Late gestation maternal dietary methyl donor and cofactor supplementation in sheep partially reverses protection against allergic sensitization by IUGR.

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Perinatal exposures are associated with altered risks of childhood allergy. Human studies and our previous work suggest restricted growth in utero (IUGR) is protective against allergic disease. The mechanisms are not clearly defined but reduced fetal abundance and altered metabolism of methyl donors are hypothesized as possible underlying mechanisms. We therefore examined whether late gestation maternal dietary methyl donor and co-factor supplementation of the placentally-restricted (PR) sheep pregnancy would reverse allergic protection in progeny. Allergic outcomes were compared between progeny from control pregnancies (CON, n=49), from PR pregnancies without intervention (PR, n=28), and from PR pregnancies where the dam was fed a methyl donor plus co-factor supplement from day 120 of pregnancy until delivery (PR+METHYL, n=25). Both PR and PR+METHYL progeny were smaller than CON; supplementation did not alter birth size. PR was protective against cutaneous hypersensitivity responses to ovalbumin (OVA, \(P < 0.01\) in singletons). Cutaneous hypersensitivity responses to OVA in PR+METHYL progeny were intermediate to and not different from responses of CON and PR sheep. Cutaneous hypersensitivity responses to house dust mite did not differ between treatments. In singleton progeny, upper dermal mast cell density was greater in PR+METHYL than PR or CON (each \(P < 0.05\)). The differences in the cutaneous allergic response were not explained by treatment effects on circulating immune cells or antibodies. Our results suggest that mechanisms underlying in utero programming of allergic susceptibility by IUGR and methyl donor availability may differ, and imply that late gestation methyl donor supplementation may increase allergy risk.

**Key words:** Animal models, mast cells, fetal growth, methyl donors, folic acid
Introduction

Allergic diseases are one of the main causes of non-communicable disease in the world and are estimated to affect 30-40% of the world’s population (43). Susceptibility to these diseases in postnatal life is programmed by an individual’s early life environment (18). Intriguingly, the majority of epidemiological studies suggest that restricted growth before birth is protective against postnatal development of allergy. In contrast, risks of asthma are consistently increased in this group, likely reflecting poorer lung function rather than allergy (8, 36). Birth weight, independent of gestational age, is positively associated with risk of allergic sensitization and atopic disease in many (6, 15, 26), although not all (23, 44, 48) studies of children and adults, whilst within twin pairs the risk of childhood atopic eczema is greater in the heavier birth weight twin (31). Consistent with small size for gestational age (SGA) being protective against later allergic disease, we have reported that surgical restriction of placental and fetal growth in sheep reduces cutaneous responses to challenge with allergen following a sensitization protocol (54).

Epigenetic mechanisms such as altered DNA methylation have been postulated as explaining persistent effects of early life exposures on later life health, including for allergy (5, 33). In a recent meta-analysis of epidemiological data, maternal dietary supplementation with methyl donors such as folate was associated with increased risks of wheeze in young children (51). Similarly, increased risk of food allergy was recently reported in 5 year-old children when the mother consumed folic acid supplements in late pregnancy (50). Although maternal supplementation was not associated with risk of allergic diseases including asthma, atopic dermatitis and eczema (51), the available data is variable and heavily influenced by other factors including timing and doses of supplements and evaluation of outcomes, as well as genetic and environmental factors. Randomization to folic acid supplements is not possible in human cohorts for ethical reasons given the protective effects against
neural tube defects, as well as practically due to widespread use of food fortification and pregnancy supplements.

Experimental evidence supports the hypothesis that elevated maternal methyl donor abundance can increase risk of allergy in progeny. Offspring from mice fed methyl donor and cofactor supplements from two weeks before and throughout gestation had greater specific IgE, allergic airway disease and cytokine responses to ovalbumin (OVA) following sensitization (25). This shift to a more allergic phenotype was associated with increased methylation and a decrease in the expression of runt-related transcription factor 3 in the lung tissue of the offspring (Runx3, 25). This gene down-regulates mechanisms that promote allergic airway disease by stimulating Forkhead Box P3 (FOXP3) expression, which is in turn drives function of regulatory T (Treg) cells (27). Also consistent with a positive relationship between methyl status during pregnancy and later susceptibility to allergy, marginal dietary restriction of methionine and choline during gestation and lactation in rat dams decreased the immune response to infection and decreased cutaneous hypersensitivity responses following sensitization in progeny at five months of age (52). There is some evidence that these effects of maternal methyl donor abundance during pregnancy on allergy in progeny may be through changes in methylation of immune-regulating genes, induced before birth. Increased DNA methylation of FOXP3 in T cells at birth is associated with reduced FOXP3 expression, decreased Treg function and Th2 cytokine production (29), and is predictive of allergy risk in infants at one year of age (24).

Based on the above, we hypothesized that the apparently protective effects of restricted fetal growth (IUGR) against later susceptibility to allergy may be due to reduced supply of methyl donors in utero, particularly in late gestation. Restricted placental function reduces the abundance of hepatic one-carbon methyl donors and alters postnatal DNA methylation in day 0 rat liver (13, 32), while SGA in humans is associated with increased methionine in cord blood (10). Placentally-
restricted (PR) sheep share many characteristics with IUGR humans (1, 46), including reduced immune responses to allergic sensitization (54). If a reduced supply of methyl donors in utero is an underlying mechanism that programs the fetus for later protection against allergy, we predict that increased methyl donor abundance in utero would at least partially reverse the protective effects of PR against allergic susceptibility in the sheep. We therefore hypothesized that supplementing the maternal diet of PR dams with methyl donors and cofactors during the last month of pregnancy would increase the responses to allergic sensitization in adolescent progeny compared to those of progeny from non-supplemented PR pregnancies. Because the changes in intradermal inflammatory response to antigen exposure did not correspond to IgE responses in our previous study of PR and control (CON) progeny, suggesting down-regulation of the skin response to antigen-IgE complexes in PR progeny (54), we also tested the hypothesis that PR decreases intradermal mast cell abundance in the present study.

Materials and Methods

All procedures were approved by the University of Adelaide Animal Ethics Committee (M-2009-145, M-2010-139 and M-2011-155) and conducted as per Australian guidelines (38).

Animal model.

Placental growth of ewes was restricted (PR) by surgically removing all but four visible endometrial placental attachment sites (caruncles) from each uterine horn (1, 46). CON ewes were un-operated. PR surgery and animal management were as described previously, with ewes housed in individual pens throughout late gestation and the first month of lactation, then group-housed in pens until weaning at 13 weeks post-delivery, and progeny housed in same-sex groups in small paddocks except when pen-housed for a week for subcutaneous hypersensitivity testing (54). Ewes were
weighed before supplementation at ~ day 119 of pregnancy (gestational day 119, g 119), at g 136 and after delivery of lambs. Lambs were weighed and measured (crown-rump length, shoulder height, abdominal circumference at the level of the umbilicus and thoracic circumference at the base of the sternum, skull width and length) within 24 h of birth, then weighed every second day until postnatal day 16 (d 16) and at least weekly until weaning, then every 5 weeks postnataally.

