# Development of an experimental model of maternal allergic asthma during pregnancy

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## Abstract

Maternal asthma during pregnancy adversely affects pregnancy outcomes but the underlying mechanisms are unknown. Identification of the cause/s, and the ability to evaluate interventions, is limited by the lack of an appropriate animal model. We aimed to characterise maternal and fetal effects 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 then 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 d gestational age (term ~147 d). The eosinophil influx into lungs was greater after HDM challenge in allergic ewes than after saline challenge in control ewes before mating ($P$<0.01) and in late gestation ($P$<0.001). Airway resistance increased throughout pregnancy in allergic ($P$<0.05) but not control ewes, consistent with airway smooth muscle accumulation in allergic ewes ($P$<0.01). Maternal allergic asthma decreased relative fetal weight (-12%, $P$=0.038) and altered placental morphology. Expression of surfactant protein B mRNA was 48% lower in fetuses from allergic ewes than controls ($P$=0.045), with a similar trend for surfactant protein D (-50%, $P$=0.053). Thus, allergic asthma in pregnant sheep 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.

## 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). In women of reproductive age the prevalence of asthma is higher than in the general population: 12% (95% 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. Maternal asthma is a well-established risk factor for numerous adverse pregnancy outcomes including preeclampsia (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; 1.03, 2.20) and this increase in risk was evident in both term and preterm neonates (Hodyl *et al.*, 2014). Risks of neonatal respiratory distress syndrome (RDS) and transient tachypnea 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 25$^{th}$ week of gestation (Murphy *et al.*, 2005; Murphy *et al.*, 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. 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 need for an animal model of maternal asthma in which mechanistic studies can be undertaken. 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 HDM allergen (Bischof *et al.*, 2003; Snibson *et al.*, 2005; Bischof *et al.*, 2008), 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). We hypothesised that sensitisation to HDM and airway allergen challenges prior to mating would similarly establish an asthmatic phenotype in pregnant sheep, and that this would have effects on pregnancy and fetal development consistent with observations from human pregnancy. 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 (MARP/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 (Figure 1). Sheep in the sensitised group were immunised with HDM using four subcutaneous (s.c.) injections of 50 μg of solubilised HDM extract (*Dermatophagoides pteronyssinus*; CSL Ltd, VIC, Australia) in sterile 0.9% NaCl, and aluminium hydroxide as adjuvant (1:1), with injections given at 2 week intervals (Bischof *et al.*, 2003). Peripheral blood was collected by venepuncture prior to the commencement of HDM immunisations and again 7 days (d) after the final immunisation, to determine HDM-specific serum IgE levels and allergic status of immunised sheep; time-matched samples were collected from control sheep. HDM-specific IgE levels were determined in duplicate samples by ELISA, with optical density (OD) read at 450 nm ($A_{450}$) (Bischof *et al.*, 2003; Bischof *et al.*, 2008). Of the 31 sensitised sheep, there were 17 that showed a two-fold or greater increase in IgE levels after HDM immunisation, and these were defined as allergic (Bischof *et al.*, 2003).

Allergic and control sheep then received weekly endoscopic airway challenges with HDM or saline, respectively (described below), for eight weeks before timed mating with Merino rams (Figure 1). Estrus was synchronised using intravaginal sponges containing 300 mg of the synthetic progestagen flugestone acetate (Eazi-breed CIDR device, Zoetis Australia Pty Ltd, NSW, Australia) for a 12-d period. Mating dates were recorded, and pregnancy status and fetal number were determined by ultrasound at 40-45 d gestational age (dGA; term ~147 dGA).

Throughout pregnancy, allergic sheep received endoscopic airway challenges with HDM every two weeks, and non-allergic sheep received endoscopic airway challenges with saline every four weeks (Figure 1). All animals were housed and handled as one group. Sheep were housed outdoors in small paddocks during allergen sensitisation and airway challenges until approximately 90-100 dGA. During this period, sheep grazed natural pastures and were supplemented with lucerne hay. Pregnant sheep were housed indoors in individual pens for the remainder of the experimental period, in a facility with a 12 h:12 h dark-light cycle, and were fed 0.8-1.0 kg lucerne chaff and 0.85 kg ewe and lamb pellets (Rumevite, Ridley AgriProducts, VIC, Australia) daily, with water available *ad libitum*.

