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Distinct Sex-Specific Gene Expression Changes in the Placenta in Association with Childhood Allergy

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Abstract

Background: The prevalence of allergic disease has risen significantly during recent years. A major component of the susceptibility to allergic disease is determined in prenatal life, when the placenta plays a central role in fetal growth and development. In this study, we aimed to identify the patterns of gene expression in the placenta that may program early immune function to increase susceptibility to allergy.

Methods: A set of immune genes known to be associated with asthma, allergy and inflammation were selected for analysis by quantitative real-time polymerase chain reaction (qRT-PCR) on placental tissue from infants who did or did not develop an allergy by 2 years of age. Analysis was performed on males and females separately for each allergy type including eczema, rhinitis or asthma.

Results: Of 11 candidate allergy-associated genes tested by qRT-PCR, 4 were found to be associated with the development of specific childhood allergy types ($P < 0.05$). These included *MMP9* for both males and females that developed eczema, *TLR7* for females that developed eczema, *KITL1* for males that developed rhinitis and ORMDL3 for females that developed asthma.

Conclusions: This study has identified altered expression of placental genes involved in inflammation in association with the development of specific allergies in childhood. The current data provide supporting evidence implicating the placenta in programming the fetal immune system in early life.

Keywords

Human, Placenta, Allergy, Child, Gene, Immune, Eczema, Rhinitis, Asthma

Introduction

There has been an epidemic rise in allergic disease since the second-half of the twentieth century, particularly in Western countries [1,2]. Diseases such as eczema, allergic rhinitis and food allergies now represent significant burdens to human health. Australia has one of the highest rates of allergic disease, with allergic sensitisation evident in up to 40% of children and asthma accountable as the most common cause of chronic disease in childhood [3,4]. It is now clear that developmental events play a critical role in determining susceptibility to allergy [5-7]. Exposures during pregnancy, when developing fetal systems are particularly vulnerable to environmental influences, could have significant effects on the programming of disease susceptibility [8].

The placenta plays a central role in fetal growth and development and acts as the immunological and metabolic interface between the mother and fetus. It has been demonstrated that levels of immune mediators detected in the fetus, of which the placenta is the major source, correlate with subsequent development of allergy [9].

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Furthermore, reduced mRNA levels of regulatory T cell (TREG) marker, FOXP3, have been reported in the placentae of infants who subsequently developed an allergic disease [10]. Thus, the placenta has great potential to program the fetal immune system, potentially increasing the susceptibility to allergy after birth. The concept of altered placental gene expression influencing fetal programming is supported by a recent study in mice. Knockout of the placenta-specific insulin-like growth factor-2 (*Igf2*) P0 transcript (*Igf2*-P0 KO) created an imbalance between fetal nutritional demand and placental supply of nutrients, leading to the offspring displaying significantly increased anxiety in later life [11]. Defining the molecular mechanisms in the placenta that may be altered in association with childhood allergy is vital for identifying causal pathways of altered fetal immune programming that increase the risk of allergy susceptibility.

Previous work by our team has demonstrated that the human placenta functions in a sex specific manner with significant differences in global gene and protein expression [12]. In particular there are significant differences in the placental immune response to the presence of maternal allergy [13] and its regulation by glucocorticoids between male and female placentae [14] with female placentae appearing more sensitive to an immune challenge and glucocorticoids relative to male placentae. These findings suggest that any placental immune dysregulation that leads to the susceptibility to allergy in later life may be vary in a sex specific manner.

In this study, we hypothesised that childhood susceptibility to allergy is increased by significant alterations in the *in utero* environment which include sex specific alterations in placental function that may program the development of the fetal immune system. We aimed to identify genetic pathways altered in the placentae of children who subsequently developed allergy in early childhood and to explore whether there are differences in relation to the allergic phenotype a child develops. We also aimed to determine whether there are sex differences in the expression profile. To do this, we selected a set of genes with immune functions known to be associated with asthma, allergy and inflammation for analysis on placental tissue from male or female infants who did or did not develop an allergy in early childhood. While the immune genes examined in this study were not exhaustive of those previously associated with allergy, we have chosen a select number of strong candidates for investigation in this study based on preliminary findings of placental microarray work comparing children with and without allergy by 4 years of age. From these, we have identified several immune genes that are associated with the development of specific childhood allergy phenotypes.

