

Development of an experimental model of maternal allergic asthma during pregnancy

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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

controls, with a similar trend for surfactant protein D. Thus, allergic asthma in pregnant sheep modifies placental phenotype, and inhibits fetal growth and lung development consistent with observations from human pregnancies. Preconceptional allergen sensitisation and repeated airway challenges in pregnant sheep therefore provides an animal model to identify mechanisms of altered fetal development and adverse pregnancy outcomes caused by maternal asthma in pregnancy.

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Abbreviations BAL, bronchoalveolar lavage; BM, airway basement membrane; dGA, days gestational age; ET, endotracheal; HDM, house dust mite; IUGR, intrauterine growth restriction; RDS, respiratory distress syndrome; R_L , lung resistance; RR, risk ratio; SP, surfactant protein; TTN, transient tachypnoea of the newborn.

Introduction

Asthma is a chronic inflammatory disease of the airways, characterised by reversible airflow limitation or bronchial hyper-responsiveness resulting in respiratory symptoms such as wheezing, shortness of breath and coughing. Asthma has a variety of causes and is phenotypically diverse, but allergic sensitisation is considered the most common initiating factor (Bousquet *et al.* 2010). Allergic asthma is characterised by an initial airway epithelial response to airborne allergens causing allergen specific IgE activation of lung mast cells (reviewed by Agache *et al.* 2012). This causes a release of histamine and the subsequent recruitment of leukocytes from the bloodstream to the airway and results in increased hyper-responsiveness of smooth muscle cells to contractile agents, the adhesion, migration and activation of inflammatory cells to the submucosa, and the secretion of mucus. This manifests as acute exacerbations recognised by increased breathlessness, wheezing, coughing and decreased lung function. Persistent chronic inflammation of the lung leads to airway remodelling with thickening of the bronchial smooth muscle and airway obstruction. However, the effects of pregnancy on many of these responses are unknown.

In women of reproductive age the prevalence of asthma is higher than in the general population: 12% (95% confidence interval (CI): 9.7–14.3%) of females aged 15–29 years and 10.8% (95% CI: 9.4–12.3%) of women aged 30–44 years have asthma (Australian Bureau of Statistics, 2012). Thus, asthma is one of the most common chronic diseases to affect pregnant women. Pregnancy is recognised as a major challenge in the management of asthma as it can alter the course of asthma severity and its treatment, which in turn can affect pregnancy outcomes. Maternal asthma is a well-established risk factor for numerous adverse pregnancy outcomes including pre-eclampsia (risk ratio (RR) 1.54; 95% CI 1.32–1.81), preterm birth (RR 1.41; 95% CI 1.22–1.61), intrauterine growth restriction (RR 1.46; 95% CI 1.22–1.75) (Murphy

et al. 2011), peripartum maternal cardiomyopathy (8% vs. 2%; $P < 0.0001$) (Kao *et al.* 2013), placental abruption, and antenatal or postnatal haemorrhage (Clifton *et al.* 2009; Hodyl *et al.* 2014). In addition to adverse pregnancy outcomes, maternal asthma is a major risk factor for poor neonatal outcome. Analysis of a large retrospective cohort of over 200,000 singleton pregnancies demonstrated that infants of asthmatic mothers were at increased risk of hospitalisation (RR 1.50; 95% CI 1.03–2.20) and this increase in risk was evident in both term and pre-term neonates (Hodyl *et al.* 2014). Risks of neonatal respiratory distress syndrome (RDS) and transient tachypnoea of the newborn (TTN) are also elevated in infants of mothers who had asthma during pregnancy (Mendola *et al.* 2014), suggesting that maternal asthma impairs fetal lung development.

Asthma worsens during pregnancy in over 50% of women (Murphy *et al.* 2005). Asthma exacerbation during pregnancy (defined as any asthma-related event that involves a hospital admission, an unscheduled doctor visit, a course of oral steroids, an increase in medication use and/or decreased peak expiratory flow rate) is the most significant risk factor for fetal morbidity and mortality in progeny of asthmatic mothers (Murphy *et al.* 2005). Such exacerbations are common, particularly in women whose asthma is more severe, occurring in 8%, 47% or 65% of pregnant women with mild, moderate or severe asthma, respectively (Murphy *et al.* 2005). Exacerbations can occur at any time during gestation but are most likely to occur in the second and third trimesters, between weeks 17 and 34, with peak incidence around the 25th week of gestation (Murphy *et al.* 2005, 2006).

Despite a well-established association between maternal asthma and adverse pregnancy outcomes, knowledge of the mechanisms underlying these effects remains limited, largely because investigations have been restricted to clinical studies. Numerous studies on the placenta of pregnancies complicated by asthma suggest there are significant alterations in pathways associated with growth that may contribute to reduced fetal development

(reviewed by Clifton, 2010) but the impact of these placental alterations on fetal organ systems are unknown and require animal studies to examine mechanisms in more depth. Understanding the relative contributions of asthma severity, exacerbations and treatment to adverse pregnancy, fetal and neonatal outcomes from investigations of clinical cohorts is difficult since it is ethically impossible to randomise women to no treatment for their asthma during pregnancy. Mechanistic information to guide the development of targeted therapies to prevent adverse outcomes is often impossible to obtain from humans. Therefore, there is a need for an animal model of maternal asthma in which mechanistic studies can be undertaken to define the mechanisms by which maternal asthma can affect fetal development, to test the impact of treatment on maternal asthma and fetal development and for the purpose of testing new interventions. Sheep have long been used to study pregnancy, fetal development and perinatal physiology, and a number of medical interventions now routinely used in contemporary obstetrics and neonatology were translated into practice from sheep experiments (Liggins, 1969; Gunn *et al.* 1997; Roelfsema *et al.* 2004).

It is possible to induce allergy and asthma in non-pregnant sheep using house dust mite (HDM) allergen (Bischof *et al.* 2003, 2008; Snibson *et al.* 2005), which results in a similar lung phenotype to human asthma (James *et al.* 2012), with eosinophil infiltration, progressive loss of lung function, increased airway collagen deposition and thickening of bronchial smooth muscle (Bischof *et al.* 2003; Snibson *et al.* 2005; Meeusen *et al.* 2009). HDM allergens are the most prevalent allergens associated with asthma, and have both direct and indirect effects on immune cells, airway epithelial and airway structural cells, thereby mediating pathways to allergic sensitisation, airway inflammation, decline in lung function and airway wall remodelling (Gandhi *et al.* 2013; Calderón *et al.* 2015). Mouse models investigating maternal allergic airway exposure and the impact on fetal outcomes have been reported (Hylkema & Blacquiére, 2009; Cook-Mills, 2015); however, there are limitations when using mouse models as the mother and fetus cannot be chronically instrumented and sample collection is often limited to post mortem. Additionally, airway structure and function in mice, compared to the larger sheep airways, is poorly aligned to the human lung (Allen, 2009; Meeusen *et al.* 2009). We therefore established the HDM sheep model of experimental asthma upon which the current study is based, and which is considered a relevant model for allergic airways disease (Collie, 2003; Sharma *et al.* 2003; Meeusen *et al.* 2009). Given the increased risk of adverse maternal outcomes, including worsening of asthma control and cardiomyopathy, and of adverse fetal outcomes, including intrauterine growth restriction (IUGR), we hypothesised that sensitisation to

HDM and airway allergen challenges prior to mating in the sheep would result in an eosinophilic allergic phenotype localised to the lung leading to decreased maternal lung function with airway remodelling and induce adverse changes in maternal heart structure and gene expression. We further hypothesised that the presence of an allergic asthmatic phenotype in the maternal system would decrease fetal growth, alter placental phenotype and adversely affect fetal organ development, including lung maturation. The aim of the present study was to develop a sheep model of asthma in pregnancy that reproduces maternal and fetal responses to maternal asthma in human pregnancy. This will provide an experimental model in which to answer mechanistic questions and evaluate interventions not possible in clinical or epidemiological human studies.