Outcomes were studied in only singleton-bearing ewes from each group. Singleton-bearing non-allergic animals (control, n=9) included 5 sheep from the non-sensitised group plus 4 sheep from the sensitised group who did not show increases in IgE after the sensitisation protocol, and the allergic group consisted of 11 singleton-bearing sheep (Figure 1).

***Catheterisation of ewes and fetuses***

Surgery was performed at 96-108 dGA (102 ± 1 dGA, mean ± SEM) on control (n=9) and allergic (n=11) ewes identified by ultrasound as pregnant with singleton fetuses (Figure 1). Food was withheld for 24 h prior to surgery, while drinking water remained unrestricted. Prophylactic antibiotics (1g ampicillin sodium; Aspen Pharmacare Australia, 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 sodium thiopentone (Pentothal; Boehringer-Ingelheim, Australia) and maintained by inhalation of 1.5-3% isoflurane (Delvet, Ceva, 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 well-being 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, VIC, Australia) was inserted into the lung via the nasal passage (Bischof *et al.*, 2003). In allergic ewes, 5 ml of 100 μg/ml HDM extract (in sterile saline) was delivered as a bolus infusion into each of the right and left caudal lobes of the lung, whilst control non-allergic ewes were similarly challenged with the same volume of saline (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, 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., 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 and blood pressure 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 μg/ml) for allergic sheep, or saline for control sheep, was nebulised for 15 min at 20 breaths per minute (bpm) (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 intra-thoracic 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, MA, USA), Rapid Flow™ Nebulizer bowl (Allersearch Laboratories, Oakhurt USA) and Vitalair Rapid Nebulizer Pump (Gardner Denver Thomas, Inc., Sheboygan, 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 per minute, inspiratory time 1.5 s, and a tidal volume ($V_T$) 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 extra-thoracic 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 (PFA) and processed for embedding in paraffin. Consecutive tissue sections cut at 4 µm were stained with haematoxylin and eosin (H&E), 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 mm$^2$ 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 (beta 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 (M value, factor=0.3-0.4 (Vandesompele *et al.*, 2002; Soo *et al.*, 2012; Hellemans & Vandesompele, 2014)). The relative expression of 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; Zhang *et al.*, 2013; Lie *et al.*, 2014)), cortisol availability (GR, MR, 11βHSD1 and 11βHSD2 (Gentili *et al.*, 2009)), inflammation (TNFα and IL1β), fibrosis (TGFβ, 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 qRT-PCR (Table 1) using Fast SYBR® Green Master Mix (Applied Biosystems, 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 optimized 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). Gene primers for surfactant protein (SP) -A, -B, -C and -D were as previously published (Westover & Moss, 2012), and *Rps29* rRNA was used as the reference gene because its levels were stable across fetal lung samples. *Rps29* was amplified using the following primers (F: CAGGGTTCTCGCTCTTGC 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 mean ± 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 (Figure 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 (Figure 2). In late pregnancy, macrophages comprised a greater proportion of immune cells in BAL collected pre-challenge, in allergic compared to control sheep ($P<0.05$, Figure 2). After challenge in late pregnancy, eosinophils comprised a greater proportion of immune cells in BAL and macrophages comprised a lower proportion of immune cells in BAL, in allergic compared to control sheep (each $P<0.001$, Figure 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 ($P<0.001$, Figure 2). The abundance of neutrophils in BAL was lower in pregnant compared to non-pregnant sheep overall ($P<0.05$, Figure 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 (Figure 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 (Figure 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 (Figure 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 ($P=0.056$, Figure 4). Chronic HDM challenge of the airways in allergic pregnant sheep did not alter total mast cell numbers within the parenchyma and airway wall (Figure 4 E-F). However, there was a trend to greater numbers of degranulated mast cells within the airway walls in allergic compared to control pregnant sheep (controls: 0.15 ± 0.03 cells/mm BM; allergic 0.72 ± 0.29 cells/mm BM, $P=0.093$).

Maternal body weight and absolute and relative organ weights did not differ between control and allergic sheep (Table 2, Figure 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 (Figure 6A) compared to placentae of control ewes, Type B ($P=0.037$) and Type C ($P=0.047$) placentomes comprised greater proportions of total placentome numbers in the allergic group (Figure 6B). Additionally, the total weight of Type A placentomes was lower in placentae from allergic than in those from control ewes (Figure 6C).