Methods and Materials

Study participants

This work was approved by the Government of South Australia (SA) Health Human Research Ethics Committee (TQEH/LMH/MH)

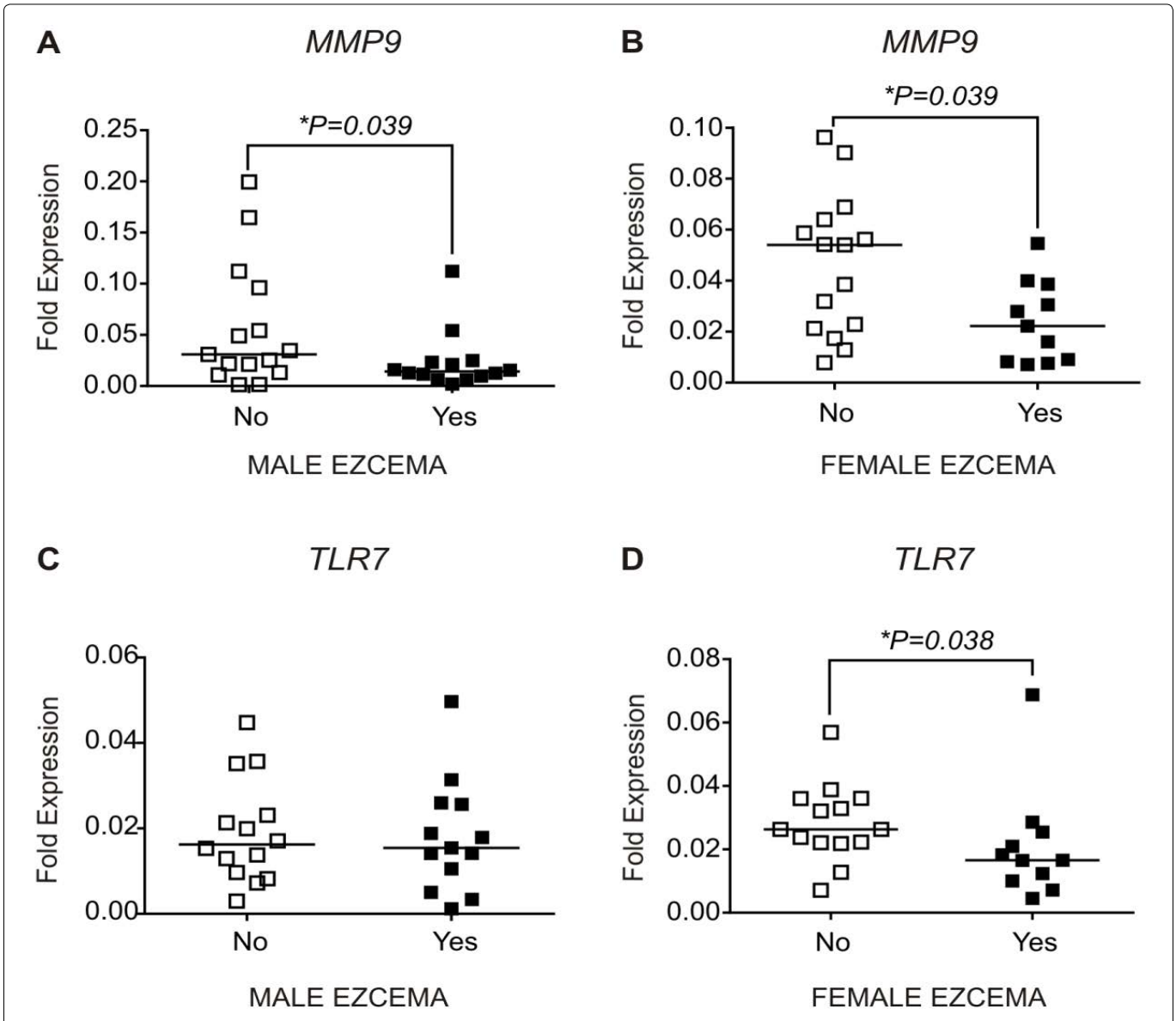

Figure 1: Gene expression levels in placentae of offspring with eczema compared to placentae from offspring without eczema. The solid bar indicates the median. Genes are expressed relative to β-actin. Male no eczema n = 14, male eczema n = 14, female no eczema n = 15, female eczema n = 11.* indicates significantly different to no allergy, P < 0.05 (Mann-Whitney U test).

and the University of Adelaide Human Research Ethics Committee (2009045). Women were recruited from the antenatal clinic at the Lyell McEwin Hospital (Elizabeth, South Australia, Australia) during the first trimester of their pregnancy following written, informed consent. Women who smoked during pregnancy were excluded. Women were assessed throughout pregnancy at 12, 18, 30 and 36 weeks for atopic status, diet and fetal growth. Placentae were collected within 45 minutes of delivery and multiple samples from the central and peripheral regions of the maternal side of placenta were pooled together, snap frozen and stored at -80°C.

