ml (TNF- α).

Fetal lung morphology

Fetal lung tissue was sampled from the right cranial lung lobe, which was fixed by instillation of neutral buffered formalin at 20 cm H₂O, paraffin-embedded and sectioned (5 μ m) for structural and immunohistochemical analyses. Tissue sections were stained with hematoxylin and eosin, and the ratio of tissue to air space volumes for each lung lobe determined by point-counting in a total of 15 randomly-selected high-power fields sampled from two lung sections (Westover *et al.*, 2012). All lung structural and immunohistochemical analyses were conducted blinded and 15-20 randomly-selected fields from two sections were counted for each outcome. Lung sections were immunostained to identify leukocytes

(anti-ovine CD45, MCA2220GA, AbD Serotec), as previously described (Westover *et al.*, 2012).

To identify type II alveolar epithelial cells (SP-C⁺ cells), paraffin-embedded 5- μ m sections were deparaffinised, rehydrated, and washed in phosphate-buffered saline (PBS) solution. Endogenous peroxidase activity was blocked by incubating sections in 3% H₂O₂ for 15 min before non-specific binding was blocked by incubation in PBS with 10% normal goat serum and 2% bovine serum albumin for 30 min. The sections were incubated with rabbit anti proSP-C primary antibody (Merck Millipore AB3786, 1:1000 diluted in Dako antibody diluent) overnight at 4°C. Sections were washed in PBS solution containing 0.1% Tween-20 and then incubated with biotinylated goat anti-rabbit IgG secondary antibody (VECTOR[®], BA-1000, 1:200 dilution, Vector Laboratories, UK) for 60 min at room temperature. After 3 washes in PBS/0.1%Tween-20, the sections were incubated with ABC (VECTOR, PK-6100, 1:200 dilution) for 45 min. Sections were washed 3 times in PBS and then stained by 3,3 diaminobenzidine tetrahydrochloride to detect antibody binding. All sections were counterstained with hematoxylin.

To identify macrophages (CD163⁺ cells), paraffin-embedded 5- μ m sections were deparaffinised, rehydrated, and washed in Tris-buffered saline (TBS) solution. Antigen retrieval was performed by immersing slides in 0.01M sodium citrate buffer (pH 6) and heating in a microwave for 20 min. Endogenous peroxidase activity was blocked by incubating sections in 3% H₂O₂ for 20 min before non-specific binding was blocked by incubation in TBS with 10% normal goat serum and 2% bovine serum albumin for 30 min. The sections were incubated with mouse ant-human CD163 primary antibody (Acris, SM2160P, 1:2000 diluted in Dako antibody diluent) overnight at 4°C. Primary antibody was omitted from negative control sections. Sections were washed in TBS solution containing 0.1% Tween-20 and then incubated with biotinylated goat anti-mouse IgG secondary

antibody (VECTOR, BA-9200, 1:200 dilution) for 60 min at room temperature. After 3 washes in TBS/0.1%Tween-20, the sections were incubated with ABC (VECTOR, PK-6100, 1:200 dilution) for 45 min. Sections were washed in TBS and then stained by 3,3 diaminobenzidine tetrahydrochloride to detect antibody binding. All sections were counterstained with hematoxylin.

Fetal immune phenotype

The fetal spleen, extra-thoracic (neck) thymus and intra-thoracic (chest) thymus were collected and weighed, and samples of fetal spleen and chest thymus were then collected into cold Hank's buffered saline solution (HBSS 1470 1x, GIBCO) for cell staining and analysis by flow cytometry. To isolate cells, spleen and thymus samples were cut into small pieces and cells passed through a 70-µm filter cup (BD Biosciences, CA, USA) with several 5 mL-washes of ice-cold HBSS. Tris-buffered ammonium chloride (0.17 µM Tris, 0.16 µM ammonium chloride, pH 7.2) was added to spleen cell isolates to lyse and facilitate removal of red blood cells (Bischof *et al.*, 2003). Cell samples were assessed by light microscopy to determine cell viability and cell number (trypan blue), then stained with conjugated monoclonal antibodies (mAb) for CD4 (AF647-conjugated anti-ovine CD4 mAb, in-house, and FITC-conjugated anti-ovine CD4 mAb, AbD Serotec), CD5 (FITC-conjugated anti-ovine CD5 mAb, AbD Serotec), CD8 (PE-conjugated anti-ovine CD8 mAb, AbD Serotec), CD14 (AF647-conjugated anti-human CD14 mAb, AbD Serotec), CD25 (FITC-conjugated anti-ovine CD25 mAb, AbD Serotec), CD44 (FITC-conjugated anti-ovine CD44 mAb, AbD Serotec), major histocompatibility complex (MHC) class I (FITC-conjugated anti-ovine MHC I mAb, AbD Serotec) or MHC class II (FITC-conjugated anti-ovine MHC II mAb, AbD Serotec) for flow cytometry analysis, as detailed elsewhere (Bischof *et al.*, 2003; Melville *et al.*, 2017). Each preparation was labelled with a single antibody, except for 3-colour flow cytometry, where cells were labelled concurrently using mAbs for CD4, CD5 and CD8. Lymphocytes were gated on forward-scatter (FSC) and side-scatter (SSC) characteristics (Bischof *et al.*,

2003), and 10,000 events acquired on a BD LSR II flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Data were analysed using FlowJo™ software (TreeStar, San Carlos, CA, USA). Fluorescence-activated cell sorting (FACS) analyses was completed for a minimum of 4 fetuses per treatment for each antibody and tissue combination.

Statistical Analysis

Normally-distributed outcomes in fetuses from control and allergic sheep were compared by one-way ANOVA, and other outcomes were compared by Mann-Whitney U-test, using SPSS 24.0 for Windows (IBM, Armonk, USA). Data are presented as mean $\pm$ SEM for normally-distributed data and as median (inter-quartile range) for non-normally-distributed data unless otherwise stated. A P-value <0.05 was accepted as significant.