Absolute measures of fetal weight (-10% in allergic cf. control group) and size did not differ between treatments (Table 2, Figure 5B). However, fetal weight relative to maternal weight was 12% lower in the allergic group ($P$=0.038, Figure 5C). The singleton fetuses collected at post-mortem comprised 2 males and 3 females from control ewes, and 3 males and 4 females from allergic ewes.

Maternal allergy decreased fetal lung gene expression of SP-B ($P$=0.045, Figure 7), and this also approached significance for SP-D expression ($P$=0.053, Figure 7). Expression of SP-A and SP-C did not differ between fetuses of control and allergic ewes (Figure 7).

## Discussion

We have established a sheep model of maternal asthma in pregnancy, which demonstrates effects on fetal growth and development consistent with effects of maternal asthma in humans. Fetal body weight (relative to maternal weight) was 12% lower in asthmatic 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 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).

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 is 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).

Our findings of 10% and 12% reductions in absolute and relative fetal weights respectively in response to maternal allergic asthma is consistent with findings in human studies. 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; Murphy *et al.*, 2005; Murphy *et al.*, 2006; Murphy *et al.*, 2011; Namazy *et al.*, 2013; Mendola *et al.*, 2014). Several studies in human pregnancy indicate that the fetal response to maternal asthma is dependent on fetal sex. Thus, in the presence of maternal asthma, there is a ~12% reduction in female birth weight when compared to female neonates from non-asthmatic mothers, while there is no difference in birth weight of male infants overall (Murphy *et al.*, 2003). Critically, if the mother experiences an exacerbation during pregnancy then birth weight of male fetuses is decreased dramatically, with an associated rise in the rates of intra-uterine growth restriction (IUGR), preterm deliveries and stillbirths, whilst exacerbations do 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; Miller *et al.*, 2012; Wang *et al.*, 2013; 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 and to determine the underlying mechanisms.

We demonstrated a significant alteration in placental phenotype in the presence of maternal allergic asthma in this study. 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 change in placentome distribution with fewer Type A and more Types B, C and D (Penninga & Longo, 1998). Maternal undernutrition in early-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 d after mating) shifts placental phenotype from Type A towards Type D placentomes (Steyn *et al.*, 2001). Thus, placental changes induced by maternal asthma likely 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). The change in placental phenotype observed in the present study might also alter prostaglandin production near term, as developmental changes in protein content of the rate-limiting enzyme for prostaglandin synthesis vary between placentome sub-types (Braun *et al.*, 2011). However, we do not yet know whether this would decrease gestational age at spontaneous delivery, consistent with increased rates of preterm birth in human pregnancies complicated by asthma (Murphy *et al.*, 2011). Consistent with maternal asthma altering placental phenotype in the present study, maternal asthma is associated with increased risks of placental complications including placental abruption and placenta praevia in human pregnancies (Wang *et al.*, 2014).

Maternal allergic asthma in sheep also has effects on fetal lung development consistent with the increased risk of RDS and TTN in human neonates whose mothers have asthma,

compared to the general population (Mendola *et al.*, 2014). 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 principle cause of respiratory distress syndrome (RDS) and contributes to the pathogenesis of transient tachypnoea of the newborn (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). 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 maternal allergic asthma *per se* impairs development of the fetal lungs.

Our development and validation of this new and unique model of allergic asthma in pregnancy lays the foundation for future studies focussed 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 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, clinically-used treatments and novel interventions to be assessed in detail.

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*Competing interests*

No authors have competing interests in relation to this work.

*Author contributions*

*In vivo* studies were carried out in the Department of Physiology, Monash University. Author contributions to the study were as follows: Conception and design of experiments (VLC, TJMM, KLG, BSM, JLM, RD, MJW, RJB); collection, assembly, analysis and interpretation of data (all authors); drafting and critical revision of article for important intellectual content (VLC, TJMM, ALW, KLG, BSM, JLM, RD, MJW, RJB).