Follow-up data was collected on infants every 12 months from 6 months of age until 36 months of age. Allergy (asthma, rhinitis and eczema, but not food allergy) was determined by their general practitioner and/or allergy specialist and reported to the parent, who then completed a modified version of the International Study of Asthma and Allergy in Childhood (ISAAC) questionnaire [15,16], delivered at each follow-up visit. ISAAC is a collaborative project which has developed a standardized methodology to describe the prevalence and severity of asthma, rhinitis and eczema in children throughout the world [15,16]. Key questions from the ISAAC questionnaire were used to gather data on symptoms of asthma, allergic rhinitis, and atopic eczema. Rhinitis was defined if the parents reported "Yes" to the question, "Has your child ever had hayfever?", or if parents responded yes to both questions, "In the last 12 months, has your child had a problem with sneezing, or a runny, or a blocked nose when he/she DID NOT have a cold or the flu?" and "In the last 12 months, has this nose problem been accompanied by itchy/watery eyes?" Eczema was defined if the parents reported "Yes" to any one of the following questions: "In the last 12 months, has your child had a dry itchy rash at any time?" and "Has your child ever had eczema?" Asthma was defined at the 36 month visit if the parents reported "Yes" to the question, "Has your doctor ever told you that your child has asthma?"

RNA extraction

Total RNA was extracted from frozen placental tissue using the Trizol method as previously described [14,17]. RNA concentration was measured using an IMPLEN spectrophotometer, and RNA quality and integrity was assessed using a 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). Only RNA samples demonstrating intact 28S and 28S rRNA peaks and a high RNA integrity number (RIN) (> 6) were included in the study. Reverse transcription was performed on 1 µg of total RNA using the iScript cDNA Synthesis kit (Biorad, Hercules, CA, USA) according to the manufacturer's instructions.

Quantitative RT-PCR

Taqman Gene Expression Assays (Life Technologies, Carlsbad, CA, USA) were used to measure expression of putative allergy-associated genes (identified following microarray analysis, data