Results

Fetal and maternal plasma cortisol and antibodies and amniotic fluid cytokines

Maternal plasma cortisol concentrations were decreased in pregnancies complicated by allergic asthma, but fetal plasma cortisol did not differ between control and allergic groups (Table 1). Amniotic fluid concentrations of IL-6, IL-10, IL-13 and TNF- α did not differ between control and allergic groups (Table 1). TNF- α was only detectable in three (2 control, 1 allergic) samples. Maternal and fetal plasma concentrations of HDM-specific total Ig, IgE, IgA and IgM did not differ between control and allergic groups (Table 1). HDM-specific IgG₁ concentrations were at background levels in maternal and fetal plasma samples and are not reported. Levels of HDM-specific total IgG and IgE in fetal plasma samples were similarly at background levels (Table 1).

Fetal weight and lung morphology

As previously reported (Clifton *et al.*, 2015), absolute body weights were similar in fetuses from control and allergic ewes (Control: 4.11 ± 0.28 kg; Allergic: 3.69 ± 0.14 kg), and fetal weight relative to maternal weight was 12% lower in the allergic group (Control: 0.106 ± 0.005 ; Allergic: 0.093 ± 0.003 kg, $P = 0.038$). Weights of fetal lungs in absolute terms Absolute (Control: 137 ± 12 g; Allergic: 137 ± 7 g) and relative to fetal body weight (Control: $3.22 \pm 0.14\%$; Allergic: $3.72 \pm 0.20\%$) were similar in fetuses from control and allergic ewes. The ratio of lung tissue to airspace was similar in fetuses from control and allergic ewes (Fig 1A-C). The density of SP-C⁺ cells was 30% lower in lung tissue from fetuses of allergic ewes, compared to control fetuses ($P < 0.001$, Fig 1D-F). The densities of leukocytes (CD45⁺; Fig 1G-I) and macrophages (CD163⁺; Fig 1J-L) in lungs from fetuses of allergic and control ewes were similar.

Fetal immune phenotype

Absolute (Control: 5.93 ± 0.78 g; Allergic: 6.07 ± 0.48 g) and relative (Control: $0.144 \pm 0.016\%$; Allergic: $0.164 \pm 0.012\%$) spleen weights were similar in fetuses from control and allergic ewes. Total thymus weight was similar in fetuses of allergic and control ewes in absolute terms (control 19.9 ± 1.2 g; allergic 19.2 ± 1.2 g) and relative to body weight (control 0.47 ± 0.04 %; allergic 0.51 ± 0.02 %). Absolute and relative separate weights of intra-and extra-thoracic thymus were also similar in fetuses of allergic and control ewes (data not shown). The percentage of lymphocytes positive for CD44 was greater in the thymus of fetuses from allergic compared to control ewes (Fig 2, $P = 0.032$). The percentage of lymphocytes positive for CD44 in cell isolates from spleen did not differ between treatments (Fig 2). The percentages of lymphocytes in cell isolates from either thymus or spleen that were CD5⁺, CD14⁺, CD25⁺, MHC1⁺ or MHCII⁺ did not differ between treatments (data not shown). The percentages of lymphocytes from thymus or spleen that were CD4⁺ CD8⁺, CD4⁺

CD8⁺, CD4⁺ CD8⁺, or CD4⁺ CD8⁺ did not differ with treatment overall, or within CD5⁺ and CD5⁺ sub-populations (data not shown).

Discussion

Maternal allergic asthma in sheep induced changes to fetal lung and immune development that might be expected to increase risks of neonatal lung disease and program an allergic phenotype in progeny. We saw lower densities of type II alveolar epithelial cells in lung of fetuses from allergic compared to control ewes, which is likely to explain the lower expression of the surfactant protein genes previously reported for these animals (Clifton *et al.*, 2015). We also observed an increased proportion of thymic lymphocytes positive for the cell adhesion molecule, 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.

Our results indicate that effects of maternal allergic asthma on the fetal lung and immune phenotypes in sheep are likely to be due to exposure to an inflammatory *in utero* environment and are not due to fetal exposure to the allergen itself, since HDM-specific antibodies were not observed in the fetuses. Despite elevated circulating levels of HDM-specific IgE post-sensitisation (Clifton *et al.*, 2015), used as the basis for selection of allergic ewes before airway challenges and mating, we did not see higher HDM-specific IgE concentrations in ewes in late pregnancy. This was probably because this final maternal plasma sample was collected several weeks after the last HDM challenge, when IgE concentrations will be declining, in combination with inter-animal variation and limited animal numbers in this final stage of the study. Importantly, the lack of HDM-specific antibodies in fetal plasma in the present study confirms previous reports that the ovine placenta provides a complete barrier to immunoglobulin transfer from mother to fetus, unless the placenta is

significantly damaged (Poitras *et al.*, 1986). The absence of HDM-specific IgE in fetal plasma of allergic ewes further suggests that the HDM antigen itself did not cross the placenta, since fetal sheep can mount antibody responses to foreign allergens from mid-gestation (Fahey & Morris, 1978). The complete placental barrier to antibody transport in sheep contrasts with humans, rats and mice, where antibodies are transferred across the placenta (Kane & Acquah, 2009). Allergens including Der p 1, the main allergenic component of HDM, also cross the human placenta (Macchiaverni *et al.*, 2015) and could therefore potentially contribute to development of fetal allergy. The available evidence suggests that increased progeny susceptibility to allergy is induced by a maternal inflammatory state, rather than fetal exposure to allergen/s or allergen-specific antibodies. Increased allergic susceptibility in the offspring of sensitized mouse dams is not specific for the allergens to which the pregnant mother was sensitized (Hamada *et al.*, 2003) suggesting that fetal immune responses are induced by the maternal allergic response itself, even in this species where antibodies and/or antigens can cross the placenta. Species differences in placental antibody and antigen transfer is therefore not likely to affect utility of animal models to investigate developmental programming of allergy. We also did not see evidence of fetal inflammatory responses at late gestation, as amniotic cytokine concentrations and fetal plasma cortisol were similar in control and allergic pregnancies.