Neonatal absolute neonatal growth rates (AGR) and fractional growth rates (FGR) between birth and 30 days (covering the period of catchup growth in PR lambs) were calculated by linear regression (28). The present study included progeny from a subset of PR ewes who were fed a dietary methyl supplement from g 120 until delivery (PR+METHYL). PR ewes were alternately allocated to treatments in identification tag number order, stratified by expected litter size. During pregnancy and lactation, ewes were fed 1 kg Rumevite pellets daily (Ridley AgriProducts, Melbourne, Australia), with ad libitum access to lucerne chaff and water. Individual daily intakes were calculated as the weight difference between amounts fed and refusals remaining the following morning, from day 110 of pregnancy until delivery of lambs. Daily ewe energy intakes were calculated based on average energy content of Australian lucerne chaff (9 MJ/kg) and manufacturer’s specifications for pellets (9.5 MJ/kg). The methyl supplementation consisted of 2 g rumen-protected methionine (Mepron, Evonik Degussa GmbH, Hanau, Germany), 1.2 g dustless sulfur, 0.7 mg of cobalt as 5% dustless cobalt and 300 mg folic acid (F7876, Sigma, St Louis, USA), and was given in the diet by top-dressing a small amount of lucerne chaff (~200 g) with the daily supplement dose when ewes received their morning feed at 0800 to 0900 hours. The dose of folic acid in the methyl supplementation reflects a relatively low flux through the rumen and was based on doses previously fed to dairy cattle, which resulted in a respective ~doubling of maternal plasma and liver folate concentrations (17).

Methionine was given as Mepron, in a form that provides rumen by-pass protection of >70% (2), and at doses per body weight which in cattle increases by 20% the flux of methionine through the transmethylation pathway to produce the methyl donor S-adenosylmethionine (45). Sulfur and cobalt doses were selected to ensure that availability of these minerals would not limit ruminal
bacterial production of sulfated amino acids (in particular methionine) or production of vitamin B\textsubscript{12}, each of which can be limited by feeding diets deficient in these minerals in sheep which in turn increases circulating homocysteine (47). The remainder of the morning ration was offered once the lucerne chaff and supplement were consumed. Ewes were weighed on the day prior to commencing supplement (g 119) and ~ half way through the supplementation period on g 135. In order to assess the effects of PR and the supplement on maternal abundance and in utero transfer of these nutrients to the lamb, blood samples were collected by jugular venipuncture from the ewe and neonatal lamb for later measurement of folate, methionine and cysteine. Blood samples were collected at least 2 h after delivery to minimize disruption of perinatal behaviors, and were collected within 24 h of delivery. Blood was collected into lithium heparin or EDTA tubes on ice, centrifuged, and plasma stored at -80°C for later analysis. In the present manuscript, we have restricted analyses to those samples collected within 4 h of birth (n = 33 ewes, 44 lambs) to avoid potential dilution of effects over time, since ewes did not receive supplements after delivery. Immune function in progeny was studied in 24 CON males (2 singletons, 20 twins, 2 triplets), 25 CON females (7 singletons, 18 twins), 11 PR males (6 singletons, 5 twins), 17 PR females (12 singletons, 4 twins, 1 triplet), 10 PR+METHYL males (5 singletons, 5 twins) and 15 PR+METHYL females (8 singletons, 7 twins). Allergic sensitization to OVA and house dust mite (HDM) was carried out from 20-26 weeks of age, with cutaneous hypersensitivity testing at 28 weeks of age. Our sheep were most likely peri-pubertal, since cross-bred progeny of Australian Merino sheep attain puberty at an average age of 35 weeks, although this varies with season and nutritional status (12).

**Analyses of 1-carbon metabolites in plasma**

Plasma vitamin B\textsubscript{12} and folate concentrations were measured in plasma harvested from blood collected using EDTA as anti-coagulant, using a commercially available kit (Simultrac Radioassay Vitamin B\textsubscript{12} \textsuperscript{[57]Co]/Folate \textsuperscript{[125]I} Kit; MP Biomedicals), as described previously for sheep (47).
Unfortunately plasma vitamin B\textsubscript{12} concentrations were above the standard range for 50\% of lamb samples and 85\% of ewe samples and sufficient volume was not available to allow dilution and re-analysis, so no data could be included for plasma vitamin B\textsubscript{12} concentrations. Folate concentrations below the minimal detectable concentration (1.0 ng/mL) were entered as 0.9 ng/mL for analysis, and those with values above the upper standard of 20.0 ng/mL were entered as 20.1 ng/mL for analysis.

Plasma methionine and S-adenosyl-methionine concentrations were measured by LC-MS in plasma harvested from blood collected using heparin as anti-coagulant. Water-soluble metabolites were extracted as following: 200 µL ice-cold methanol was added to 50 µL plasma in Eppendorf tube, vortexed for 10 seconds and incubated at -20 °C for 20 min. Samples were then centrifuged at 13200 rpm for 15 min and the supernatant taken as extract. The extracts were dried under nitrogen flow and reconstituted in LC-MS grade water using a ratio of 1 mL water for 50 µL plasma. Samples were analyzed on two separate instrument platforms to cover both positive charged and negative charged metabolites. Negative charged metabolites were analyzed via reverse-phase ion-pairing chromatography coupled to an Exactive orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA). The mass spectrometer was operated in negative ion mode with resolving power of 100,000 at m/z 200, scanning range being m/z 75-1000. Liquid chromatography was performed on a Synergy Hydro-RP column (100 mm × 2 mm, 2.5 µm particle size, Phenomenex, Torrance, CA) with a flow rate of 200 µL/min as described previously (30). The LC gradient was 0 min, 0% B; 2.5 min, 0% B; 5 min, 20% B; 7.5 min, 20% B; 13 min, 55% B; 15.5 min, 95% B; 18.5 min, 95% B; 19 min, 0% B; 25 min, 0% B. Solvent A is 97:3 water:methanol with 10 mM tributylamine and 15 mM acetic acid; solvent B is methanol. Positive charged metabolites were analyzed on a Q Exactive Plus mass spectrometer coupled to Vanquish UHPLC system (ThermoFisher Scientific, San Jose, CA). The mass spectrometer was operated in positive ion mode with resolving power of 140,000 at m/z 200, scanning range being m/z 75-1000. The LC separation was achieved on an Agilent Poroshell 120 Bonus-RP column (150 x 2.1 mm, 2.7 µm particle size). The gradient was 0 min, 50 µL/min, 0% B; 6 min, 50 µL/min, 0% B; 12 min, 200 µL/min, 70% B; 14 min, 200 µL/min, 100% B; 18 min, 200 µL/min,
100% B; 19 min, 200 µL/min, 0% B; 24 min, 200 µL/min, 0% B; 25 min, 50 µL/min, 0% B. Solvent A is 10 mM ammonium acetate + 0.1% acetic acid in 98:2 water:acetonitrile and solvent B is acetonitrile (42). Data were analyzed using MAVEN software (34).

Immunization, sensitization and cutaneous hypersensitivity testing.

In vivo immune outcomes in the majority of these CON and PR progeny have been reported previously (54); the present manuscript adds additional animals and investigates the effects of maternal methyl donor and cofactor supplementation and the treatment groups were studied concurrently. The sheep were immunized at 5 and 9 weeks of age with anti-Clostridial vaccine (Ultravac 5-in-1; Pfizer Animal Health, West Ryde, Australia) as part of routine management. Sheep were separately immunologically sensitized with 50 µg HDM allergen (CSL Ltd, Parkville, Australia) and 100 µg OVA (A2512, Sigma, Missouri, USA) with aluminum hydroxide as adjuvant (1:1) by subcutaneous injections at 20, 22, 24 and 26 weeks of age (3, 4, 54). These allergens are common allergens in humans and sheep can be sensitized to each of these to assess allergic susceptibility (3, 54). In humans, several proteins in HDM are causal for allergic asthma whilst sensitization to the single protein OVA is a cause of food allergy, and responses to these two allergens can differ in other animal models (7). Immediate and delayed cutaneous responses (cutaneous hypersensitivity, skin prick test, SPT) to intradermal injections of 50 µL saline (negative control), histamine (10 µg/mL; histamine diphosphate, Sigma), HDM (100 µg/mL) and OVA (10 µg/mL) were assessed at 28 weeks of age (54). Skin wheal responses were measured with digital calipers at 0.5, 4, 24 and 48 h after injections, and an average diameter across two perpendicular readings of ≥3 mm was classified as a positive skin reaction (54).