Methods

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 (National Health and Medical Research Council of Australia, 2013).

Merino ewes (1–2 years of age) were allocated randomly to either non-immunised control ($n = 9$) or sensitised ($n = 31$) groups (Fig. 1). Sheep in the sensitised group were immunised with HDM using four subcutaneous injections of 50 μg of solubilised HDM extract (*Dermatophagoides pteronyssinus*; CSL Ltd, Parkville, VIC, Australia) in sterile 0.9% NaCl, and aluminium hydroxide as adjuvant (1:1), with injections given at 2 week intervals (Bischof *et al.* 2003). Peripheral blood was collected by venepuncture prior to the commencement of HDM immunisations and again 7 days after the final immunisation, to determine HDM-specific serum 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 enzyme-linked immunosorbent assay (ELISA), with optical density read at 450 nm (A_{450}) (Bischof *et al.* 2003, 2008). Of the 31 sensitised sheep, there were 17 that showed a twofold or greater increase in IgE levels after HDM immunisation, and these were defined as allergic (Bischof *et al.* 2003).

Allergic and control sheep then received weekly endoscopic airway challenges with HDM or saline, respectively (described below), for 8 weeks before timed mating with Merino rams (Fig. 1). Oestrus was synchronised using intravaginal sponges containing 300 mg of the synthetic progestagen flugestone acetate (Eazi-breed CIDR device, Zoetis Australia Pty Ltd, Rhodes, NSW, Australia) for a 12 day period. Mating dates were recorded, and pregnancy

status and fetal number were determined by ultrasound at 40–45 days gestational age (dGA; term ~147 dGA).

Throughout pregnancy, allergic sheep received endoscopic airway challenges with HDM every 2 weeks, and non-allergic sheep received endoscopic airway challenges with saline every 4 weeks (Fig. 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, Melbourne, 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 five sheep from the non-sensitised group plus four 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 (Fig. 1).

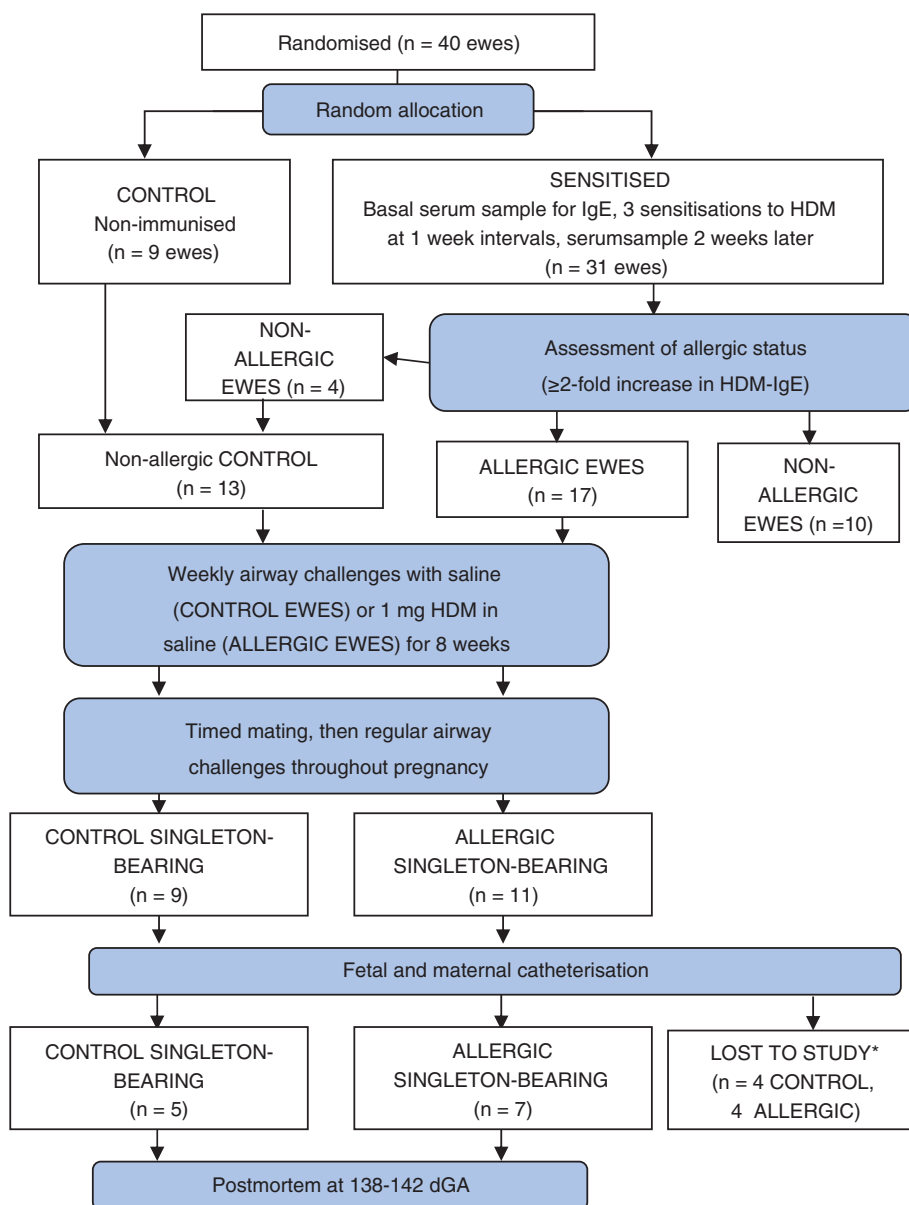


Figure 1. Study design

*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).