Maternal allergic asthma decreased numbers of surfactant-producing type II alveolar epithelial cells in the near-term fetal lung, although lungs were of normal weight and gross structure (Clifton *et al.*, 2015). The decreased density of type II alveolar epithelial cells is consistent with lower lung expression of the gene for surfactant protein B and similar trends for reduced levels of other surfactant protein genes (although they did not reach statistical significance, Clifton *et al.*, 2015). These results suggest that uncontrolled maternal allergic asthma delays maturation of the fetal pulmonary surfactant system. These observations in fetal sheep are consistent with increased risk of neonatal respiratory distress in human

infants from asthmatic mothers (Mendola *et al.*, 2014). Interventions to enhance surfactant production or differentiation/maturation of type II epithelial cells might therefore protect neonates born to mothers with poorly controlled asthma from the increased risk of neonatal lung disease. Administration of antenatal corticosteroids to women at risk of preterm birth is routine, to induce precocious maturation of the fetal lungs and prevent neonatal respiratory distress in preterm infants (Roberts & Dalziel, 2006). Mechanisms have been elucidated in sheep, where antenatal cortisol administration to the late gestation fetus increases numbers of type II alveolar cells as well as surfactant protein gene expression in the fetal lung (Flecknoe *et al.*, 2004). Use of antenatal corticosteroids is being trialled in other populations with increased risk of neonatal respiratory complications, such as in infants delivered at term by Caesarean section, with promising results (Nada *et al.*, 2016). American College of Obstetricians and Gynecologists recommendations on Antenatal Corticosteroid Therapy for Fetal Maturation do not differentiate between asthmatic and non-asthmatic women (American College of Obstetricians and Gynecologists, 2017), and antenatal corticosteroids are not contraindicated in asthmatic women. However, this therapy has not yet been formally evaluated for preventing neonatal lung disease in the setting of maternal asthma, suggesting a promising avenue for further research to improve neonatal outcomes in this infant population.

The apparent delay in fetal lung maturation induced by maternal asthma contrasts with the effects of intrauterine inflammation (chorioamnionitis) on fetal lung development. Human infants born after chorioamnionitis have a lower risk of neonatal respiratory distress syndrome than non-exposed infants, suggesting accelerated lung maturation (Thomas & Speer, 2011). Experiments in sheep (Moss *et al.*, 2002; Kuypers *et al.*, 2012) and other experimental animals (Prince *et al.*, 2004; Schmiedl *et al.*, 2011) show that intrauterine inflammation increases production of pulmonary surfactant by the preterm lungs. Clearly, the effects of localized intrauterine inflammation and a chronic maternal inflammatory state, as

exists in asthma, have differing effects on fetal development. Further elucidation of the effects of these prenatal exposures on development of fetal tissues including lungs, and identification of the underlying mechanisms, requires further investigation.

To the best of our knowledge, this is the first demonstration that fetal or progeny CD44 is altered by *in utero* exposure to maternal allergy. We observed a greater proportion of CD44⁺ thymic lymphocytes in fetuses from allergic ewes than in fetuses from control ewes. The fact that this effect was observed for lymphocytes isolated from thymus but not spleen might suggest that maternal allergic asthma affects predominantly the phenotype of immature T cells that remain in the thymus. However, additional studies are needed to identify which population of lymphocytes express CD44 in these fetal sheep. In particular, the identification of T regulatory (Treg) lymphocytes (CD4⁺CD25⁺FOXP3⁺) is likely to be informative. FOXP3 expression in Treg cells is positively associated with their capacity to suppress inflammation (Liu *et al.*, 2010). This transcription factor is epigenetically regulated and its function correlates negatively with methylation in the Treg-specific demethylated region (Liu *et al.*, 2010), suggesting a pathway for programming of Treg function by the prenatal environment. 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 (Meng *et al.*, 2016), suggesting this marker may be a useful predictor of immune function. CD44 staining was not localized in the present study; however, CD44⁺ cells are predominantly seen in the medulla (with few positive cells in the cortex of the thymus) in control fetuses and fetuses following intrauterine inflammation (Melville *et al.*, 2012). CD44 expression allows lymphocyte adhesion to endothelial cells, and is essential for migration of progenitor cells into the thymus (Wu *et al.*, 1993) and activated T cells from the circulation into sites of inflammation (DeGrendele *et al.*, 1997). 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 sensitized mice (Camp *et al.*, 1993; Katoh *et al.*,

2003). Also consistent with a role for CD44 in allergic inflammation is the reported increase in CD44 expression on lymphocytes in bronchoalveolar lavage following allergen challenge in sheep (Bischof *et al.*, 2003). These results suggest a pathway for developmental programming of allergy. If the increased frequency of 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 (Lim *et al.*, 2010; Illi *et al.*, 2014).