Circulating blood cell counts and serum antibody concentrations.
Peripheral blood was collected by jugular venipuncture prior to sensitizations at 20 weeks of age and immediately prior to cutaneous hypersensitivity tests at 28 weeks of age. Serum was stored at -80 °C until analysis of HDM- and OVA-specific total Ig, IgG, IgE, IgA and IgM at both time points, and Clostridial-specific total Ig at 28 weeks of age, in duplicate by antigen-specific ELISA (3, 4, 54).

Antibody responses to sensitization were classified as positive when they increased at least 2-fold relative to basal concentrations (3, 4). Peripheral blood was collected into EDTA-coated tubes at 18 weeks of age (two weeks prior to sensitizations, in 64% of the cohort) and 33 weeks of age (~7 weeks after final sensitization, in all animals) for quantification of total red and white blood cells, and of white blood cell subtypes (54).

Quantitation of intradermal mast cells.

Postmortems were conducted at 52 weeks of age. Sheep were humanely killed by an intravenous overdose of thiopentone (Troy Laboratories, New South Wales, Australia). A full-thickness section of skin (~1 cm²) was dissected from mid-way down the left side of the sheep over the third rib and fixed in 10% neutral-buffered formalin. Skin samples were subsequently embedded in paraffin and 5 µm-thick cross-sections of skin tissue were stained with toluidine blue (0.5% toluidine blue in 0.5 M HCl, pH 0.8) for 30 min before light counterstaining with fresh 2% eosin in distilled water (modified from 53). The slides were scanned (NanoZoomer 2.0-HT (C9600-13) digital slide scanner, Hamamatsu Photonics, Japan) then fields spanning the depth of the upper dermis and perpendicular to the skin surface were digitally captured by random systematic sampling (0.299 mm²/field, 22-47 fields per sample, NDP.view NanoZoomer Digital Pathology software version 1.2.36, Hamamatsu Photonics, Japan). Numbers of cells with metachromatic purple staining and mast cell morphology such as the presence of small purple granules (Figure 3A) were counted in all fields (ImageJ, version 1.48, National Institutes of Health, USA).
Statistical analysis.

Average weekly ewe feed intakes were analyzed using a Generalized Linear Mixed Models (GLMM) framework that examined the effects of treatment (CON, PR, PR+METHYL), litter size (singleton birth vs. multiple birth), and week of pregnancy, treating the dam as the experimental unit. Continuous and binary outcomes in progeny were analyzed using a GLMM framework that examined the effects of treatment (CON, PR, PR+METHYL), litter size (singleton birth vs. multiple birth), and sex, treating the dam as the experimental unit and sibling data as repeated measures on each dam.

Concentrations of 1-carbon metabolites in neonatal lamb plasma were analyzed using a similar GLMM model but excluding lamb sex, which did not affect concentrations of these metabolites. Ewe weights and concentrations of 1-carbon metabolites in ewe plasma were analyzed using a mixed model framework including treatment and litter size and including the ewe as a random factor. Where treatment effects or trends were apparent ($P < 0.1$), we compared means for each treatment by the least significant difference method based on *a priori* questions to 1) determine the effects of placental restriction (CON cf. PR treatments), 2) determine effects of maternal methyl donor and cofactor supplementation in progeny from PR pregnancies (PR cf. PR+METHYL treatments), and 3) assess whether maternal methyl donor and cofactor supplementation restored values to those of controls (CON cf. PR+METHYL treatments). The distributions of continuous variables were assessed for normality, and a square root, log or inverse transformation was applied as required. Binary outcomes (responders or non-responders in antibody fold-changes and SPT) were also analyzed within this framework, assuming a binomial distribution and utilizing a logit link function. Interaction effects were non-significant for all binomial outcomes, and the final models used for these outcomes included only main effects. Relationships between circulating concentrations of 1-carbon metabolites in ewes and their neonatal lambs were analyzed through the calculation of Pearson’s correlation coefficient, using all available ewe-lamb pair data. Relationships between all other continuous variables were analyzed through the calculation of Pearson’s correlation coefficient,
restricted to singleton birth sheep to remove effects of clustering due to ewes. Data were analyzed
using SPSS version 20.0 (SPSS Inc., Chicago, USA) and are presented as estimated means ± SE. P <
0.05 was accepted as significant. Interactions are not described unless significant.

Results

Circulating concentrations of 1-carbon metabolites

In both ewes and lambs, plasma folate (Figure 1A) was higher in PR+METHYL animals compared to
both CON (ewes: \( P = 0.001 \), lambs: \( P = 0.006 \)) and PR groups (ewes: \( P = 0.014 \), lambs: \( P = 0.006 \)).

Plasma folate was higher in ewes who had delivered twins than in those who had delivered singleton
lambs (\( P = 0.043 \)), but did not differ between twin and singleton lambs (\( P > 0.8 \), Figure 1A). Maternal
and neonatal lamb plasma concentrations of folate were positively correlated overall (Figure 1A),
and within CON pairs (\( R = 0.870 \), \( P < 0.001 \), \( n = 24 \)), with a similar trend within PR+METHYL pairs (\( R =
0.557 \), \( P = 0.060 \), \( n = 9 \)), but not within PR pairs (\( R = 0.100 \), \( P > 0.4 \), \( n = 7 \)).

Plasma methionine (Figure 1B) tended to be higher in PR+METHYL ewes compared to CON (\( P = 0.054 \))
or PR ewes (\( P = 0.059 \)), was higher in PR+METHYL than CON lambs (\( P = 0.015 \)) and tended to be
higher in PR than CON lambs (\( P = 0.086 \)). Plasma methionine concentrations tended to be higher in
ewes who had delivered twins than in those who had delivered singleton lambs (\( P = 0.082 \)), and
were higher in twin than singleton lambs (\( P = 0.014 \), Figure 1B). Maternal and neonatal lamb plasma
concentrations of methionine were positively correlated overall (Figure 1B), and within CON (\( R =
0.421 \), \( P = 0.025 \), \( n = 22 \)) and PR+METHYL pairs (\( R = 0.724 \), \( P = 0.006 \), \( n = 11 \)), but not within PR pairs
(\( R = -0.268 \), \( P > 0.2 \), \( n = 7 \)).
Plasma S-adenosyl-methionine (Figure 1C) was lower in PR than CON ewes \((P = 0.001)\), and tended to be lower in PR+METHYL than CON ewes \((P = 0.057)\). In lambs, plasma S-adenosyl-methionine (Figure 1C) tended to be higher in PR+METHYL \((P = 0.061)\) and PR groups \((P = 0.070)\), compared to CON (Figure 1C). Plasma S-adenosyl-methionine concentrations did not differ between litter size groups in either ewes or lambs (Figure 1C). Maternal and neonatal lamb plasma concentrations of S-adenosyl-methionine were not significantly correlated overall (Figure 1C), or within CON \((R = 0.395, P = 0.069, n = 22)\), PR \((R = 0.692, P = 0.085, n = 7)\), or PR+METHYL pairs \((R = 0.094, P > 0.7, n = 11)\).