Catheterisation of ewes and fetuses

Surgery was performed at 96–108 dGA (102 ± 1 dGA, mean $\pm$ SEM) on control ($n = 9$) and allergic ($n = 11$) ewes identified by ultrasound as pregnant with singleton fetuses (Fig. 1). Food was withheld for 24 h prior to surgery, while drinking water remained unrestricted. Prophylactic antibiotics (1 g ampicillin sodium; Aspen Pharmacare Australia) were administered intravenously (i.v.) to the ewe on the day of surgery. General anaesthesia was induced by i.v. injection of 15–20 mg kg⁻¹ sodium thiopentone (Pentothal; Boehringer-Ingelheim, Australia) and maintained by inhalation of 1.5–3% isoflurane (Delvet, Ceva Animal Health, Glenorie, NSW, Australia). Indwelling catheters were placed into the maternal and fetal carotid arteries and jugular veins, and amniotic fluid (Moss *et al.* 2003; Westover & Moss, 2012) to enable maternal physiological responses to airway challenges to be recorded and fetal wellbeing to be monitored.

Endoscopic airway challenges and characterisation of maternal immune responses

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, Melbourne, VIC, Australia) was inserted into the lung via the nasal passage (Bischof *et al.* 2003). In allergic ewes, 5 ml of 100 μ g ml⁻¹ 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 (Bischof *et al.* 2003; Meeusen *et al.* 2009).

Bronchoalveolar lavage (BAL) fluid samples were collected from the right caudal lobe before the first airway challenge, and then prior to (0 h) and 48 h after airway allergen challenges (Bischof *et al.* 2003) conducted pre-mating, at mid-pregnancy (50–65 dGA; 55 ± 1 dGA) and in late pregnancy (118–132 dGA; 123 ± 1 dGA). Challenges at mid- and late pregnancy were delivered as aerosols (see below). BAL cells were fixed and stained in Turk's solution (Merck Millipore, Melbourne, VIC, Australia) and total white blood cells counted using a haemocytometer. Cytospots of BAL samples were prepared on glass microscope slides, stained with Kwik-Diff (Thermo Electron Corp., Waltham, MA, USA) and white blood cell subpopulations counted using light microscopy.

White blood cell subpopulations were counted in all animals (as detailed above) in maternal blood smears collected immediately before and 24 h and 48 h after challenge in late pregnancy.

Characterisation of maternal lung function responses to aerosolised airway challenges

Allergen-specific airway responses to HDM challenge in allergic sheep were measured at early (8–28 dGA;

19 ± 2 dGA), mid- and late pregnancy (at the same ages as BAL collection as detailed above). Airway responses to saline challenge were measured in early and late pregnancy in control sheep. Similar to airway challenges above, sheep were restrained, unsedated, in a custom-made sling. Lung function was assessed following aerosolised HDM or saline airway challenges. A solution of HDM extract (100 μ g ml⁻¹) for allergic sheep, or saline for control sheep, was nebulised for 15 min at 20 breaths min⁻¹ (Koumoundouros *et al.* 2006). In order to measure lung resistance (R_L), oesophageal and tracheal catheters were placed via the nostrils for measurement of extra- and intrathoracic pressures, respectively. A fibre-optic endoscope was used to guide placement of the catheters, with the tracheal catheter being inserted via a cuffed endotracheal (ET) tube. The ET tube placed through the nostril into the trachea enabled a closed respiratory loop to be established for airway delivery and lung function measures. The aerosol delivery system consisted of a breathing circuit, a Harvard Ventilator (ventilator Model 55-0723; Harvard 289 Apparatus, Holliston, MA, USA), Rapid Flow Nebulizer bowl (Allersearch Laboratories, Oakhurst, NJ, USA) and Vitalair Rapid Nebulizer Pump (Gardner Denver Thomas, Inc., Sheboygan, WI, USA). The nebuliser was connected to the end of the breathing circuit via a T-piece to the ET tube, during aerosol challenges only, with the ventilator set at 20 breaths min⁻¹, inspiratory time 1.5 s and a tidal volume of 300 ml. The oesophageal and tracheal catheters were connected to differential pressure transducers (GE Druck, ThermoFisher Scientific, Scoresby, VIC, Australia), recorded via LabChart software (ADInstruments, Bella Vista, NSW, Australia), and R_L was derived from breath-by-breath analysis of intra- and extrathoracic pressures for a period of 25 min after aerosol challenge (Koumoundouros *et al.* 2006).

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) (Vatnick *et al.* 1991).

Maternal lung structure

Tissue was sampled from the left caudal lobe of the maternal lung, fixed in 4% paraformaldehyde and

Table 1. Sequences of oligonucleotide primers used for quantitative real-time RT-PCR for maternal cardiac tissues

Accession no.	Gene	Forward (F) and reverse (R) primer sequences
NM_001160026.1	<i>BNP</i>	F: CCTGCTTCTCCTCTTCTTGC R: TAGACGGTCCAACAGCTCCT
X55152	<i>TNFα</i>	F: ACACCATGAGCACCAAAAGC R: AGGCACCAGCAACTTCTGGA
NM_001009465.2	<i>IL1β</i>	F: TGCCTACGAACATGTCTCCGTGA R: TGCTCTCTGCTCTGGAGTTTGCAT
NM_001034494.1	<i>PCNA</i>	F: ACTCCACTGTCTCTACAGTAA R: CGATCTTGGGAGCCAAATAGT
XM_005197116.1	<i>Ki67</i>	F: TCAGTGAGCAGGAGGCAGTA R: GGAAATCCAGGTGACTTGCT
NM_001014912.1	<i>HO 1</i>	F: CTGGTGATGGCGTCTTTGTA R: CAGCTCCTCTGGGAAGTAGA

processed for embedding in paraffin. Consecutive tissue sections cut at 4 μm were stained with haematoxylin and eosin, Masson's trichrome or immunostained for airway mast cells (Snibson *et al.* 2005; Bischof *et al.* 2008). To assess airway wall remodelling in asthmatic sheep, collagen and smooth muscle area contents of the airway wall were determined by digital colour analysis of Masson's trichrome-stained histological sections (Snibson *et al.* 2005). Digital images were scanned using an Aperio Image Capture device and Imagescope software (Leica Biosystems Imaging Inc., Vista, CA, USA). Image analysis of 5–10 bronchioles and small bronchi, ranging from 200 to 2000 μm in diameter (calculations based on airway basement membrane perimeter), were performed on Masson's trichrome-stained lung sections using FIJI Image-J software (Schindelin *et al.* 2012), with all measurements standardised to airway basement membrane (BM) length to adjust for airway size (Snibson *et al.* 2005). Lung tissues immunostained for tryptase⁺ mast cells were scanned as above and total number of stained cells were counted in 10 scanned images of lung parenchyma (presented as cells per square millimetre of lung parenchyma) and in separate counts around the airway wall of 5–10 bronchioles and small bronchi (total and degranulated mast cells per BM length, as detailed above).

Maternal heart measurements and gene expression

The heart was weighed, dissected, and each ventricular free wall was weighed separately. A sample of the left ventricular free wall was snap frozen in liquid nitrogen. RNA was isolated from samples of the left ventricular free wall (~50 mg) of each ewe, and cDNA was synthesised as previously described (Wang *et al.* 2011).