Unexpectedly, given the evidence for increased allergic susceptibility in progeny of asthmatic or allergic human mothers (Lim *et al.*, 2010; Illi *et al.*, 2014) and progeny of allergic mice (Hamada *et al.*, 2003; Hubeau *et al.*, 2006), exposure to chronic maternal allergic asthma did not alter expression of the majority of immune mediators we examined in tissues of the late gestation fetal sheep, including CD4, CD8 and MHC II. Maternal allergic asthma did not affect fetal weight of the thymus or spleen, nor the proportions of CD4⁺ and CD8⁺ lymphocytes in the fetal thymus and spleen in late gestation, suggesting there is not Th1 or Th2 polarisation of the immune system. Thymus size is decreased in human pregnancies complicated by funisitis (Di Naro *et al.*, 2006), but our results suggest that the chronic mild inflammatory state in allergic asthma is not sufficient to suppress growth of fetal immune tissues. In mice, *in utero* exposure to chronic maternal allergic asthma increased the number of splenic CD4⁺ lymphocytes that were FOXP3⁻ and CD25⁻, although numbers of CD4⁺CD25⁺FOXP3⁺ Treg cells were unchanged (Hubeau *et al.*, 2007). In the sheep, *in utero* exposure to intra-amniotic LPS in late gestation reduced the proportion of fetal thymocytes positive for CD8 and MHC II, and increased the CD4:CD8 ratio seven days after exposure (Melville *et al.*, 2012). This inflammatory exposure was initiated later in gestation than the present study, and in response to intrauterine rather than chronic maternal inflammation. Differences in immune programming between these models may also reflect impacts of repeated hypoxic exposure, secondary to asthmatic exacerbations.

Strengths of this study include the consistency between the allergic asthmatic phenotype induced by this protocol in sheep and that seen in human asthmatics (Bischof *et al.*, 2003; Bischof *et al.*, 2008; Clifton *et al.*, 2015). Reduced fetal growth in allergic ewes (Clifton *et al.*, 2015) is also consistent with effects of poorly controlled asthma in humans (Murphy, 2015; Grzeskowiak *et al.*, 2016). Importantly, developmental timing and maturity at birth are more similar in sheep to that of humans than are rodents, so sheep provide an excellent model in which to investigate programming of postnatal health and function by *in utero* exposures (Morrison *et al.*, 2018). One limitation of this study is that animal numbers were not sufficient to allow investigation of sex-specific responses. Lung structure and surfactant protein gene expression are similar in male and female sheep fetuses in late gestation (Ishak *et al.*, 2014), and we saw no sex differences in the protective effects of surgically-restricted placental growth and function (and hence restricted fetal growth) on later susceptibility to allergy in adolescent sheep in a previous study (Wooldridge *et al.*, 2014). However, effects of maternal asthma on fetal growth in humans are sex-specific (Murphy *et al.*, 2003), and we therefore recommend that subsequent studies are powered to detect sex-specific effects of maternal asthma on fetal growth and development and progeny respiratory and immune function.

Overall, decreased numbers of type II alveolar cells and surfactant protein expression indicate that maternal allergic asthma impairs lung maturation, which may explain increased risk of neonatal lung disease in infants of mothers whose asthma was poorly controlled in pregnancy. Our finding of increased CD44 expression in lymphocytes from the thymus might be one mechanism underlying programming of an allergic phenotype by maternal allergic asthma, most likely downstream of maternal responses and not reflecting antigen sensitisation of the fetus. Recent studies show that improved asthma control during pregnancy not only improves maternal well-being, but also improves outcomes for neonates

and reduces asthma risk in children. Understanding mechanisms for effects of maternal asthma in order to develop and evaluate interventions remains vital. However, approximately 50% of pregnant women have poorly controlled asthma (Grzeskowiak *et al.*, 2016). This may be due, at least in part, to problems with compliance with ongoing use of inhaled corticosteroids. Women are often concerned about the effects of asthma medications on the fetus, which may reduce their compliance (Murphy, 2015) and lead to inadequate asthma control. In other women, continued, or even increased use of inhaled corticosteroids may be insufficient to achieve adequate control of their disease, given the worsening asthma phenotype experienced in 35-50% of pregnancies (Murphy, 2015; Grzeskowiak *et al.*, 2016). Further research is needed to determine if these changes in lung and immune phenotype in our sheep model of allergic asthma lead to impaired neonatal lung function and increased susceptibility to allergic disease in the affected offspring, and to evaluate fetal and progeny impacts of interventions.

Additional information

Competing Interests

None.

Author contributions

The experiments described in this manuscript were performed at Monash University (in vivo studies and flow cytometry) and the Hudson Institute for Medical Research (lung structure and cell types). All authors have contributed to conception and design of the work (VLC, TJMM, BSM, JLM, RDM, MJW, RJB, KLG), acquisition, analysis or interpretation of data for the work (ALW, VLC, TJMM, HL, MJ, SA, JLM, MJW, RJB, KLG), and/or drafting the work or revising it critically for important intellectual content (ALW, VLC, TJMM, BSM, JLM, MJW, RJB, KLG), have approved the final version of the manuscript, and agree to be accountable

for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This work was supported by the Jack Brockhoff Foundation (Grant # 3699 to RJB, TJMM, KLG, JLM, RDM, VLC, BSM and MJW) and the Victorian Government Operational Infrastructure Support Program (TJMM, MJW, RJB). TJMM (APP1043294), and VLC (APP1041918 and APP1136100) are supported by NHMRC Senior Research Fellowships, and BSM (APP1083009) is supported by NHMRC Career Development Fellowship. JLM is supported by a NHMRC Career Development Fellowship (APP1066916) and an Australian Research Council Future Fellowship (FT170100431). ALW was supported by Australian Postgraduate Awards from the Australian Government, and ALW by a Healthy Development Adelaide Scholarship.

Acknowledgements

The authors thank Monash Animal Services for care of animals, and Stacey Holman, Gary Nguyen, Bahar Liravi, Dasom Kim and Pamela Sim for assisting with airway challenges, post mortems and sample analyses. We thank Courtney McDonald for assistance optimizing FACS protocols.