**Ewe phenotype, birth size and neonatal growth**

Ewe phenotype: Ewe weights did not differ between treatment groups before supplementation at g 119 or ~half-way through supplementation at g 136 \((P > 0.3\) for each). At g 119 of pregnancy, ewes who subsequently delivered two or three lambs tended to be heavier than those who delivered singletons (singleton-bearing ewes: 87.3 ± 1.8 kg; multiple-bearing ewes: 91.9 ± 1.9 kg, \(P = 0.088)\). The multiple-bearing ewes were heavier than singleton-bearing ewes at g 136 (singleton-bearing ewes: 93.1 ± 1.8 kg; multiple-bearing ewes: 99.4 ± 1.9 kg, \(P = 0.017)\). Absolute and daily weight gains between these gestational ages were similar between treatments and litter sizes \((P > 0.4\) for each).

On a weekly average basis, ewe intakes of lucerne chaff \((1618 ± 65 g/day)\), pellets \((924 ± 22 g/day)\) and energy \((23.3 ± 0.7 MJ DE/day)\) did not change from day 113 to day 147 of pregnancy, nor differ between treatments or between ewes carrying singleton or multiple birth pregnancies \((P > 0.1\) for all main effects and interactions). Gestation length ranged between 139-151 days, and differed between treatments (overall: \(P = 0.035\), singleton birth: \(P < 0.002\), Table 1). Within singleton birth sheep, PR pregnancies \((145.9 ± 0.4 d)\) had gestation lengths that were 1.8 d shorter than CON pregnancies \((147.7 ± 0.6 d, P = 0.014)\), and PR+METHYL pregnancies \((145.7 ± 0.4 d)\) were 2.0 d shorter than CON pregnancies \((P = 0.019)\). Gestation lengths were similar between PR and PR+METHYL pregnancies. Overall, pregnancies with male offspring \((146.7 ± 0.4 d)\) were 1.0 d longer
than pregnancies with female offspring (145.7 ± 0.3 d, \( P = 0.014 \)). In singleton birth sheep, pregnancies with male offspring (147.1 ± 0.5 d) were 1.1 d longer than pregnancies with female offspring (146.0 ± 0.3 d, \( P = 0.014 \)). Gestation length was not affected by litter size, either overall or in singleton birth sheep.

Birth size: Birth weight differed between treatment groups (overall: \( P < 0.001 \), singleton birth: \( P = 0.001 \)), such that birth weight was reduced by PR (overall: by 17% \( P < 0.002 \), singleton birth: by 24%, \( P = 0.004 \)) and PR+METHYL (overall: by 26%, \( P < 0.001 \), singleton birth: by 35%, \( P < 0.001 \)), in comparison to CON lambs (overall outcomes in Table 1; singleton birth CON: 6.37 ± 0.43 kg, PR: 4.83 ± 0.27 kg, PR+METHYL: 4.12 ± 0.30 kg). Birth weight was not significantly different between PR and PR+METHYL sheep, both overall and in singleton birth sheep (overall: \( P = 0.098 \); singleton birth: \( P = 0.081 \)). Overall, birth weight of males was greater than that of females (males: 5.16 ± 0.19 kg; females: 4.46 ± 0.14 kg, \( P < 0.001 \)), although birth weight did not differ between sexes within singleton birth sheep. Other measures of size at birth were similar in male and female lambs (\( P > 0.05 \)), except that skull width was greater in males than in females (males: 6.41 ± 0.05 cm; females: 6.19 ± 0.04 cm, \( P < 0.001 \)). Birth weight of singleton birth sheep was greater than that of multiple birth sheep (singleton birth: 5.10 ± 0.18 kg; multiple birth sheep: 4.52 ± 0.16 kg, \( P = 0.017 \)) with similar effects of litter size for other measures of length, abdominal and thoracic circumferences and skull size at birth, but no effect of litter size on shoulder height (\( P > 0.05 \)). Most other measures of body size at birth were lower in PR+METHYL and PR lambs than CON lambs, with few differences between PR and PR+METHYL groups (Table 1). Skull length at birth was lower in PR+METHYL than PR lambs overall (\( P = 0.017 \)). In singleton birth offspring, PR+METHYL offspring had lower shoulder heights than either CON (\( P = 0.012 \)) or PR (\( P = 0.029 \)) offspring, but PR offspring did not differ from CON offspring (singleton birth CON: 41.3 ± 1.7 cm, PR: 39.1 ± 0.9 cm, PR+METHYL: 36.3 ± 0.9 cm). Also within singletons, PR+METHYL sheep had smaller skull widths compared to CON (\( P < 0.001 \)) or PR (\( P = 0.005 \)) sheep, and this measure did not differ between PR and CON (singleton birth CON: 6.41 ± 0.05 cm; females: 6.19 ± 0.04 cm, \( P < 0.001 \)).
6.67 ± 0.12 cm, PR: 6.41 ± 0.08 cm, PR+METHYL: 6.06 ± 0.09 cm). Within sheep from multiple birth
litters, effects of treatment on skull width differed with sex (treatment × sex: \( P < 0.038 \)). There was
no treatment effect on skull width within males from multiple birth litters, but in females from
multiple birth litters, PR lambs had smaller skull widths than CON lambs (\( P = 0.034 \)) and PR+METHYL
offspring did not differ from either CON or PR offspring (multiple birth female CON: 6.22 ± 0.04 cm,
PR: 6.03 ± 0.08 cm, PR+METHYL: 6.08 ± 0.06 cm).

Neonatal growth: We have reported previously that in singletons within this cohort, fractional
growth rates (FGR) in the first month of life were higher in PR than CON lambs, while absolute
growth rates (AGR) were similar between these groups (28). In the cohort including CON, PR and
PR+METHYL lambs, absolute growth rates in the first 30 d after birth did not differ between
administration groups (CON: 0.329 ± 0.013 kg/d, PR: 0.329 ± 0.012 kg/d, PR+METHYL: 0.327 ± 0.012 kg/d,
\( P > 0.9 \)). Males grew faster than females in absolute terms (males: 0.346 ± 0.011 kg/d, females:
0.311 ± 0.008 kg/d, \( P = 0.010 \)). Not unexpectedly, AGR was higher in singleton lambs than in lambs
from multiple births (singleton lambs: 0.343 ± 0.010 kg/d, multiple-birth lambs: 0.314 ± 0.009 kg/d, \( P
= 0.042 \)). No interactions between factors affected AGR in the first month of life. Neonatal FGR in the
first month of life differed with treatment (\( P < 0.001 \)), but was not different between sexes (\( P > 0.4 \))
or litter size groups (\( P > 0.9 \)), nor were interactions between main factors significant (all \( P > 0.4 \)).
Neonatal FGR differed between all groups, being higher in PR+METHYL (8.1 ± 0.3 %/d) than CON (6.0
± 0.3 %/d, \( P < 0.001 \)) or PR (7.3 ± 0.3 %/d, \( P = 0.045 \)), and higher in PR than CON (\( P = 0.005 \)).

Circulating red and white blood cell counts.