The reference genes (β actin, hypoxanthine phosphoribosyltransferase 1 and tyrosine 3-monooxygenase (YWAHZ)) were chosen (Duffield *et al.* 2009; Passmore

et al. 2009; Wang *et al.* 2013; Hellemans & Vandesompele, 2014) based on expression analysis using the geNorm component of the qBase (Biogazelle, Zwinjnaarde, Belgium) relative quantification analysis software (Hellemans *et al.* 2007) because their expression was stable across samples (maximum value, factor = 0.3–0.4; Vandesompele *et al.* 2002; Soo *et al.* 2012; Hellemans & Vandesompele, 2014). The relative expression of messenger ribonucleic acid (mRNA) transcripts of molecules involved in physiological hypertrophy (IGF1, IGF1R and IGF2; Gentili *et al.* 2009; Zhang *et al.* 2010), pathological hypertrophy (ANP, BNP, IGF2R and AT1R; Lie *et al.* 2013, 2014; Zhang *et al.* 2013), cortisol availability (GR, MR, 11 β HSD1 and 11 β HSD2; Gentili *et al.* 2009), inflammation (TNF α and IL1 β), fibrosis (TGF β , collagen type II, MMP 2, TIMP 1, TIMP 2 and TIMP 3; Zhang *et al.* 2010; Wang *et al.* 2015), proliferation (PCNA and Ki67), reactive oxygen species (HO1) and glucose and fatty acid uptake (GLUT1, GLUT4, FATP1 and CPT1; Gentili *et al.* 2009; Wang *et al.* 2013; Nicholas *et al.* 2014) were measured by quantitative real-time RT-PCR (qRT-PCR) (Table 1) using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) on a ViiA7 Fast Real-time PCR system (Applied Biosystems) as previously described (Wang *et al.* 2011; Soo *et al.* 2012; McGillick *et al.* 2013).

Fetal lung phenotype

Tissue was sampled from the left caudal lobe of the fetal lung, avoiding large airways and blood vessels, and snap frozen in liquid nitrogen. Total RNA was extracted and qRT-PCR was used to measure gene expression under optimised primer-specific conditions as described previously (Westover & Moss, 2012), using 1 μg of RNA with Superscript III First Strand Synthesis system kit for real-time PCR, as specified by the manufacturer

(Life Technologies, Waltham, MA, USA). Gene primers for surfactant protein (SP) -A, -B, -C and -D and cytokines (interleukin (IL)-1 β , IL-6, IL-8 and IL-10) were as previously published (Wallace *et al.* 2009; Westover *et al.* 2012), and for IL-10 primer sequences were F (forward): GCTGTCATCGTTTCTGCCC; R (reverse): CTCTCTTCACCTGCTCCACC). *Rps29* rRNA was used as the reference gene because its levels were stable across fetal lung samples, and it was amplified using the primers F: CAGGGTTCTCGCTCTTGC and R: ACTGGCGGCACATATTGAG. Messenger RNA levels were normalised to expression of the reference gene *Rps29* rRNA for each fetus, and gene expression in fetuses from the allergic group was expressed relative to the mean mRNA abundance for that gene in the control fetuses.

Statistical analyses

Continuous outcomes in control and allergic sheep were compared by one-way ANOVA.

Changes in R_L were analysed by repeated measures ANOVA for effects of gestational age and treatment. Immune cell percentages in BAL pre- and post-challenge, in allergic and control sheep, and across gestational ages were compared by Holm–Sidak's multiple comparisons test. Relative mRNA levels between groups were compared using non-paired *t* tests. Statistical tests were performed

using SPSS version 21 and data are presented as means $\pm$ SEM.

Results

Maternal immune responses to challenge

Prior to mating and in late pregnancy, BAL samples collected before the airway challenge (0 h) from control and allergic sheep comprised similar proportions of macrophages, lymphocytes, neutrophils and eosinophils (Fig. 2). In allergic sheep, the proportion of eosinophils in BAL increased markedly 48 h after airway allergen challenge at each time point (each $P < 0.01$), whilst eosinophil proportions did not rise after saline challenge in control sheep (Fig. 2). In late pregnancy, macrophages formed a greater proportion of immune cells in BAL collected pre-challenge, in allergic compared to control sheep ($P < 0.05$, Fig. 2). After challenge in late pregnancy, eosinophils formed a greater proportion of immune cells in BAL and macrophages formed a lower proportion of immune cells in BAL, in allergic compared to control sheep (each $P < 0.001$, Fig. 2). In allergic sheep in late pregnancy, the proportions of eosinophils increased in post-challenge compared to pre-challenge BAL samples ($P < 0.001$), whilst the proportions of macrophages decreased ($P < 0.001$, Fig. 2). The abundance of

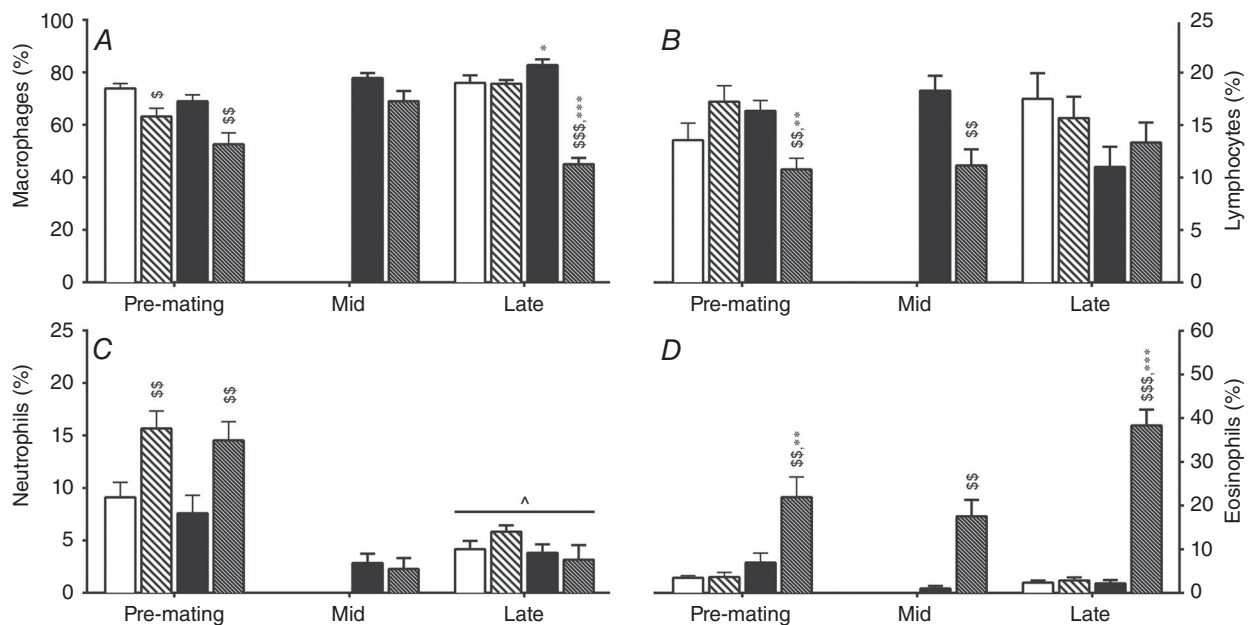


Figure 2. Immune cell populations in BAL fluid before and 48 h after airway challenge

Data are numbers of macrophages (A), lymphocytes (B), neutrophils (C) and eosinophils (D) as a proportion of all immune cells and are shown as means $\pm$ SEM. Control animals are shown in open bars, and allergic animals in filled bars, samples collected prior to airway challenge are in plain bars and samples collected 48 h after airway challenge are in hatched bars. Differences between control and allergic animals at the same time relative to challenge are shown by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; differences between pre- and 48 h post-challenge values within a group at each gestational age are shown by \$ $P < 0.05$, \$\$ $P < 0.01$ and \$\$\$ $P < 0.001$; overall differences between gestational ages are indicated by ^ $P < 0.05$.

neutrophils in BAL was lower in pregnant compared to non-pregnant sheep overall ($P < 0.05$, Fig. 2).