Translational Perspective

Asthma is the most common chronic disease in pregnant women and has adverse effects on the neonate and progeny, in addition to its well-established association with increased risks of adverse pregnancy outcomes. Babies of women with asthma are more likely to have respiratory problems at birth, with 50% higher risks of hospitalisation or death. Later, children of asthmatic mothers have a 3-fold greater risk of developing asthma than children of non-

asthmatic mothers. We sought to explore the underlying mechanisms, and hypothesized that fetal lung structure and immune phenotype in late gestation fetal sheep would be impaired in our sheep model of maternal allergic asthma during pregnancy. The density of type II alveolar epithelial cells, which produce surfactant proteins, was 30% lower in the lungs of fetuses from allergic ewes than controls. This likely contributes to lower lung expression of surfactant proteins in these fetuses, which we have reported previously. Together, these findings suggest that antenatal corticosteroid treatment of the mother, which promotes lung maturation and surfactant production, may be an effective intervention to improve neonatal outcomes of asthmatic mothers. We also found a 3.5-fold higher proportion of CD44⁺ lymphocytes in the thymus of fetuses from allergic ewes than control ewes. If the elevated lymphocyte expression of CD44 persists postnatally, this would confer greater susceptibility to allergic diseases in progeny of asthmatic mothers, consistent with observations in humans. Further studies are planned to evaluate neonatal and long-term respiratory and immune phenotypes in progeny after maternal asthma and antenatal corticosteroids.

(248 words)

Table 1. Cytokine and HDM-specific antibody concentrations near term.

	Control	Allergic
Number of animals	5	7
<i>Cortisol</i>		
Maternal plasma (ng/ml) ^{\$}	13.6 ± 4.8 ^a	3.6 ± 1.5 ^b
Fetal plasma (ng/ml) ^{\$}	4.3 ± 1.0	3.6 ± 0.4
<i>Cytokines</i>		
Amniotic IL-6 (ng/ml)	1.36 ± 0.58	1.80 ± 0.73
Amniotic IL-10 (BU/ml)	0.45 ± 0.13	0.77 ± 0.17

Amniotic IL-13 (ng/ml)	4.06 ± 1.85	4.26 ± 1.66
Amniotic TNF-α (ng/ml) ^{\$}	0.307 ± 0.420	0.205 ± 0.542
<i>HDM-specific antibodies</i>		
Total Ig, maternal plasma (A ₄₅₀ - background) ^{\$}	0.140 ± 0.091	0.186 ± 0.121
Total Ig, fetal plasma (A ₄₅₀ - background)	-0.003 ± 0.004	-0.003 ± 0.004
IgE, maternal plasma (A ₄₅₀ - background) ^{\$}	0.034 ± 0.023	0.097 ± 0.032
IgE, fetal plasma (A ₄₅₀ - background)	-0.002 ± 0.002	-0.002 ± 0.002
IgA, maternal plasma (A ₄₅₀ - background) ^{\$}	0.018 ± 0.010	0.024 ± 0.011
IgA, fetal plasma (A ₄₅₀ - background) ^{\$}	0.002 ± 0.002	0.009 ± 0.010
IgM, maternal plasma (A ₄₅₀ - background)	0.084 ± 0.013	0.102 ± 0.010
IgM, fetal plasma (A ₄₅₀ - background) ^{\$}	0.015 ± 0.011	0.002 ± 0.004

Data are expressed as mean ± SEM. AU, arbitrary units; BU, biological units. Means were compared by 1-way ANOVA, or by ^{\$}Mann-Whitney U-test for non-normally-distributed data. Groups with different superscripts differ (P=0.01)..

Figure legends

Figure 1. Lung structure in fetuses of control and allergic ewes. Representative immunohistochemistry showing lung tissue from fetuses of control (left-hand column, n=5) and allergic ewes (middle column, n=7), and data from individual control (open circles) and allergic (filled circles) fetuses, with mean $\pm$ SEM shown in bars (right-hand column). Scale bars indicate 50 μ m. ***, $P < 0.001$. Lung sections were stained with haematoxylin and eosin for point-counting of tissue and airspace (A - C), and immunohistochemistry was used to identify SPC⁺ type II alveolar cells (D - F), CD43⁺ leukocytes (G - I) or CD163⁺ macrophages (J - L). Insets (panels D, G, J) show negative control sections.