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

At 33 weeks of age, treatment and sex did not affect levels of circulating red blood cells (overall mean: $10.4 \pm 0.1 \times 10^9$/L), white blood cells (overall mean: $8.6 \pm 0.2 \times 10^9$/L) and white blood cell subtypes (overall mean for monocytes: $1.3 \pm 0.1 \times 10^9$/L; eosinophils: $0.1 \pm 0.0 \times 10^9$/L; basophils: $0.1 \pm 0.0 \times 10^9$/L either overall or within singleton birth sheep and did not affect concentrations of circulating neutrophils or eosinophils in singleton birth sheep. Treatment and litter size did not affect circulating concentrations of lymphocytes or neutrophils either overall or within singleton birth sheep. Overall, males had greater circulating concentrations of neutrophils than females (males: $3.71 \pm 0.23 \times 10^9$/L, females: $2.88 \pm 0.18 \times 10^9$/L, $P = 0.005$), but within singleton birth sheep this was not statistically significant. The circulating concentration of lymphocytes at 33 weeks of age was greater in females than in males, both overall (females: $4.29 \pm 0.21 \times 10^9$/L, males: $3.20 \pm 0.27 \times 10^9$/L, $P = 0.005$) and in singleton birth sheep (females: $4.34 \pm 0.32 \times 10^9$/L; males: $3.00 \pm 0.48 \times 10^9$/L, $P = 0.043$).

**Antibody responses.**

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

**HDM-specific total Ig, IgG, IgA and IgM antibodies:** The proportion of animals that showed positive plasma HDM-specific antibody responses (≥2-fold increase after sensitization), and the fold-

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382 monocytes: $1.3 \pm 0.1 \times 10^9$/L; eosinophils: $0.1 \pm 0.0 \times 10^9$/L; basophils: $0.2 \pm 0.0 \times 10^9$/L either overall or within singleton birth sheep, and litter size did not affect these concentrations in overall analyses.

385 At 33 weeks of age, treatment and sex did not affect levels of circulating red blood cells (overall mean: $10.4 \pm 0.1 \times 10^9$/L), white blood cells (overall mean: $8.6 \pm 0.2 \times 10^9$/L) and white blood cell subtypes (overall mean for monocytes: $1.3 \pm 0.1 \times 10^9$/L; eosinophils: $0.1 \pm 0.0 \times 10^9$/L; basophils: $0.1 \pm 0.0 \times 10^9$/L either overall or within singleton birth sheep and did not affect concentrations of circulating neutrophils or eosinophils in singleton birth sheep. Treatment and litter size did not affect circulating concentrations of lymphocytes or neutrophils either overall or within singleton birth sheep. Overall, males had greater circulating concentrations of neutrophils than females (males: $3.71 \pm 0.23 \times 10^9$/L, females: $2.88 \pm 0.18 \times 10^9$/L, $P = 0.005$), but within singleton birth sheep this was not statistically significant. The circulating concentration of lymphocytes at 33 weeks of age was greater in females than in males, both overall (females: $4.29 \pm 0.21 \times 10^9$/L, males: $3.20 \pm 0.27 \times 10^9$/L, $P = 0.005$) and in singleton birth sheep (females: $4.34 \pm 0.32 \times 10^9$/L; males: $3.00 \pm 0.48 \times 10^9$/L, $P = 0.043$).

Antibody responses.

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

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

**HDM-specific IgE antibodies**: In the entire cohort, the proportion of animals with positive plasma HDM-specific IgE responses was greater in PR sheep than CON sheep (CON: 37.7 ± 8.0%, PR: 78.4 ± 8.3%, *P = 0.001*) or PR+METHYL sheep (33.8 ± 9.8%, *P = 0.001*) and did not differ between CON and PR+METHYL sheep, nor with sex or litter size. Similarly in singleton birth sheep, the proportion of animals with positive HDM-specific IgE responses was greater in PR sheep than CON sheep (CON: 5.4 ± 6.1%, PR: 63.5 ± 14.3%, *P = 0.001*) with a similar trend for PR+METHYL sheep (39.0 ± 16.0%, *P = 0.050*) and did not differ between PR and PR+METHYL sheep. In singleton birth sheep, the proportion of HDM-specific IgE responders was greater in females than in males (females: 51.4 ± 13.1%, males: 13.1 ± 9.5%, *P = 0.042*). In analyses of the entire cohort, effects of treatment on the fold increase in HDM-specific IgE differed depending on levels of other factors (treatment × sex × litter size *P = 0.059*). In males alone, the fold-increase in HDM-specific IgE did not differ between treatments or litter sizes. In females, effects of treatment on the fold-increase in HDM-specific IgE differed with litter size (treatment × litter size: *P = 0.005*). In singleton birth females, the fold-increase in HDM-specific IgE differed between treatments (*P = 0.015*) such that both PR (*P = 0.027*) and PR+METHYL (*P = 0.005*) sheep had greater fold-increases in HDM-specific IgE than CON sheep (CON: 1.39 ± 0.76-fold, PR: 3.04 ± 0.59-fold, PR+METHYL: 3.93 ± 0.67-fold). Fold-increases in HDM-specific IgE did not differ between singleton birth female PR sheep and singleton birth female PR+METHYL sheep.

**OVA-specific total Ig and IgM**: The proportion of animals with positive OVA-specific total Ig or IgM antibody responses in plasma (≥2-fold increase after sensitization) did not differ between treatments, overall (total Ig: 97.0%; IgM: 4.3% responders) or within singleton birth sheep (total Ig: 92.3%; IgM: 8.1% responders).
OVA-specific IgE: The proportion of animals with positive plasma OVA-specific IgE antibody responses (≥2-fold increase after sensitization) did not differ between treatments, overall (7.0% responders) or within singleton birth sheep (5.1% responders). In multiple birth sheep, the fold increase in OVA-specific IgE differed between treatments (P = 0.046), such that PR+METHYL sheep had lower fold-increases in OVA-specific IgE than PR sheep (PR: 1.77 ± 0.19-fold, PR+METHYL: 1.13 ± 0.18-fold, P = 0.015).

OVA-specific IgG\textsubscript{1}: The proportion of animals with positive plasma OVA-specific IgG\textsubscript{1} antibody responses (≥2-fold increase after sensitization) did not differ between treatments, overall (67.3% responders) nor within singleton birth sheep (66.7% responders). Fold-increases in OVA-specific IgG\textsubscript{1} did not differ between treatments, sexes or litter size overall. Within singleton birth sheep, there was a treatment effect (P = 0.030) such that PR+METHYL singleton birth sheep had greater fold-increases in OVA-specific IgG\textsubscript{1} than PR singleton birth sheep (PR: 2.74 ± 0.50-fold, PR+METHYL: 4.10 ± 0.54-fold, P = 0.010). Fold-increases in OVA-specific IgG\textsubscript{1} in CON singleton birth sheep (2.74 ± 0.88-fold) did not differ from either PR or PR+METHYL singleton birth sheep. There was no effect of sex.

OVA-specific IgA: The proportion of animals with positive plasma OVA-specific IgA antibody responses (≥2-fold increase after sensitization) did not differ between treatments, overall (2.9% responders) or within singleton birth sheep (no responders). The effect of treatment on the fold increase in OVA-specific IgA differed between sexes (treatment × sex: P = 0.018). In females, treatment affected fold-increases in OVA-specific IgA (P = 0.024), such that PR females had greater fold-increases in OVA-specific IgA than CON females (CON: 0.96 ± 0.09-fold, PR: 1.37 ± 0.10-fold, P = 0.009). PR+METHYL females (1.21 ± 0.09-fold) also tended to have greater fold-increases in OVA-specific IgA when compared with CON females (P = 0.053), but did not differ from PR females; similar treatment differences were observed in females from multiple birth litters when analyzed
separately. Also within females, litter size affected this antibody response, with multiple birth females having greater fold-increases in OVA-specific IgA compared with singleton females (multiple birth females: 1.33 ± 0.08-fold, singleton birth females: 1.03 ± 0.07-fold, \( P = 0.019 \)). There were no effects in males. Females had greater fold increases in OVA-specific IgA than males in multiple birth sheep (males: 0.97 ± 0.13, females: 1.33 ± 0.09, \( P = 0.027 \)).