There was no difference in the numbers or proportions (%) of immune cell types in peripheral blood when examined prior to airway challenge in control *versus* allergic sheep before mating or in late pregnancy (data not shown). In allergic sheep in late pregnancy, there was a decline in the percentage of lymphocytes and monocytes, and a corresponding increase in eosinophils, in peripheral blood at 48 h after airway allergen challenge compared to pre-challenge, but these proportions did not change with gestation in control sheep (data not shown).

Maternal physiological responses to challenge

There was no change in R_L after saline challenge in controls in early or late gestation (Fig. 3). HDM allergen challenge increased R_L (relative to baseline R_L) throughout gestation in 6 of the 7 allergic sheep. The increase in R_L in allergic sheep was greater in mid- and late pregnancy compared to early pregnancy (Fig. 3, $P < 0.05$). In late pregnancy, airway responses to HDM challenge in allergic sheep were greater than the response to saline challenge in control sheep (Fig. 3, $P < 0.01$).

Maternal outcomes at post mortem

Morphometric image analysis of maternal lung revealed significant increases in airway smooth muscle accumulation around the airways in allergic compared to control pregnant sheep, without significant changes in collagen deposition ($P = 0.056$, Fig. 4). Chronic HDM challenge of the airways in allergic pregnant sheep did not alter total mast cell numbers within the parenchyma

and airway wall (Fig. 4E and F), nor the numbers of degranulated mast cells within the airway walls (controls: 0.15 ± 0.03 cells (mm BM) $^{-1}$; allergic: 0.72 ± 0.29 cells (mm BM) $^{-1}$, $P = 0.093$).

Maternal body weight and absolute and relative organ weights did not differ between control and allergic sheep (Table 2, Fig. 5A). Maternal cardiac expression of genes involved in physiological and pathological hypertrophy, cortisol availability, inflammation, fibrosis, proliferation, glucose uptake and reactive oxygen stress also did not differ between groups (Table 3).

Placental and fetal outcomes at post mortem

Total placental weight, the number of placentomes and average placentome weight did not differ between groups (Table 4), but there were differences in placental phenotype. Whilst placentae from allergic ewes had similar absolute numbers of Type A ($P = 0.070$), Type B ($P = 0.076$) and Type C ($P = 0.073$) placentomes (Fig. 6A) compared to placentae of control ewes, Type B ($P = 0.037$) and Type C ($P = 0.047$) placentomes formed greater proportions of total placentome numbers in the allergic group (Fig. 6B). Additionally, the total weight of Type A placentomes was lower in placentae from allergic than in those from control ewes (Fig. 6C).

Absolute measures of fetal weight (-10% in allergic cf. control group) and size did not differ between treatments (Table 2, Fig. 5B). However, fetal weight relative to maternal weight was 12% lower in the allergic group ($P = 0.038$, Fig. 5C). The singleton fetuses collected at post mortem comprised two males and three females from control ewes, and three males and four females from allergic ewes.

Fetal lung gene expression

Maternal allergy decreased fetal lung gene expression of SP-B ($P = 0.045$), but not SP-A, SP-C or SP-D ($P = 0.053$) expression (Fig. 7A). Expression of IL-1 β , IL-6, IL-8 and IL-10 did not differ between fetuses of control and allergic ewes (Fig. 7B).

Discussion

We have established a sheep model of maternal asthma in pregnancy, which demonstrates effects on maternal lung function, fetal and placental growth and lung development that are consistent with effects of maternal asthma in humans. The alterations in maternal inflammation and lung morphology and function seen in response to HDM exposure indicated that an allergic phenotype of asthma was induced in the pregnant ewes. Fetal body weight (relative to maternal weight) was 12% lower in asthmatic

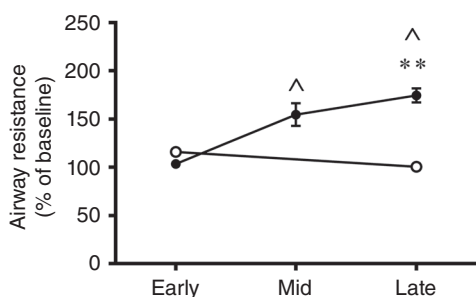


Figure 3. Responses to airway challenge in pregnancy

Mean percentage change from baseline lung resistance (R_L) in allergic sheep challenged with HDM (filled circles) and control sheep challenged with saline (open circles), at early, mid and late stages in pregnancy. Results for each time point represent the group mean of the maximum change in resistance over the first 30 min after challenge. Differences between control and allergic animals at the same gestational age are shown by $**P < 0.01$. Difference in allergic animals at late pregnancy compared to early pregnancy is shown by $^{\wedge}P < 0.05$.

sheep pregnancies compared to controls, consistent with the magnitude of fetal growth restriction observed in the presence of maternal asthma in human pregnancy (Murphy *et al.* 2011; Namazy *et al.* 2013; Mendola *et al.* 2014). Placental phenotype in late pregnancy was altered, consistent with accelerated maturation of the placenta (Alexander, 1964), suggesting placental adaptation to compensate for impaired nutrient and/or oxygen supply in asthmatic pregnancies. Maternal asthma also decreased expression of surfactant proteins in fetal lung, which would be expected to impair neonatal lung function. These data provide novel insights into the impact of maternal lung allergy on fetal–placental physiology and suggest this model may be important for discovering the mechanisms underlying the association of maternal asthma with greater risks of respiratory distress syndrome (RDS) and transient tachypnoea of the newborn (TTN) in human babies (Mendola *et al.* 2014).