712 **Table 1.** Sequences of oligonucleotide primers used for quantitative real-time RT-PCR for
713 maternal cardiac tissues.
714

| Accession no. | Gene | Forward (F) and reverse (R) primer sequences |
|---|---|---|
| NM_001160026.1 | *BNP* | F- CCTGCTTCTCCTCTTCTTGC |
| | | R- TAGACGGTCCAACAGCTCCT |
| X55152 | *TNF$\alpha$* | F- ACACCATGAGCACCAAAAGC |
| | | R- AGGCACCAGCAACTTCTGGA |
| NM_001009465.2 | *IL1$\beta$* | F- TGCCTACGAACATGTCTTCCGTGA |
| | | R- TGCTCTCTGTCCTGGAGTTTGCAT |
| NM_001034494.1 | *PCNA* | F- ACTCCACTGTCTCCTACAGTAA |
| | | R- CGATCTTGGGAGCCAAATAGT |
| XM_005197116.1 | *Ki67* | F- TCAGTGAGCAGGAGGCAGTA |
| | | R- GGAAATCCAGGTGACTTGCT |
| NM_001014912.1 | *HO 1* | F- CTGGTGATGGCGTCTTTGTA |
| | | R- CAGCTCCTCTGGGAAGTAGA |

715

716 **Table 2**. Maternal body and organ weights at post-mortem.

717

| | 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 |

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

720

721 **Table 3**. Mean normalised expression of molecules that regulate cardiac growth and
722 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 |

723 Data are mean normalised expression ± SEM.

724

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

726

| | 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 |
| Brain:liver ratio | 0.64 ± 0.07 | 0.67 ± 0.04 |
| 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 |

727

728 Data are mean ± SEM. Unless otherwise noted, organ weights given as % are relative to fetal
729 body weight. Circ., circumference

730 **Figure legends**

731

732 **Figure 1.** Study design. *Lost to study: 4 allergic and 4 control singleton-bearing ewes were
733 lost to study due to non-pregnancy (detected at surgery; n= 1 allergic ewe), sick on farm (n=
734 1 control), failure to recover post-surgery (n= 0 control, 1 allergic ewes), fetal death (n= 1
735 control, 0 allergic ewes) or premature delivery (n= 2 control, n= 2 allergic ewes).

736

737 **Figure 2:** Immune cell populations in BAL fluid before and 48 h after airway challenge. Data
738 are numbers of (A) macrophages, (B) lymphocytes, (C) neutrophils and (D) eosinophils as a
739 proportion of all immune cells and are shown as mean +- SEM. Control animals are shown in
740 open bars, and allergic animals in filled bars, samples collected prior to airway challenge in
741 plain bars and samples collected 48 h after airway challenge are in hatched bars. Differences
742 between control and allergic animals at the same time relative to challenge are shown by *
743 $P<0.05$, ** $P<0.01$, and *** $P<0.001$, differences between pre- and 48h-post challenge
744 values within a group at each gestational age are shown by $ $P<0.05$, $$ $P<0.01$, and $$$
745 $P<0.001$, and overall differences between gestational ages are indicated by ^ $P<0.05$.

746

747 **Figure 3.** Responses to airway challenge in pregnancy. Mean percentage change from
748 baseline lung resistance ($R_L$) in allergic sheep challenged with HDM (filled circles) and
749 control sheep challenged with saline (open circles), at early, mid and late stages in pregnancy.
750 Results for each time point represent the group mean of the maximum change in resistance
751 over the first 30 min after challenge. Differences between control and allergic animals at the
752 same gestational age are shown by **, $P<0.01$. Difference in allergic animals at late
753 pregnancy compared to early pregnancy is shown by ^, $P<0.05$.

754

755 **Figure 4.** Maternal lung tissue sections stained with Masson's trichrome in (A) control and
756 (B) allergic pregnant sheep, showing collagen (blue stained areas) and airway smooth muscle
757 (black arrows) staining. Image analysis of Masson's trichrome-stained lung sections for the
758 quantitation of (C) airway smooth muscle (ASM) and (D) collagen content relative to
759 basement membrane (BM) length. Anti-tryptase mast cell$^+$ staining (white arrows;
760 degranulated mast cells indicated with open arrows) in lung tissue of (E) control and (F)
761 allergic pregnant sheep, with cell counts presented in (G) airway parenchyma, and (H) within
762 the airway wall. Differences between control and allergic animals are shown by **, $P<0.01$.

Quantitative data are presented as data from individual animals for control (open circles) and allergic (closed circles) sheep, with mean ± SEM (n=5/group).