**Cutaneous hypersensitivity responses.**

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

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

**OVA:** In singleton-born sheep cutaneous responses at 24 h after injection of OVA differed between treatments (Figure 2B, \( P = 0.049 \)), although these groups did not differ when singleton and multiple-birth animals were combined (Figure 2A). The proportion of singleton birth PR sheep that responded positively to OVA was lower than that of CON singleton birth sheep (\( P = 0.002 \), Figure 2B). The proportion of positive cutaneous responders to OVA at 24 h was intermediate in PR+METHYL singleton birth sheep and not different from either CON or PR singleton birth sheep (Figure 2B). In the combined cohort and in singleton-born animals analyzed separately, treatment, sex and litter
size did not affect the proportion of positive cutaneous responses to OVA at 30 min (overall: 91 ± 3%; singletons: 92 ± 6%), 4 h (overall: 73 ± 5%, singletons: 76 ± 9%), or 48 h (overall: 27 ± 5%; singletons: 29 ± 8%).

Mast cell density.

Upper dermis mast cell densities (Figure 3A) were higher in sheep from multiple birth pregnancies than in those from singleton birth pregnancies (overall means, singleton birth: 43.5 ± 2.2 mast cells/mm$^2$, multiple birth: 53.7 ± 2.4 mast cells/mm$^2$, $P = 0.002$, Figure 3B) and did not differ between sexes, either overall or in singleton birth sheep. Effects of treatment tended to differ between litter size groups (treatment $\times$ litter size $P = 0.092$, Figure 3B), and we therefore analysed outcomes separately in singleton and multiple birth groups. Within singleton birth sheep, there was a treatment effect ($P = 0.040$, Figure 3B), such that PR+METHYL sheep had greater upper dermis mast cell densities than CON ($P = 0.040$) and PR ($P = 0.027$) sheep. Upper mast cell densities did not differ between CON and PR singleton birth sheep. Within multiple birth sheep, effects of treatment differed between sexes ($P = 0.020$, Figure 3B), with no treatment effects on mast cell densities evident in females. In males from multiple birth litters, mast cell densities were >50% higher in PR than CON sheep ($P < 0.001$). Wheal diameter responses during acute response to histamine (30 min and 4 h) and during delayed phase response to OVA and HDM allergens (24 and 48 h) in SPT were not correlated with mast cell densities in adult skin, overall or within singletons.

Discussion

Consistent with our hypothesis, we have shown in the present study that maternal dietary methyl donor and cofactor supplementation during late gestation in PR sheep pregnancies partially blocks the protection against adolescent susceptibility to allergic inflammation afforded by restricted
growth before birth. Cutaneous hypersensitivity responses to allergens after prior sensitization were intermediate in PR progeny whose mothers had received methyl donor and cofactor supplementation in late pregnancy, compared to progeny of un-supplemented PR and control pregnancies. Interestingly, altered antibody responses to allergic sensitization did not explain the effect of maternal methyl donor supplementation on changes in cutaneous hypersensitivity. Methyl supplementation also did not rescue the restricted birth phenotype induced by PR in this model of IUGR, implying its effects on allergic susceptibility are not due to increased fetal growth. Treatment effects on cutaneous hypersensitivity responses to allergens were also not explained by changes in newborn circulating abundance of folate, methionine and S-adenosyl-methionine. Our results suggest that the one-carbon pathway is one mechanism underlying developmental programming of the immune system and allergy, although mechanisms underlying effects of IUGR and methyl donor supplementation may differ. Whether other changes to the fetal environment characteristic of uteroplacental insufficiency in PR fetuses, such as hypoxia and elevated circulating corticosteroids (21), also program subsequent immune development remains to be evaluated.

The maternal supplementation strategy chosen for the present study was designed to increase maternal circulating abundance of both one-carbon donors and cofactors important for the one-carbon pathway. The rationale for this approach was based on previous observations of reduced one-carbon metabolite abundance and altered methylation in late gestation rat IUGR fetuses in a model of severe uteroplacental insufficiency (13, 32), and on epidemiological and experimental findings suggesting that high maternal methyl donor consumption in late gestation is associated with increased incidence of allergic disease in progeny (18, 25). Ewes in the present study received not only folic acid and rumen-protected methionine, but also cobalt and sulfur to maximize rumen bacterial synthesis of Vitamin B\textsubscript{12} and sulfated amino acids such as methionine, respectively (47). In a previous experiment we showed that this supplement increased maternal plasma folate by ~10-fold during late gestation without altering maternal plasma homocysteine concentrations, although
limited neonatal samples meant we were not able to demonstrate that this altered 1-carbon abundance in progeny (49). The present study adds evidence that maternal dietary supplementation increases folate abundance in the newborn lamb as well as the ewe, consistent with increased folate transfer to the fetal lamb. Recommended peri-conceptional folate intakes for human pregnancy are 500-600 μg/d (16). In order to compensate for loss of the majority of folate during ruminal passage, our dietary supplement in pregnant sheep contained far higher levels of folic acid (300 mg/d) than those recommended in human pregnancy. The ~2.5-fold increase in circulating folate we achieved in PR ewes was consistent with the responses in lactating cattle fed similar folic acid doses per body weight (17). The dietary supplement fed to sheep resulted in a greater relative increase in circulating folate (~150% higher in supplemented than non-supplemented PR ewes) than the 33-66% increases in serum folate in women taking 250-800 or >1000 μg/d of supplementary folic acid (14). This may in part reflect the much lower basal (non-supplemented) circulating folate concentrations in sheep compared to women. Plasma folate concentrations at delivery averaged 1.3 ng/mL (2.9 nmol/L) in control ewes and 3.4 ng/mL (7.7 nmol/L) in non-supplemented PR ewes, far lower than serum concentrations in non-supplementing pregnant women at 6-20 weeks gestation (19.5 nmol/L, 14). Despite the greater fold-increase induced by dietary supplementation, average circulating folate concentrations in supplemented pregnant PR ewes (8.8 ng/mL, 19.9 nmol/L) were still lower than those measured in late gestation or at term in populations of pregnant women where most were consuming vitamin supplements (20-37 nmol/L, 9, 19, 35). Also consistent with results of human studies (9, 19, 35), circulating folate concentrations in newborn lamb plasma was higher than that in maternal plasma, and neonatal and maternal abundance were strongly and positively correlated. This has been suggested to reflect active transport of folate from mother to fetus in humans (9). Although maternal dietary methyl donor and cofactor supplementation of PR ewes increased folate concentrations in newborn lambs, neither plasma methionine nor S-adenosyl-methionine concentrations differed between PR and PR+METHYL lambs. Indeed, greater plasma methionine concentrations in twin than singleton fetal
lams and in PR+METHYL than CON lambs, with similar and intermediate high levels in PR lambs, suggests that elevated circulating methionine in the newborn lamb is a marker of fetal restriction. This might reflect reduced methionine use in IUGR fetuses, perhaps due to reduced protein synthesis under conditions of low oxygen and nutrient availability and decreased circulating concentrations of anabolic hormones (21). Although maternal dietary supplementation increased folate abundance in the newborn lamb and partially reversed the protection against allergic sensitization observed in PR progeny, PR did not reduce folate abundance at birth. It is therefore possible that different mechanisms underlie the opposite effects of PR and maternal dietary methyl donor and cofactor supplementation on progeny susceptibility to allergy. Additional evidence that PR perturbs the fetal one-carbon pathway is required to support the hypothesis of an epigenetic mechanism underlying allergic protection in this sheep model of IUGR.