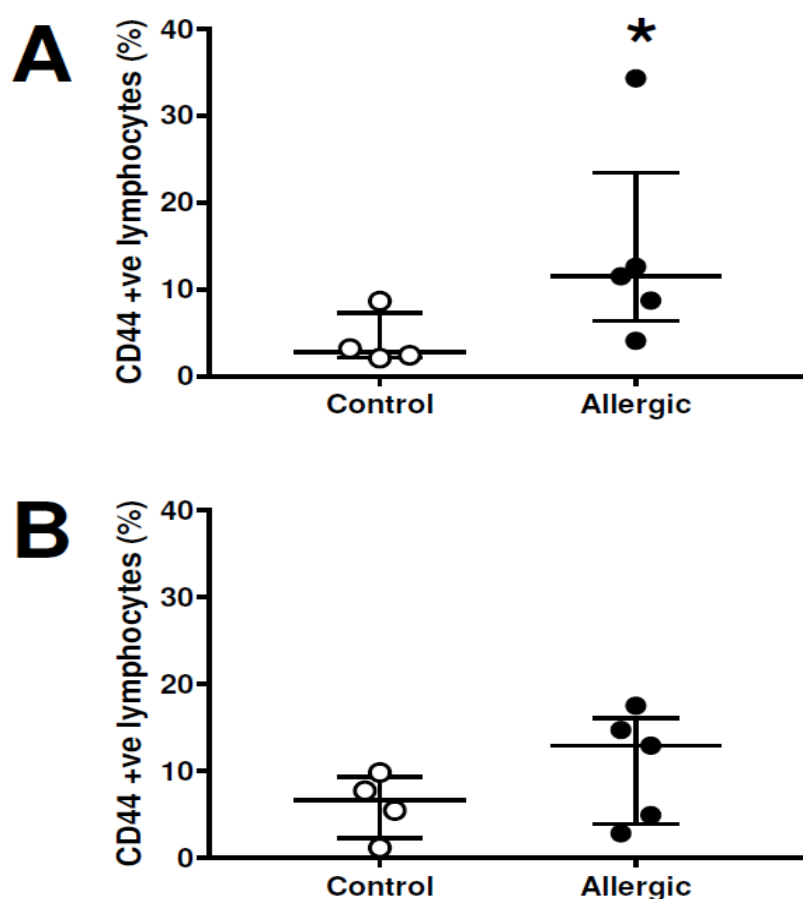
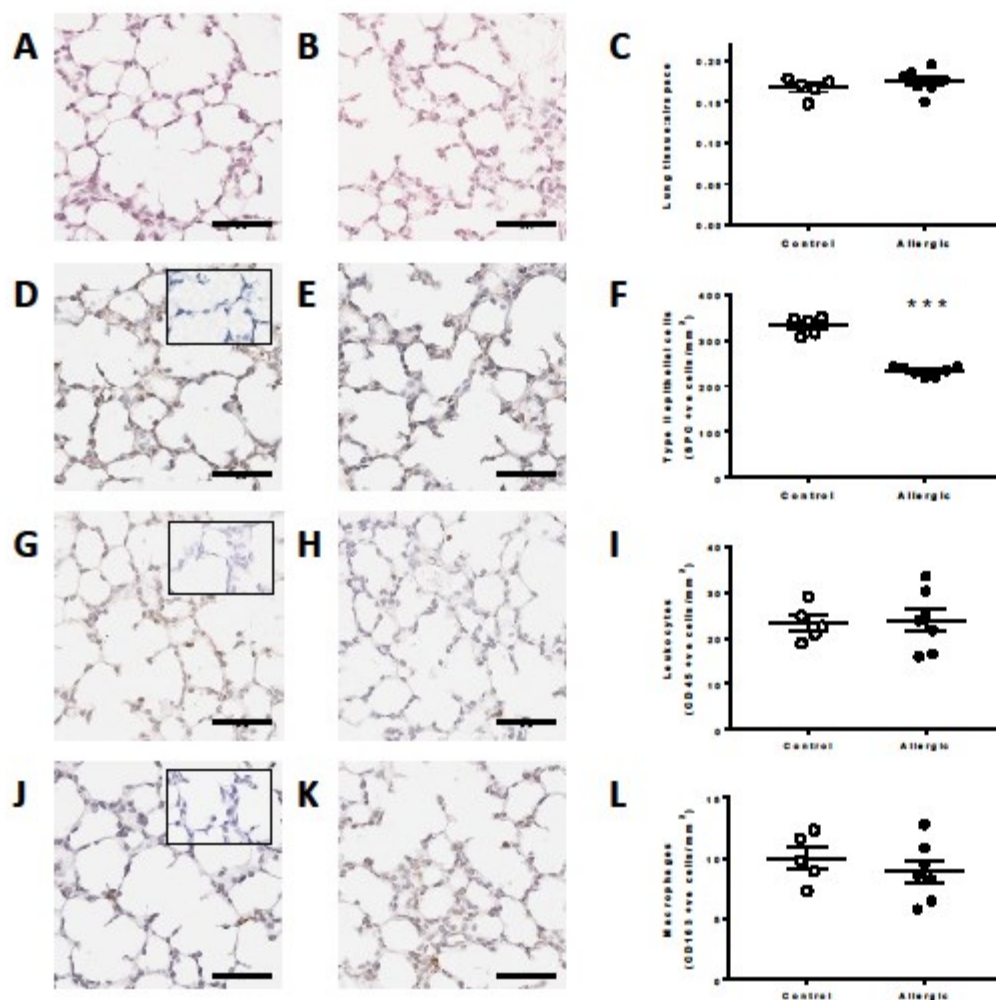


Figure 2. CD44 staining of lymphocytes isolated from spleen and thymus in fetuses of control and allergic ewes. The percentage of positive cells was measured by flow cytometry in cells isolated from thymus (A) and spleen (B). Data are shown for individual control (open circles, n=5) and allergic (filled circles, n=7) fetuses, with median and inter-quartile range shown in bars. *, $P = 0.032$



References

- American College of Obstetricians and Gynecologists. (2017). ACOG Committee Opinion. No 713. Antenatal corticosteroid therapy for preterm maturation, pp. <https://www.acog.org/Clinical-Guidance-and-Publications/Committee-Opinions/Committee-on-Obstetric-Practice/Antenatal-Corticosteroid-Therapy-for-Fetal-Maturation?IsMobileSet=false>.
- Bischof R, Snibson K, Velden J & Meeusen E. (2008). Immune response to allergens in sheep sensitized to house dust mite. *J Inflamm* **5**, 16.
- Bischof RJ, Snibson K, Shaw R & Meeusen EN. (2003). Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. *Clin Exp Allergy* **33**, 367-375.
- Bousquet J, Mantzouranis E, Cruz AA, Ait-Khaled N, Baena-Cagnani CE, Bleecker ER, Brightling CE, Burney P, Bush A, Busse WW, Casale TB, Chan-Yeung M, Chen R, Chowdhury B, Chung KF, Dahl R, Drazen JM, Fabbri LM, Holgate ST, Kauffmann F, Haahtela T, Khaltaev N, Kiley JP, Masjedi MR, Mohammad Y, O'Byrne P, Partridge MR, Rabe KF, Togias A, van Weel C, Wenzel S, Zhong N & Zuberbier T. (2010). Uniform definition of asthma severity, control, and exacerbations: document presented for the World Health Organization Consultation on Severe Asthma. *J Allergy Clin Immunol* **126**, 926-938.
- Camp RL, Scheynius A, Johansson C & Pure E. (1993). CD44 is necessary for optimal contact allergic responses but is not required for normal leukocyte extravasation. *J Exp Med* **178**, 497-507.
- Clifton VL, Engel P, Smith R, Gibson P, Brinsmead M & Giles WB. (2009). Maternal and neonatal outcomes of pregnancies complicated by asthma in an Australian population. *ANZ J Obstet Gynaecol* **49**, 619-626.
- Clifton VL, Moss TJM, Wooldridge AL, Gatford KL, Liravi B, Kim D, Muhlhausler BS, Morrison JL, Davies A, De Matteo R, Wallace MJ & Bischof RJ. (2015). Development of an experimental model of maternal allergic asthma during pregnancy. *J Physiol* **594**, 1311-1325.
- DeGrendele HC, Estess P & Siegelman MH. (1997). Requirement for CD44 in activated T cell extravasation into an inflammatory site. *Science* **278**, 672-675.
- Di Naro E, Cromi A, Ghezzi F, Raio L, Uccella S, D'Addario V & Loverro G. (2006). Fetal thymic involution: a sonographic marker of the fetal inflammatory response syndrome. *Am J Obstet Gynecol* **194**, 153-159.
- Fahey KJ & Morris B. (1978). Humoral immune responses in foetal sheep. *Immunology* **35**, 651-661.
- Flecknoe SJ, Boland RE, Wallace MJ, Harding R & Hooper SB. (2004). Regulation of alveolar epithelial cell phenotypes in fetal sheep: roles of cortisol and lung expansion. *Am J Physiol* **287**, L1207-L1214.