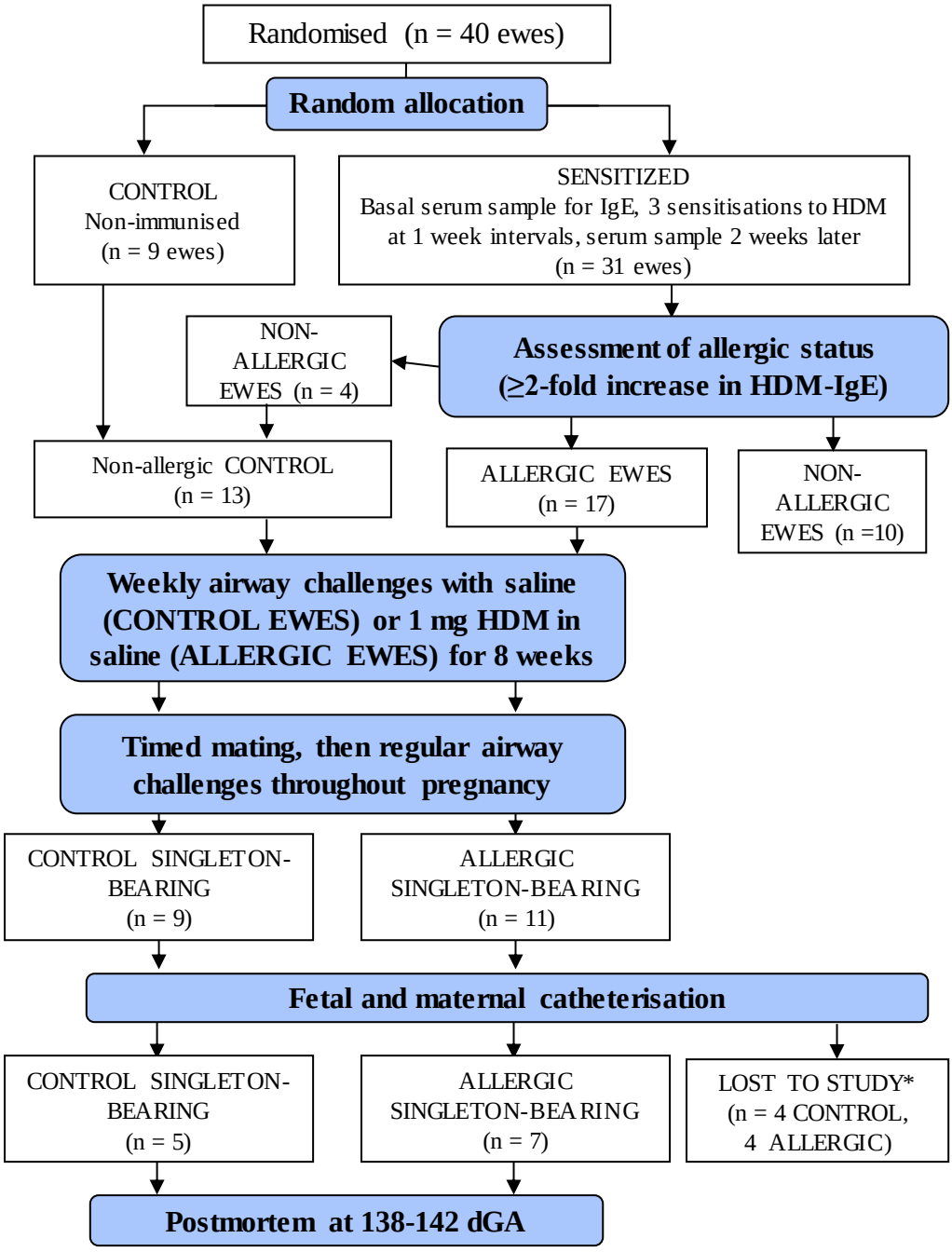
**Figure 5.** Effect of maternal asthma on (A) maternal body weight, (B) absolute fetal body weight, and (C) fetal body weight (relative to maternal weight) at ~140 dGA. Quantitative data are presented as data from individual animals for control (open circles) and allergic (closed circles) sheep, with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

**Figure 6.** Effect of maternal asthma on placental phenotype. Data are mean ± SEM. Differences between control and allergic animals are shown by *, $P<0.05$; #, $0.05<P<0.1$.