Maternal dietary supplementation with methyl donors and cofactors did not normalize the birth weight of PR sheep reported previously in this cohort (54). In fact, birth size of PR+METHYL neonates was generally slightly smaller than the PR group, and this was significant for skull size measures. This is despite the fact that the gestation lengths were normalized in supplemented PR ewes and were similar to CON ewes, and is consistent with the relatively small effect of PR on gestation length (~ 2 d shorter cf. 147 d at term in CON) not being a major contributor to their reduced size at birth. This effect of supplement also does not appear to be due to altered maternal food intake or growth, with similar food and energy intakes and weight changes during late gestation in CON, PR and PR+METHYL ewes. In healthy pregnant women, maternal supplementation with folic acid or 5-methyltetrahydrofolate from the second trimester of pregnancy until term increased birth weight and did not change gestational age at delivery (11), but this is not directly comparable to pregnancy complications such as IUGR. Interestingly, maternal folic acid supplementation protects mice from LPS-induced preterm birth, suggesting that maternal folic acid supplementation down-regulates inflammation-associated processes that promote parturition (55). The lack of effect of the
supplement on birth weight is consistent with methyl-donor abundance not being the limiting factor for fetal growth in this experimental IUGR model where supply of oxygen and nutrients is limited by reduced placental blood flow and nutrient transfer (39-41).

Consistent with our previous findings in CON and PR offspring (54), circulating immune cells (proportions and concentrations) and antibody responses to Clostridial vaccination were not affected by prenatal exposure to PR or PR+METHYL. We have previously reported that antigen-specific IgE responses to HDM but not OVA were upregulated in PR compared to CON sheep (54). Overall, maternal methyl donor supplementation reduced the proportion of IgE responders to HDM sensitization in PR progeny to similar response rates as CON progeny, although the magnitude of increases in HDM-specific IgE after sensitization were similar in PR and PR+METHYL progeny. There was a reduced magnitude of OVA-specific IgE response in PR+METHYL compared with PR sheep, but only in progeny from multiple birth litters. Thus, changes in OVA-specific IgE responses do not appear to underlie the treatment effects on cutaneous hypersensitivity responses, which we observed only within singleton birth progeny. Overall, this suggests that methyl donor supply in utero probably had limited effects on antigen-induced antibody production in our experimental model. In contrast, Hollingsworth reported that in utero exposure to a methyl-supplemented diet in mice increased antigen (OVA)-induced increases in IgE and IgG, as well as allergic responses to airway challenge with OVA (25). Interestingly, we observed that the effects of prenatal environment on response rates and size differed between antigens, with no effects of treatment on the proportion of sheep with a positive OVA-specific IgE response. In mice, OVA-sensitization of dams before pregnancy increases susceptibility of progeny to asthma development in response to systemic and airway challenge with either OVA or casein, suggesting that the programming effect of an allergic mother is not antigen-specific in this case (20). These results suggest that assessing responses to multiple antigens may be needed to fully characterize effects of the prenatal environment on allergic susceptibility.
As we have reported previously, PR progeny had reduced rates of cutaneous hypersensitivity responses to OVA, and this was antigen-specific, with no change in this response to HDM (54). This is consistent with the majority of epidemiological studies suggesting a protective effect of IUGR, SGA or LBW against development of allergic disease such as eczema in later life (18). Our new findings of intermediate rates of cutaneous hypersensitivity responses to OVA in the PR+METHYL progeny suggests that in utero supply of methyl donors may be important determinants of the inflammatory response to allergens. Others have suggested that epigenetic mechanisms underlie the persistent effects of early life exposures on allergic disease (33). Consistent with the hypothesis that increased methyl donor abundance promotes allergic susceptibility and may involve altered methylation of gene networks important for immune function, maternal supplementation with methyl donors throughout pregnancy in mice increased methylation of the Runx3 gene, which is involved in negative regulation of allergic airway disease (25). Furthermore, increased methylation of the immunoregulatory gene FOXP3 in human cord blood correlates positively with a neonate’s risk of developing allergic disease in early life (24). Whether the epigenetic state and regulation of Runx3, FOXP3 or other immune-regulating genes are altered in our sheep model is yet to be determined. We suggest that these, as well as other markers of altered 1-carbon metabolism should be the subject of further studies.

Unexpectedly, maternal methyl donor and cofactor supplementation increased mast cell density in the upper dermis of adult PR progeny, which might contribute to their partial loss of protection against cutaneous sensitization to OVA. Consistent with this hypothesis, increased cutaneous mast cell densities persisted for several months after experimentally-induced allergic dermatitis in mice and were associated with increased allergic airway hyper-reactivity following sensitization and challenge with an independent allergen (22). Whether methyl donor supplementation and metabolism alter mast cell proliferation, differentiation or lifespan, and potential epigenetic
mechanisms for such changes, requires further investigation. The similar mast cell densities in PR
and CON progeny do not, however, support our hypothesis that restricted growth in utero decreases
mast cell density in the upper dermis, based on evidence for decreased mast cell activation in PR
progeny (54), although cutaneous responses and mast cell density were measured at different ages
(28 and 52 weeks respectively). Decreased cutaneous hypersensitivity responses in PR compared to
CON sheep might therefore reflect either decreases in mast cell function, or changes in other
regulatory pathways such as altered basophil function (37). To our knowledge, this is the first report
that maternal methyl donor supplementation during pregnancy affects mast cell abundance in
progeny.

Perspectives

In the present study, maternal methyl donor and cofactor supplementation in late gestation,
reduced the protective effect of PR on cutaneous hypersensitivity responses after OVA sensitization
in singleton birth sheep. Effects of PR and maternal supplementation on allergic susceptibility in
sheep are consistent with the hypothesis that the late gestation fetal environment modulates fetal
immune development and hence progeny allergic susceptibility. These ovine models will allow the
additional studies of one-carbon metabolism, fetal immune development and methylation of genes
important for immune function that are required to define the underlying pathways for
programming of allergy by IUGR and maternal methyl supplementation. Maternal folic acid
supplementation in women before conception and during neural tube formation, which is complete
at ~28 days of gestation, is undeniably effective in reducing risks of neural tube defects in their
babies (16). However, our results add to those of previous studies in preclinical models, and to data
from epidemiological evidence, suggesting that late gestation methyl donor supplementation may
increase allergy risk in progeny. Additional studies are needed to clarify the potential risks for
progeny allergic outcomes of continued folic acid supplementation throughout human pregnancy,
particularly in high risk populations such as those with family history of allergy. We recommend that
pregnancy advice should continue to include the recommendation that maternal folic acid
supplementation be given peri-conceptionally, but that patients should be counselled regarding the
potential increased risks of progeny allergy of continuing folic acid supplementation for the entirety
of pregnancy.