The current data suggest maternal allergic asthma in sheep has specific effects on lung surfactant production. SP-B gene expression was reduced in fetuses from allergic pregnancies in the present study, with similar trends for SP-A, -C and -D, suggesting delayed maturation of the pulmonary surfactant system. Surfactant deficiency is the principal cause of RDS and contributes to the pathogenesis of TTN (Moss, 2006; Machado *et al.* 2011). RDS and TTN are common complications of prematurity, but in infants from asthmatic mothers the risk is elevated even after adjustment for gestational age (Mendola *et al.* 2014). These effects do not appear to be induced by fetal lung inflammation, because fetal lung expression of inflammatory cytokines was not elevated in our model of maternal allergic asthma. It has been speculated that associations between maternal asthma and risk of RDS and TTN might relate to genetic factors associated with asthma (Machado *et al.* 2011), but our findings show that

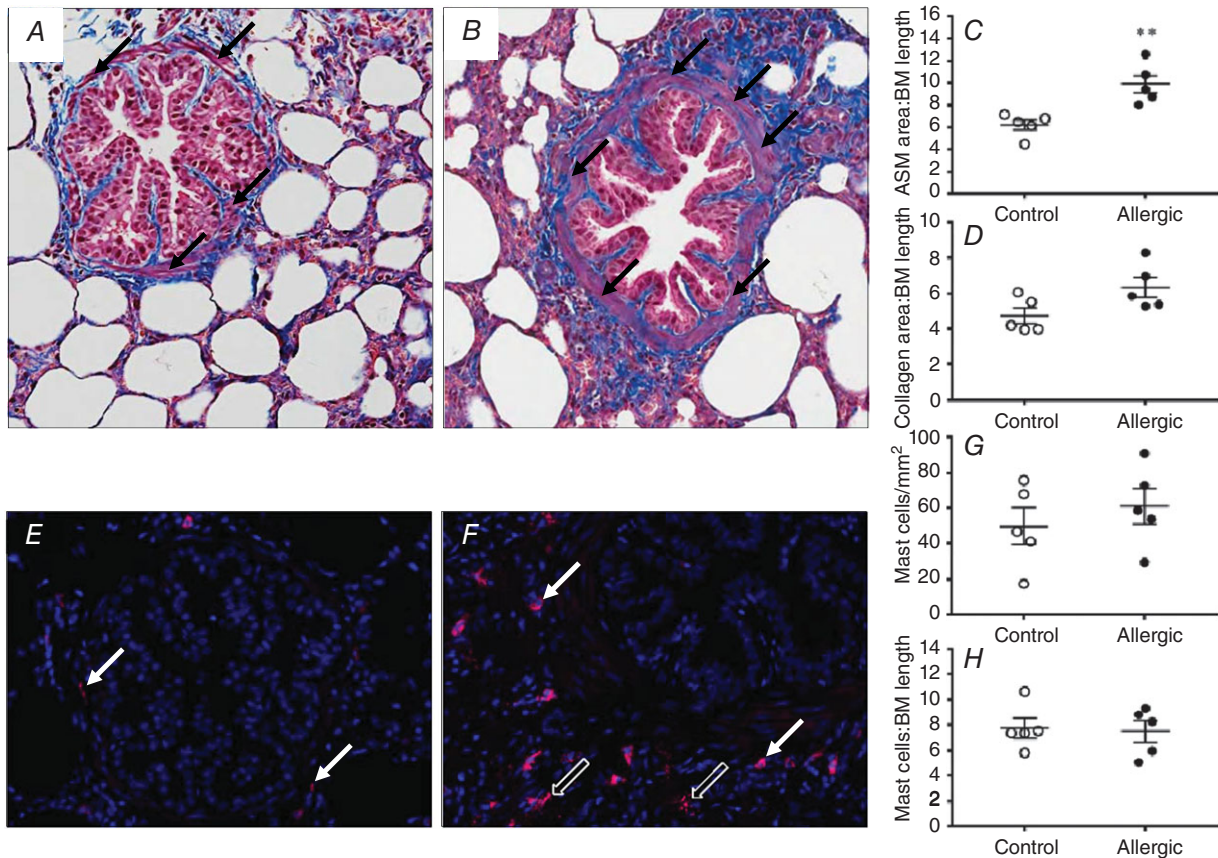


Table 2. Maternal body and organ weights at post mortem

	Control (n = 5)	Allergic (n = 7)
Body weight (kg)	38.9 ± 2.1	39.6 ± 1.4
Lung (g)	517 ± 106	476 ± 31
Lung (%)	1.27 ± 0.19	1.23 ± 0.09
Heart (g)	169 ± 8	176 ± 4
Heart (%)	0.436 ± 0.014	0.447 ± 0.016
Right ventricle (g)	35.4 ± 2.3	37.4 ± 1.2
Left ventricle (g)	61.2 ± 2.6	66.1 ± 3.7
Liver (g)	597 ± 47	560 ± 24
Liver (%)	1.50 ± 0.04	1.41 ± 0.03
Kidneys (g)	100 ± 4	109 ± 7
Kidneys (%)	0.257 ± 0.007	0.275 ± 0.013
Spleen (g)	129 ± 19	135 ± 26
Spleen (%)	0.334 ± 0.052	0.344 ± 0.071
Visceral fat (g)	418 ± 90	514 ± 92
Visceral fat (%)	1.07 ± 0.22	1.29 ± 0.21

Data are means ± SEM. Unless otherwise noted, organ weights given as % are relative to maternal body weight.

maternal allergic asthma *per se* impairs development of the fetal lungs.

The impact of maternal allergic asthma on fetal weights in the sheep model is similar to the human data and suggests the sheep model will be important in defining the growth mechanisms altered by maternal asthma. There were 10% and 12% reductions in absolute and relative fetal weights, respectively, in response to maternal allergic asthma, which is consistent with findings in human studies (Murphy *et al.* 2003). Numerous epidemiological analyses report that asthmatic mothers are at an increased risk of delivering a low birth weight infant, in particular when the asthma is severe, poorly controlled or acute exacerbations are experienced during gestation (Murphy *et al.* 2003, 2005, 2006, 2011; Namazy *et al.* 2013; Mendola *et al.* 2014). Several studies in human pregnancy indicate that the fetal response to maternal asthma was dependent on fetal sex. Thus, in the presence of maternal asthma, there was a ~12% reduction in female birth weight when compared to female neonates from non-asthmatic mothers, while there was no difference in birth weight of male infants overall (Murphy *et al.* 2003). Critically, if the mother experienced an exacerbation during pregnancy then birth weight of male fetuses was decreased dramatically, with an associated rise in the rates of IUGR, preterm deliveries and stillbirths, whilst exacerbations did not have any further impact on birth weight in females (Murphy *et al.* 2005). These findings suggest males and females respond differently to maternal asthma and implement different strategies in relation to growth. Other perinatal exposures, including experimentally induced IUGR or preterm birth, maternal antenatal glucocorticoid treatment or hypoxia, and fetal interventions such as treatment with insulin-like

growth factor 1, induce sex-specific acute and long-term responses in sheep (Owens *et al.* 2007; Gentili *et al.* 2009; Lumbers *et al.* 2009; De Matteo *et al.* 2010; Tang *et al.* 2010; Giussani *et al.* 2011; Wang *et al.* 2011, 2013; Miller *et al.* 2012; Wooldridge *et al.* 2014; Poudel *et al.* 2015). The current study was not powered to examine sex differences, and additional studies will be required to assess whether responses to maternal asthma are also sex-dependent in sheep in order to determine the underlying mechanisms.