**Figure 7.** Effect of maternal allergy (black bars) on mRNA levels of surfactant proteins A, B, C and D in fetal lung. Data are presented as mean ± SEM, expressed relative to the control group (white 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 *, $P<0.05$; #, $P=0.05$

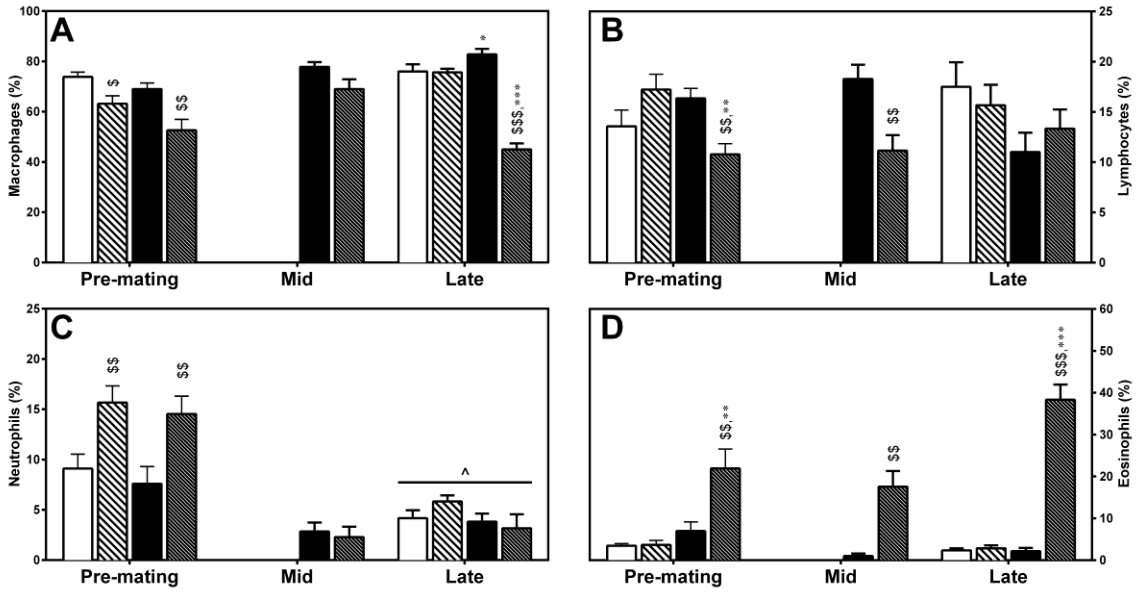
781 **Figure 1.**

782