- Grieger JA, Clifton VL, Tuck AR, Wooldridge AL, Robertson SA & Gatford KL. (2016). In utero programming of allergic susceptibility. *Int Arch Allergy Immunol* **169**, 80-92.
- Grundy D. (2015). Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology. *J Physiol* **593**, 2547-2549.
- Grzeskowiak LE, Smith B, Roy A, Dekker GA & Clifton VL. (2016). Patterns, predictors and outcomes of asthma control and exacerbations during pregnancy: a prospective cohort study. *ERJ Open Res* **2**, 00054-02015.
- Hamada K, Suzuki Y, Goldman A, Ning YY, Goldsmith C, Palecanda A, Coull B, Hubeau C & Kobzik L. (2003). Allergen-independent maternal transmission of asthma susceptibility. *J Immunol* **170**, 1683-1689.
- Hubeau C, Apostolou I & Kobzik L. (2006). Adoptively transferred allergen-specific T cells cause maternal transmission of asthma risk. *Am J Pathol* **168**, 1931-1939.
- Hubeau C, Apostolou I & Kobzik L. (2007). Targeting of CD25 and glucocorticoid-induced TNF receptor family-related gene-expressing T cells differentially modulates asthma risk in offspring of asthmatic and normal mother mice. *J Immunol* **178**, 1477-1487.
- Illi S, Weber J, Zutavern A, Genuneit J, Schierl R, Strunz-Lehner C & von Mutius E. (2014). Perinatal influences on the development of asthma and atopy in childhood. *Ann Allergy Asthma Immunol* **112**, 132-139.e131.
- Ishak N, Sozo F, Harding R & De Matteo R. (2014). Does lung development differ in male and female fetuses? *Exp Lung Res* **40**, 30-39.
- James AL, Elliot JG, Jones RL, Carroll ML, Mauad T, Bai TR, Abramson MJ, McKay KO & Green FH. (2012). Airway smooth muscle hypertrophy and hyperplasia in asthma. *Am J Respir Crit Care Med* **185**, 1058-1064.
- Kane SV & Acquah LA. (2009). Placental transport of immunoglobulins: a clinical review for gastroenterologists who prescribe therapeutic monoclonal antibodies to women during conception and pregnancy. *Am J Gastroenterol* **104**, 228-233.
- Katoh S, Matsumoto N, Kawakita K, Tominaga A, Kincade PW & Matsukura S. (2003). A role for CD44 in an antigen-induced murine model of pulmonary eosinophilia. *J Clin Invest* **111**, 1563-1570.
- Kuypers E, Collins JJ, Kramer BW, Ofman G, Nitsos I, Pillow JJ, Polglase GR, Kemp MW, Newnham JP, Gavilanes AW, Nowacki R, Ikegami M, Jobe AH & Kallapur SG. (2012). Intra-amniotic LPS and

antenatal betamethasone: inflammation and maturation in preterm lamb lungs. *Am J Physiol* **302**, L380-L389.