The placenta plays a central role in modulating fetal growth and development and has been previously shown to be altered in human pregnancies complicated by asthma

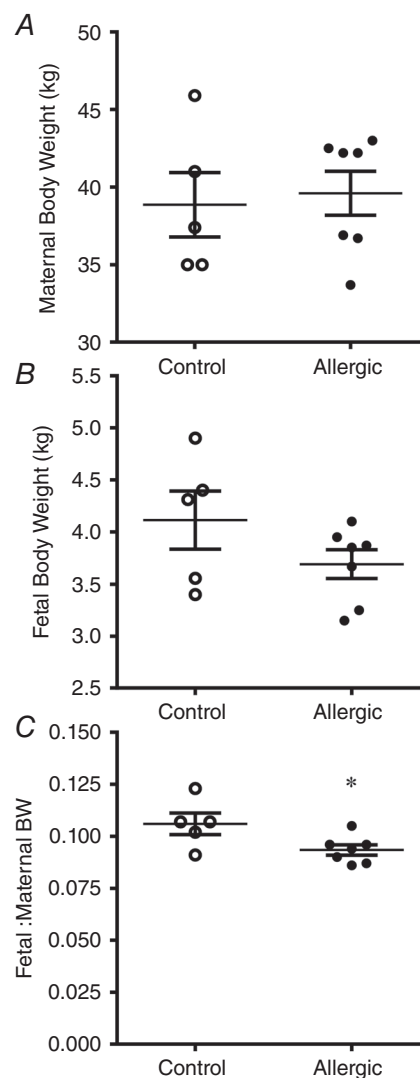


Figure 5. Effect of maternal allergic asthma on maternal body weight (A), absolute fetal body weight (B) and fetal body weight (relative to maternal weight) (C) at ~140 dGA
Quantitative data are presented as data from individual animals for control (open circles) and allergic (filled circles) sheep, with mean ± SEM shown in bars. Differences between control and allergic animals are shown by **P* < 0.05.

Table 3. Mean normalised expression of molecules that regulate cardiac growth and metabolism in control and asthmatic ewes in late gestation

	Control (n = 5)	Allergic (n = 7)
Physiological hypertrophy		
IGF1	0.058 ± 0.001	0.077 ± 0.015
IGF2	1.211 ± 0.102	1.200 ± 0.056
IGF1R	0.501 ± 0.010	0.480 ± 0.034
Pathological hypertrophy		
ANP	7.422 ± 4.696	0.485 ± 0.106
BNP	1.908 ± 1.161	0.356 ± 0.172
IGF2R	0.231 ± 0.012	0.257 ± 0.026
AT1R	0.044 ± 0.004	0.037 ± 0.003
Cortisol availability		
GR	0.535 ± 0.030	0.453 ± 0.027
MR	0.108 ± 0.003	0.100 ± 0.005
11βHSD1	0.015 ± 0.002	0.014 ± 0.001
11βHSD2	0.0009 ± 0.0002	0.0013 ± 0.0002
Inflammation		
TNFα	0.020 ± 0.005	0.016 ± 0.004
IL1β	0.011 ± 0.003	0.006 ± 0.001
Fibrosis		
TGFβ	0.212 ± 0.046	0.284 ± 0.074
Collagen type II	0.003 ± 0.001	0.002 ± 0.001
MMP2	0.104 ± 0.012	0.083 ± 0.004
TIMP1	0.259 ± 0.039	0.208 ± 0.022
TIMP2	0.179 ± 0.007	0.214 ± 0.018
TIMP3	0.852 ± 0.089	0.955 ± 0.067
Proliferation		
PCNA	0.078 ± 0.005	0.072 ± 0.002
Ki67	0.006 ± 0.002	0.004 ± 0.001
Glucose uptake		
GLUT1	0.008 ± 0.001	0.011 ± 0.002
GLUT4	0.199 ± 0.014	0.269 ± 0.027
Reactive oxygen stress		
HO1	0.050 ± 0.008	0.044 ± 0.005

Data are mean normalised expression ± SEM.

(Clifton, 2010). We demonstrated a significant alteration in placental phenotype in the presence of maternal allergic asthma in this sheep model. The increased proportion of Type B and C placentomes in the presence of maternal allergic asthma in sheep is suggestive of a more mature placental phenotype (Alexander, 1964). The shift towards Type B and Type C placentomes in response to maternal asthma in the present study is similar to the enhanced placental maturation observed in late gestation following interventions that reduce fetal growth in early gestation in sheep. For example, restriction of fetal nutrient supply by surgical reductions in uterine epithelial attachment sites before mating (and hence placentome number throughout pregnancy), increases the proportion of Type D placentomes in late gestation (Robinson *et al.* 1979; Poudel *et al.* 2015). Maternal or fetal hypoxia are also associated with a significant

Table 4. Placental phenotype and fetal size and organ weights at post mortem

	Control (n = 5)	Allergic (n = 7)
Placenta		
Placental weight	344 ± 51	323 ± 23
Total placentomes (no.)	76.4 ± 6.5	73.7 ± 4.7
Average placentome weight (g)	4.39 ± 0.37	4.40 ± 0.21
Fetal weight and size at post mortem		
Body weight (kg)	4.11 ± 0.28	3.69 ± 0.14
Crown–rump length (cm)	57.2 ± 0.9	56.1 ± 1.0
Abdominal circ. (cm)	35.0 ± 1.2	33.0 ± 0.7
Thoracic circ. (cm)	33.7 ± 1.0	32.1 ± 0.5
Head length (cm)	12.9 ± 0.4	13.3 ± 0.4
Head width (cm)	9.2 ± 0.1	9.4 ± 0.3
Fetal organ weights at post mortem		
Lung (g)	137 ± 12	137 ± 7
Lung (%)	3.22 ± 0.14	3.72 ± 0.20
Brain (g)	59.1 ± 1.0	57.3 ± 1.4
Brain (%)	1.46 ± 0.10	1.56 ± 0.05
Liver (g)	96.7 ± 11.1	86.9 ± 5.8
Liver (%)	2.33 ± 0.12	2.35 ± 0.10
Heart (g)	27.7 ± 1.8	25.3 ± 1.2
Heart (%)	0.674 ± 0.019	0.686 ± 0.022
Kidneys (g)	24.0 ± 2.1	23.3 ± 0.9
Kidneys (%)	0.582 ± 0.026	0.631 ± 0.012
Spleen (g)	5.93 ± 0.78	6.07 ± 0.48
Spleen (%)	0.144 ± 0.016	0.164 ± 0.012
Visceral fat (g)	24.2 ± 3.2	23.7 ± 0.8
Visceral fat (%)	0.580 ± 0.041	0.644 ± 0.016

Data are means ± SEM. Unless otherwise noted, organ weights given as % are relative to fetal body weight. circ., circumference.

change in placentome distribution with fewer Type A and more Types B, C and D (Penninga & Longo, 1998). Maternal undernutrition in early to mid-pregnancy (ewes fed 50% of requirements from 28 to 77 dGA) reduces the proportion of Type A and increases the proportion of Type B placentomes present near term in sheep (Heasman *et al.* 1998), and milder maternal undernutrition (ewes fed 85% of requirements for the first 70 days after mating) shifts placental phenotype from Type A towards Type D placentomes (Steyn *et al.* 2001). Thus, placental changes induced by maternal asthma probably represent an adaptation to increase placental transfer capacity and maintain fetal growth in the face of reduced oxygen supply or inflammation. The functional significance of these phenotypic changes is the subject of some debate and merits direct study; measures of cell number, vascularity and vasoreactivity are more closely related to placentome size than type (Vonnahme *et al.* 2008) and placental nutrient delivery did not correlate with the proportions of Types C and D placentomes in sheep near term (Ward *et al.*

2006). Further studies will define the role of these placental phenotypes in regulating fetal growth and survival in the presence of the maternal lung allergy.