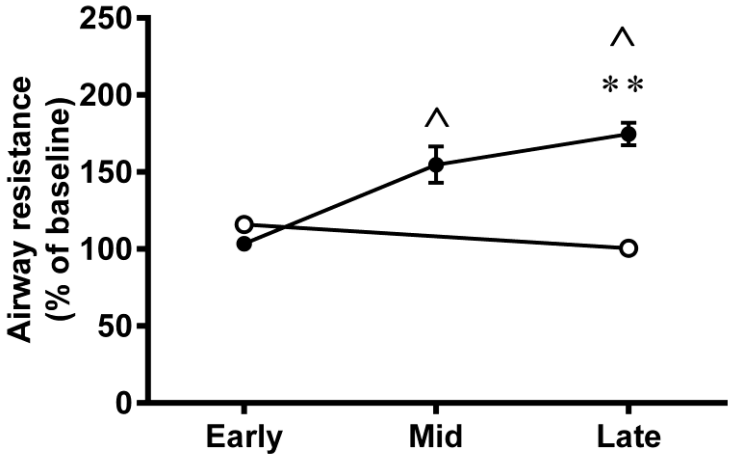



783

**Figure 2:**

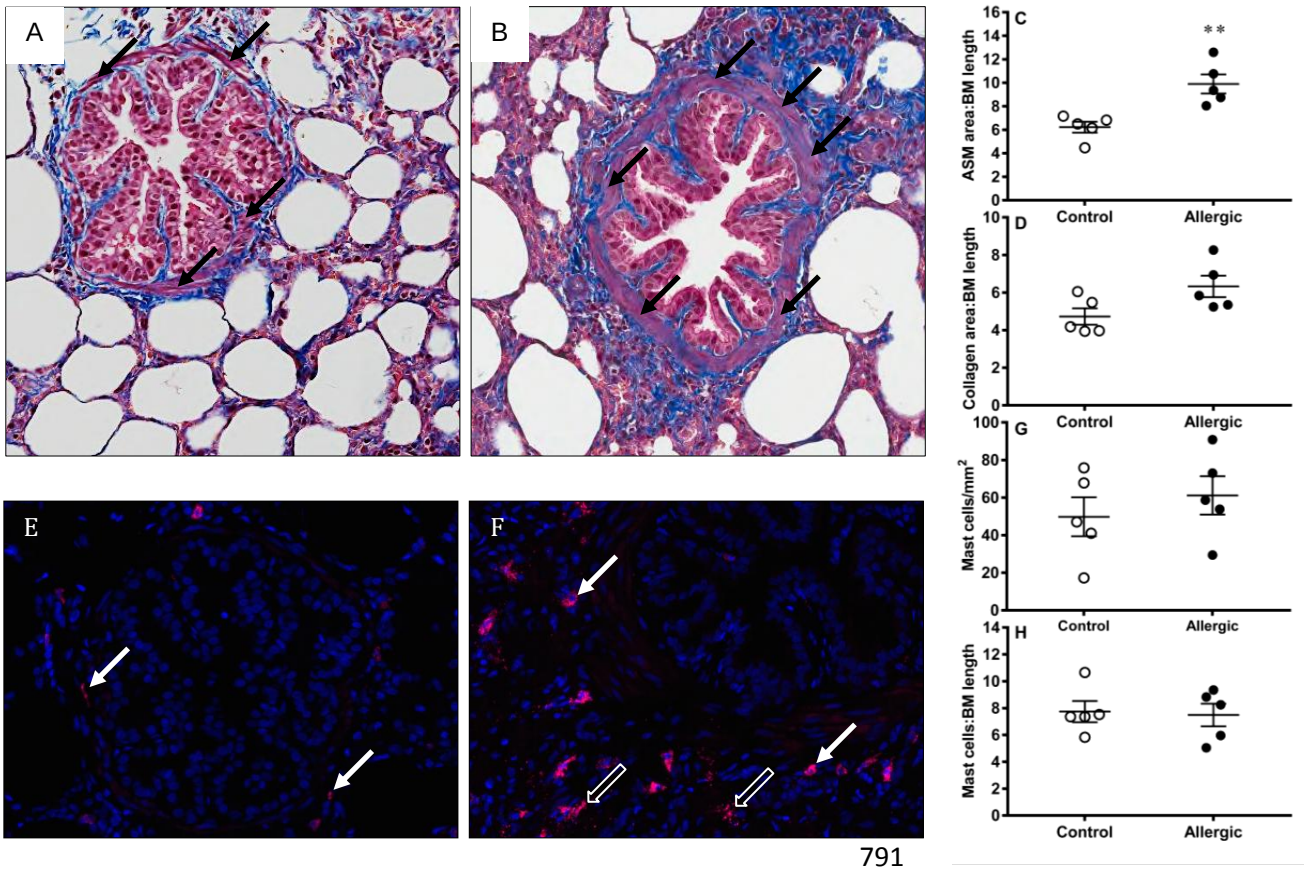

A
Macrophages (%)
100
80
60
40
20
0
$
$$
*
$$$; ***
Pre-mating
Mid
Late
B
Lymphocytes (%)
25
20
15
10
5
0
$$; **
$$
Pre-mating
Mid
Late
C
Neutrophils (%)
25
20
15
10
5
0
$$
$$
^
Pre-mating
Mid
Late
D
Eosinophils (%)
60
50
40
30
20
10
0
$$; **
$$
$$$; ***
Pre-mating
Mid
Late