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Disclosures

The authors have no conflicts of interest to disclose.
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Figure 1. Circulating 1-carbon metabolite concentrations in ewes and lambs on the day of birth in control (CON; white), placentally-restricted (PR; black) and PR with maternal dietary methyl donor and cofactor supplementation (PR+M; grey), and in singleton (fine hatching) and twin (coarse hatching) litters, and relationships between maternal and neonatal abundance. A. Plasma folate (Ewes: CON: n=15; PR: n=9; PR+M: n=9; Lambs: CON: n=24; PR: n=11; PR+M: n=9). B. Plasma methionine (Ewes: CON: n=14; PR: n=9; PR+M: n=10; Lambs: CON: n=24; PR: n=11; PR+M: n=11). C. Plasma S-adenosyl-methionine (Ewes: CON: n=14; PR: n=9; PR+M: n=10; Lambs: CON: n=24; PR: n=11; PR+M: n=11). Plasma concentrations of methionine and S-adenosyl-methionine represent relative ionic abundance obtained by LC-MS analysis. Progeny outcomes were analyzed using a Generalized Linear Mixed Models framework that examined the effects of treatment (CON, PR, PR+METHYL) and litter size (singleton birth vs. twin birth), treating the dam as the experimental unit and sibling data as repeated measures on each dam. No significant interactions were evident between treatment and litter size and data are shown as estimated means ± SE for each main factor. Where treatment effects or trends were apparent (P < 0.1), we compared means for each treatment by the least significant difference method as described under statistical analyses. Within treatments (a,b) or litter size groups (c,d) in ewes or lambs, groups that do not share a common letter differ (P < 0.05). Relationships between ewe and neonatal lamb concentrations for each metabolite, for all ewe-lamb pairs including twins, were assessed by Pearson’s correlation; overall relationships and 95% confidence intervals are shown on right-hand Figure panels while relationships within treatment groups are described in results text.

Figure 2. Proportion of positive (cross-hatched, skin wheal diameter ≥3 mm) and negative (solid color, skin wheal diameter <3 mm) responders at 24 h after intradermal challenge with ovalbumin in control (CON; white), placentally-restricted (PR; black) and PR with maternal dietary methyl
donor and cofactor supplementation (PR+M; grey) after a sensitization protocol. A. Overall cohort (CON: n=49; PR: n=28; PR+M: n=25). B. Singleton birth sheep only (CON: n=9; PR: n=18; PR+M: n=13). Binary outcomes (responders and non-responders) were analyzed using a Generalized Linear Mixed Models framework that examined the effects of treatment (CON, PR, PR+METHYL), litter size (singleton birth vs. multiple birth), and sex, treating the dam as the experimental unit and sibling data as repeated measures on each dam, assuming a binomial distribution and utilizing a logit link function. Where treatment effects or trends were apparent ($P < 0.1$), we compared means for each treatment by the least significant difference method as described under statistical analyses.

Figure 3. A. Upper dermis of skin sections from adult sheep were stained with toluidine blue; mast cells (indicated by arrows) stain metachromatically purple. Scale bar is 100 µm in length. B. Upper dermis mast cell density in singleton birth and multiple birth male (open bars) and female (closed bars) sheep in control (CON: n=49), placentally-restricted (PR: n=28) and PR with maternal dietary methyl donor and co-factor supplementation (PR+M: n=25). Outcomes were analyzed using a Generalized Linear Mixed Models framework that examined the effects of treatment (CON, PR, PR+METHYL), litter size (singleton birth vs. multiple birth), and sex, treating the dam as the experimental unit and sibling data as repeated measures on each dam. Where treatment effects or trends were apparent ($P < 0.1$), we compared means for each treatment by the least significant difference method as described under statistical analyses. Values are estimated means ± SE; *$P < 0.05$; **$P < 0.01$, ***$P < 0.005$. 
Table 1. Effect of PR and late pregnancy maternal dietary methyl donor and cofactor supplementation on body size at birth.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CON (n = 49)</th>
<th>PR (n = 28)</th>
<th>PR+METHYL (n = 25)</th>
<th>Treatment significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M:F</td>
<td>24:25</td>
<td>11:17</td>
<td>10:15</td>
<td></td>
</tr>
<tr>
<td>Gestational length (days)</td>
<td>147.0 ± 0.5</td>
<td>145.0 ± 0.4</td>
<td>146.6 ± 0.5</td>
<td>0.035*†</td>
</tr>
<tr>
<td>Birth weight, kg</td>
<td>5.62 ± 0.22</td>
<td>4.64 ± 0.20</td>
<td>4.17 ± 0.20</td>
<td>&lt;0.001*‡</td>
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<tr>
<td>Crown-rump length, cm</td>
<td>52.6 ± 1.1</td>
<td>49.7 ± 1.0</td>
<td>47.4 ± 1.0</td>
<td>0.002‡</td>
</tr>
<tr>
<td>Shoulder height, cm</td>
<td>40.4 ± 0.8</td>
<td>38.3 ± 0.6</td>
<td>37.0 ± 0.6</td>
<td>0.006*‡</td>
</tr>
<tr>
<td>Abdominal circumference, cm</td>
<td>37.3 ± 0.8</td>
<td>35.2 ± 0.8</td>
<td>33.7 ± 0.8</td>
<td>0.009‡</td>
</tr>
<tr>
<td>Thoracic circumference, cm</td>
<td>37.9 ± 0.7</td>
<td>35.9 ± 0.7</td>
<td>34.1 ± 0.7</td>
<td>0.001*‡</td>
</tr>
<tr>
<td>Skull width, cm</td>
<td>6.44 ± 0.06</td>
<td>6.29 ± 0.05</td>
<td>6.16 ± 0.05</td>
<td>0.003†^</td>
</tr>
<tr>
<td>Skull length, cm</td>
<td>13.3 ± 0.2</td>
<td>12.8 ± 0.1</td>
<td>12.3 ± 0.1</td>
<td>&lt;0.001*†‡</td>
</tr>
</tbody>
</table>

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

A  

B  

C  

Ewe plasma S-adenosyl-methionine (SAM) (ion counts)

Lamb plasma S-adenosyl-methionine (SAM) (ion counts)

R = -0.304, P = 0.056

Ewe plasma folate (ng/mL)

Lamb plasma folate (ng/mL)

R = 0.549, P < 0.001

Ewe plasma methionine (ion counts)

Lamb plasma methionine (ion counts)

R = 0.546, P < 0.001
Figure 2.

CON  PR  PR+M

A  B
Figure 3.

A

B

Singleton birth       Multiple birth

Mast cell density (cells/mm$^2$)

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>PR</th>
<th>PR+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>20</td>
<td>11</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>40</td>
<td>11</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>60</td>
<td>15</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>80</td>
<td>19</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
<td>15</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

**P < 0.05**
***P < 0.001**

A

B
### A
**Plasma Folate (ng/mL)**
- **Ewe**
  - CON: 5
  - PR: 7
  - PR+M: 10
  - Single: 8
  - Twin: 9
- **Lamb**
  - CON: 5
  - PR: 7
  - PR+M: 10
  - Single: 8
  - Twin: 9

**R = 0.549, P < 0.001**

### B
**Plasma Methionine (ion counts)**
- **Ewe**
  - CON: 5
  - PR: 7
  - PR+M: 10
  - Single: 8
  - Twin: 9
- **Lamb**
  - CON: 5
  - PR: 7
  - PR+M: 10
  - Single: 8
  - Twin: 9

**R = 0.546, P < 0.001**

### C
**S-Adenosylmethionine (ion counts)**
- **Ewe**
  - CON: 5
  - PR: 7
  - PR+M: 10
  - Single: 8
  - Twin: 9
- **Lamb**
  - CON: 5
  - PR: 7
  - PR+M: 10
  - Single: 8
  - Twin: 9

**R = -0.304, P = 0.056**
Singleton birth

CON  PR  PR+M

Mast cell density (cells/mm²)

0  20  40  60  80  100

Multiple birth

CON  PR  PR+M

B

Singleton birth  Multiple birth

Mast cell density (cells/mm²)

2 7 6 11 5 8 1915 5 2 3 7

A