- Lim RH, Kobzik L & Dahl M. (2010). Risk for asthma in offspring of asthmatic mothers versus fathers: a meta-analysis. *PLoS One* **5**, e10134.
- Liravi B, Piedrafita D, Nguyen G & Bischof RJ. (2015). Dynamics of IL-4 and IL-13 expression in the airways of sheep following allergen challenge. *BMC Pulmonary Medicine* **15**, 101. .
- Liu J, Lluís A, Illi S, Layland L, Olek S, von Mutius E & Schaub B. (2010). T regulatory cells in cord blood—FOXP3 demethylation as reliable quantitative marker. *PLoS One* **5**, e13267.
- Macchiaverni P, Ynoue LH, Arslanian C, Verhasselt V & Condino-Neto A. (2015). Early exposure to respiratory allergens by placental transfer and breastfeeding. *PLoS One* **10**, e0139064.
- Melville JM, Bischof RJ, Meeusen EN, Westover AJ & Moss TJ. (2012). Changes in fetal thymic immune cell populations in a sheep model of intrauterine inflammation. *Reprod Sci* **19**, 740-747.
- Melville JM, McDonald CA, Bischof RJ, Polglase GR, Lim R, Wallace EM, Jenkin G & Moss TJ. (2017). Human amnion epithelial cells modulate the inflammatory response to ventilation in preterm lambs. *PLoS One* **12**, e0173572.
- Mendola P, Mannisto TI, Leishear K, Reddy UM, Chen Z & Laughon SK. (2014). Neonatal health of infants born to mothers with asthma. *J Allergy Clin Immunol* **133**, 85-90 e81-84.
- Meng SS, Gao R, Yan BD, Ren J, Wu F, Chen P, Zhang J, Wang LF, Xiao YM & Liu J. (2016). Maternal allergic disease history affects childhood allergy development through impairment of neonatal regulatory T-cells. *Respir Res* **17**, 114.
- Morrison JL, Berry MJ, Botting KJ, Darby JRT, Frasch MG, Gatford KL, Giussani DA, Gray CL, Harding R, Herrera EA, Kemp MW, Lock MC, McMillen IC, Moss TJ, Musk GC, Oliver MH, Regnault TRH, Roberts CT, Soo JY & Tellam RL. (2018). Improving pregnancy outcomes in humans through studies in sheep. *Am J Physiol* **315**, R1123-R1153.
- Morten M, Collison A, Murphy VE, Barker D, Oldmeadow C, Attia J, Meredith J, Powell H, Robinson PD, Sly PD, Gibson PG & Mattes J. (2018). Managing Asthma in Pregnancy (MAP) trial: FENO levels and childhood asthma. *J Allergy Clin Immunol* **142**, 1765-1772 e1764.
- Moss TJ, Newnham JP, Willett KE, Kramer BW, Jobe AH & Ikegami M. (2002). Early gestational intra-amniotic endotoxin: lung function, surfactant, and morphometry. *Am J Respir Crit Care Med* **165**, 805-811.

- Murphy VE. (2015). Managing asthma in pregnancy. *Breathe (Sheff)* **11**, 258-267.
- Murphy VE, Clifton VL & Gibson PG. (2006). Asthma exacerbations during pregnancy: incidence and association with adverse pregnancy outcomes. *Thorax* **61**, 169-176.
- Murphy VE, Gibson P, Talbot PI & Clifton VL. (2005). Severe asthma exacerbations during pregnancy. *Obstet Gynecol* **106**, 1046-1054.
- Murphy VE, Gibson PG, Giles WB, Zakar T, Smith R, Bisits AM, Kessell CG & Clifton VL. (2003). Maternal asthma is associated with reduced female fetal growth. *Am J Respir Crit Care Med* **168**, 1317-1323.
- Murphy VE, Wang G, Namazy JA, Powell H, Gibson PG, Chambers C & Schatz M. (2013). The risk of congenital malformations, perinatal mortality and neonatal hospitalisation among pregnant women with asthma: a systematic review and meta-analysis. *Br J Obstet Gynaecol* **120**, 812-822.
- Nada AM, Shafeek MM, El Maraghy MA, Nageeb AH, Salah El Din AS & Awad MH. (2016). Antenatal corticosteroid administration before elective caesarean section at term to prevent neonatal respiratory morbidity: a randomized controlled trial. *Eur J Obstet Gynecol Reprod Biol* **199**, 88-91.
- Poitras BJ, Miller RB, Wilkie BN & Bosu WT. (1986). The maternal to fetal transfer of immunoglobulins associated with placental lesions in sheep. *Can J Vet Res* **50**, 68-73.
- Powell H, Murphy VE, Taylor DR, Hensley MJ, McCaffery K, Giles W, Clifton VL & Gibson PG. (2011). Management of asthma in pregnancy guided by measurement of fraction of exhaled nitric oxide: a double-blind, randomised controlled trial. *Lancet* **378**, 983-990.
- Prince LS, Okoh VO, Moninger TO & Matalon S. (2004). Lipopolysaccharide increases alveolar type II cell number in fetal mouse lungs through Toll-like receptor 4 and NF-kappaB. *Am J Physiol* **287**, L999-L1006.
- Roberts D & Dalziel S. (2006). Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database Syst Rev* **3**, Art. No.: CD004454. DOI: 004410.001002/14651858.CD14004454.pub14651852.
- Schatz M, Harden K, Forsythe A, Chilingar L, Hoffman C, Sperling W & Zeiger RS. (1988). The course of asthma during pregnancy, post partum, and with successive pregnancies: a prospective analysis. *J Allergy Clin Immunol* **81**, 509-517.

Schatz M, Zeiger RS, Hoffman CP, Harden K, Forsythe A, Chilingar L, Saunders B, Porreco R, Sperling W, Kagnoff M & et al. (1995). Perinatal outcomes in the pregnancies of asthmatic women: a prospective controlled analysis. *Am J Respir Crit Care Med* **151**, 1170-1174.

Schmiedl A, Behrens J, Zscheppang K, Purevdorj E, von Mayersbach D, Liese A & Dammann CE. (2011). Lipopolysaccharide-induced injury is more pronounced in fetal transgenic ErbB4-deleted lungs. *Am J Physiol* **301**, L490-L499.

Thomas W & Speer CP. (2011). Chorioamnionitis: important risk factor or innocent bystander for neonatal outcome? *Neonatology* **99**, 177-187.

Wang G, Murphy VE, Namazy J, Powell H, Schatz M, Chambers C, Attia J & Gibson PG. (2014). The risk of maternal and placental complications in pregnant women with asthma: a systematic review and meta-analysis. *J Mat Fetal Neonatal Med* **27**, 934-942.

Westover AJ, Hooper SB, Wallace MJ & Moss TJ. (2012). Prostaglandins mediate the fetal pulmonary response to intrauterine inflammation. *Am J Physiol* **302**, L664-L678.

Wooldridge AL, Bischof RJ, Meeusen EN, Liu H, Heinemann GK, Hunter DS, Giles LC, 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* **306**, R441-R446.

Wu L, Kincade PW & Shortman K. (1993). The CD44 expressed on the earliest intrathymic precursor population functions as a thymus homing molecule but does not bind to hyaluronate. *Immunol Lett* **38**, 69-75.