787 **Figure 3.**



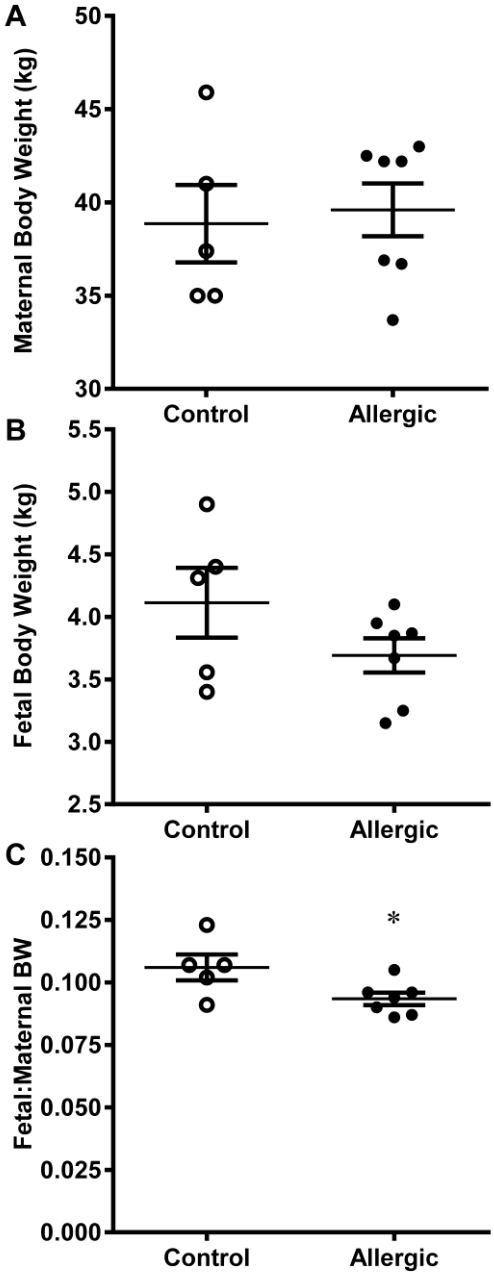

788

**Figure 4.**


A
B
E
F
C
ASM area:BM length
**
Control
Allergic
D
Collagen area:BM length
Control
Allergic
G
Mast cells/mm²
Control
Allergic
H
Mast cells:BM length
Control
Allergic

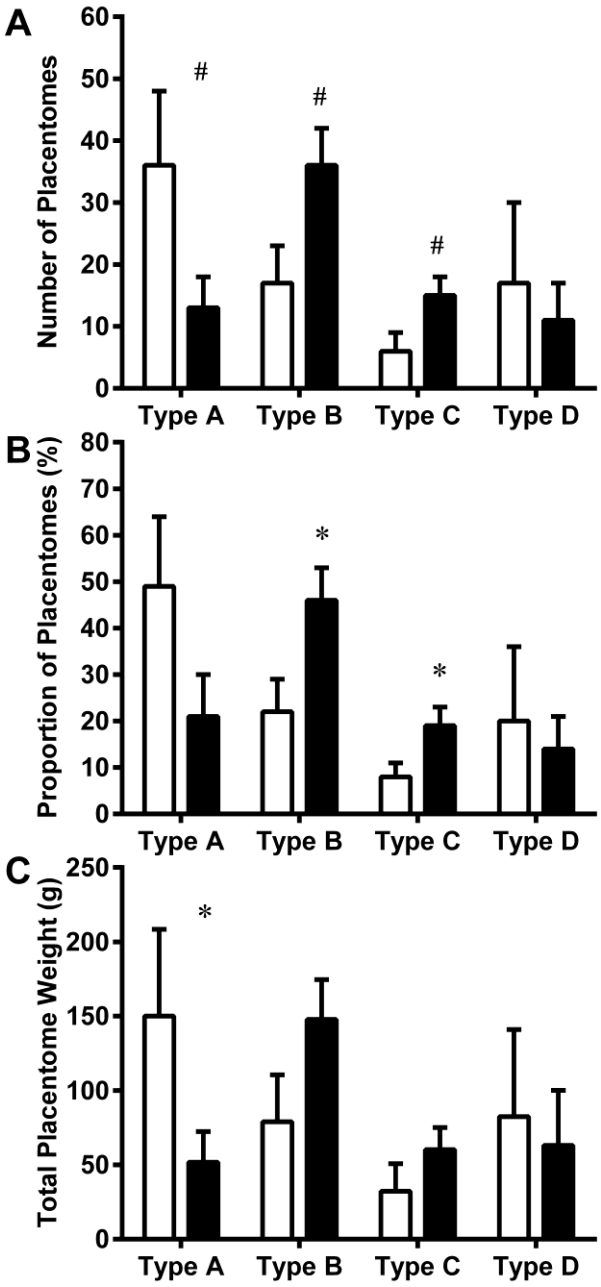
793 **Figure 5.**

794




795

796 **Figure 6.**




797
798

**Figure 7.**