Similar to our previous work in non-pregnant sheep (Bischof *et al.* 2003; Snibson *et al.* 2005; Meeusen *et al.* 2009), 55% of animals were responsive to HDM sensitisation, showing elevation of HDM-specific IgE. Our work shows that conception rates are not affected by HDM sensitisation. These sheep subsequently exhibited symptoms consistent with allergic asthma, including an increase in systemic allergen-specific IgE levels, increased circulating and airway (BAL) eosinophils, decreased lung function and features of airway wall remodelling, but no changes in cardiac gene expression. Similar immune responses and structural changes are typical of the human asthmatic lung (James *et al.* 2012), though data from human pregnancies complicated by asthma are limited to lung function and circulating immune cells (Murphy

et al. 2003; Osei-Kumah *et al.* 2010). In allergic sheep, the inflammatory response to HDM challenge in the lung worsened as pregnancy progressed, with increasing eosinophil induction in response to each challenge, and lung function continued to decline with repeated allergen exposures during pregnancy. Direct comparisons of pregnant and non-pregnant animals during the same sensitisation and airway challenge regimes are needed to confirm whether this is a pregnancy-induced worsening of asthma, as occurs in human asthma during pregnancy (Murphy *et al.* 2005). Interestingly, other studies suggest maternal asthma worsens in relation to the sex of the fetus and in particular in the presence of a female fetus. Previous studies report a higher incidence of maternal hospitalisations for asthma in women pregnant with a female (Bakhireva *et al.* 2008) and reduced maternal lung function and increased requirement for inhaled corticosteroids in women pregnant with a female (Murphy *et al.* 2003), compared to women carrying a male fetus. However, this remains controversial as a large epidemiological study of over 11,000 pregnancies did not find any difference in maternal exacerbations or daily inhaled corticosteroid dose between asthmatic women pregnant with a male or female fetus (Firoozi *et al.* 2009). The sheep model of maternal allergic asthma will allow us to directly compare male and female asthmatic pregnancies in future, larger studies.

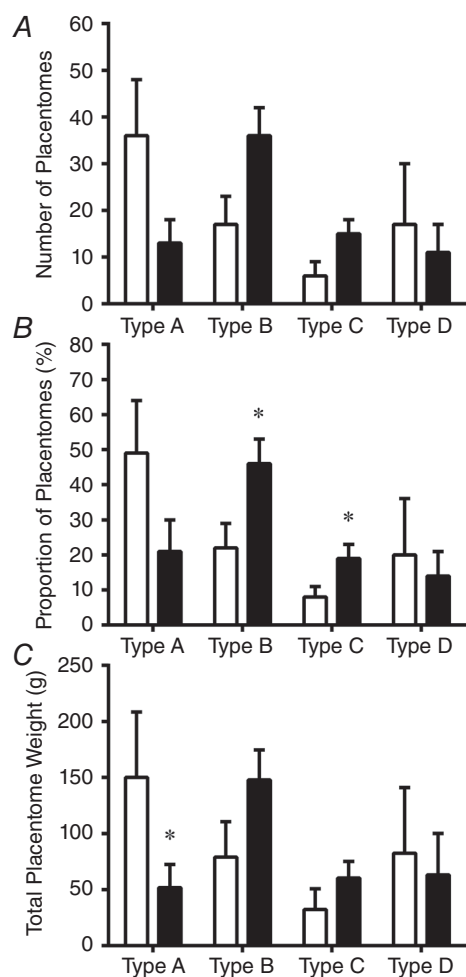


Figure 6. Effect of maternal allergic asthma on placental phenotype

Data are means $\pm$ SEM. Differences between control and allergic animals are shown by * $P < 0.05$.

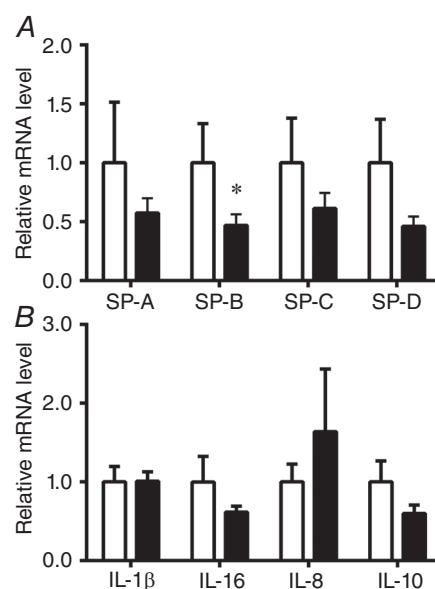


Figure 7. Effect of maternal allergic asthma (filled bars) on mRNA levels of surfactant proteins A, B, C and D (A) and cytokines IL-1 β , IL-6, IL-8 and IL-10 (B) in fetal lung

Data are presented as means $\pm$ SEM, expressed relative to the control group (open bars) at each age. All data are corrected for the expression level of the reference gene *Rps29*. Differences between control and allergic animals are shown by * $P < 0.05$.

Our development and validation of this new and unique model of allergic asthma in pregnancy lays the foundation for future studies focused on understanding mechanisms through which maternal asthma during pregnancy increases the risk of adverse pregnancy and neonatal outcomes. This model also provides the opportunity to test interventions that may prevent adverse outcomes of asthma in pregnancy by evaluating treatment responses in the mother and fetus and assessing the short and long-term effects of asthma treatments on the offspring. A major strength of this model is that fetal sheep can be instrumented for physiological studies throughout the final third of gestation. The establishment of this model of maternal allergic asthma in the sheep will therefore enable detailed fetal physiological responses to asthma, exacerbations, clinical treatments and novel interventions to be assessed in detail.

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Additional information

Competing interests

No authors have competing interests in relation to this work.

Author contributions

V.L.C., T.J.M.M., K.L.G., A.L.W., M.J.W., J.L.M., B.S.M., R.DeM. and R.J.B. were central to the conception and design, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content and final approval of the version to be published. B.L., D.K. and A.D. were involved in the analysis and interpretation of the data and final approval of the version to be published. The experiments were carried out at the Department of Physiology, Monash University, Clayton, VIC, Australia.

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