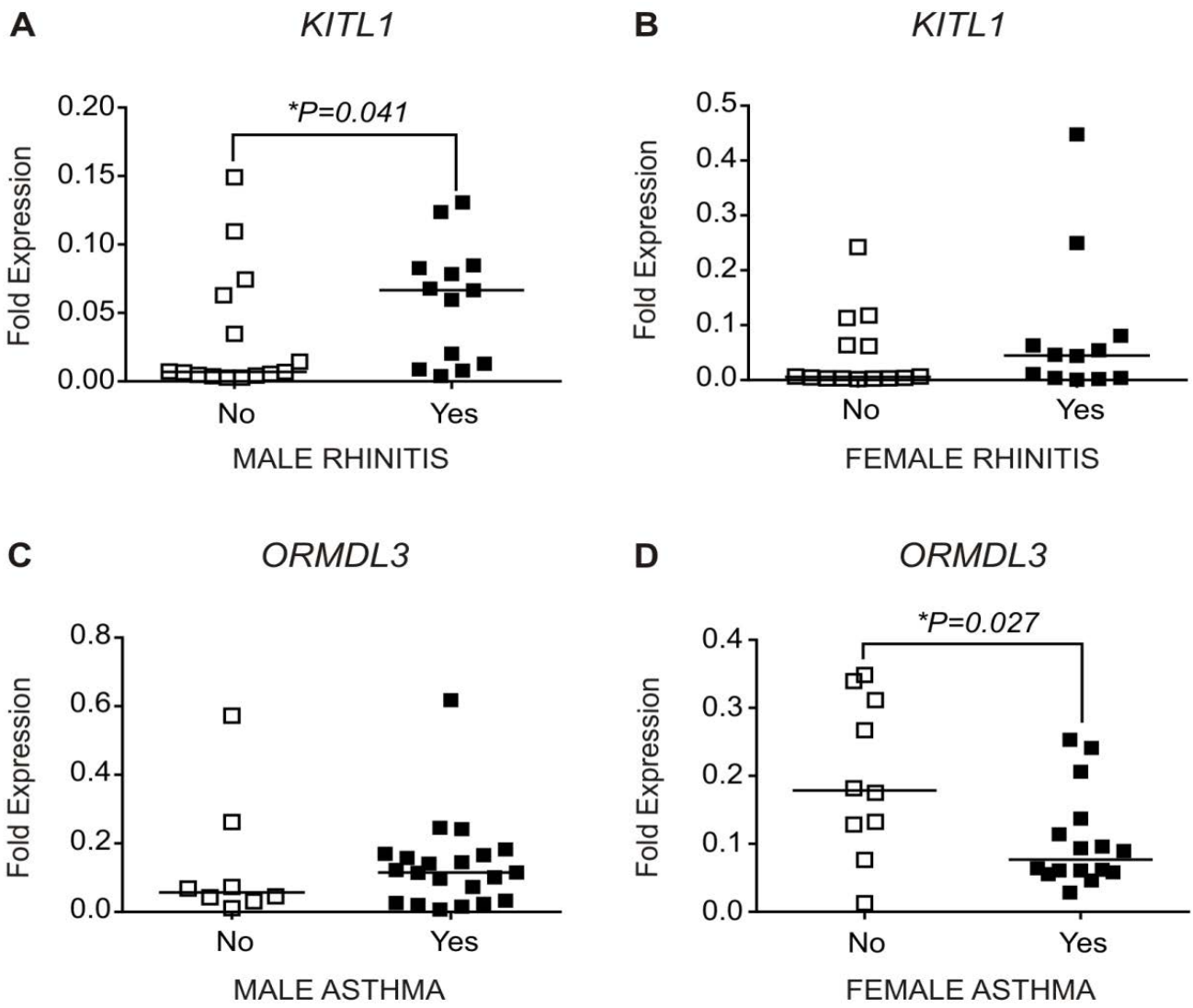

Figure 2: Gene expression levels in placentae of offspring with either rhinitis or asthma compared to placentae from offspring without rhinitis or asthma. The solid bar indicates the median. Genes are expressed relative to β-actin. Male no rhinitis n = 15, male rhinitis n = 13, female no rhinitis n = 14, female rhinitis n = 12, male no asthma n = 7, male asthma n = 21, female no asthma n = 10, female asthma n = 16. * indicates significantly different to no allergy, P < 0.05 (Mann-Whitney U test).

Table 1: Maternal characteristics during pregnancy and neonatal characteristics

	RHINITIS				ASTHMA				ECZEMA			
	No		Yes		No		Yes		No		Yes	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
n	15	14	13	12	7	10	21	16	14	15	14	11
Maternal age (yr)	26.3 ± 5.9	27.8 ± 6.0	27.8 ± 6.6	27.9 ± 6.2	29.7 ± 5.6	25.9 ± 5.6	25.1 ± 3.1	29.1 ± 7.5	26.5 ± 6.1	26.0 ± 5.8	27.2 ± 6.6	28.7 ± 6.0
Maternal BMI (pregnancy)	26.3 ± 5.0	27.3 ± 4.0	28.8 ± 6.2	28.7 ± 6.6	28.6 ± 5.8	26.1 ± 4.0	25.4 ± 5.1	30.9 ± 6.1	27.6 ± 4.8	25.9 ± 4.2	28.2 ± 5.8	29.5 ± 7.1
Gravida	2.1 ± 1.3	2.3 ± 1.3	2.0 ± 1.1	1.9 ± 1.2	2.4 ± 1.8	1.8 ± 0.7	1.9 ± 0.9	2.4 ± 1.3	2.2 ± 1.4	2.2 ± 1.3	1.8 ± 0.9	2.0 ± 1.2
Parity	1.9 ± 1.3	2.0 ± 1.0	1.5 ± 0.9	1.7 ± 0.8	2.3 ± 1.8	1.6 ± 0.7	1.5 ± 0.8	2.0 ± 1.1	1.9 ± 1.4	1.9 ± 1.0	1.5 ± 0.9	1.7 ± 1.0
% maternal asthma	80.0	42.9	53.4	58.3	85.7	30.0	61.9	62.5	71.4	46.6	64.2	54.5
Gestational age (days)	278 ± 10	278 ± 8	277 ± 10	276 ± 12	276 ± 9	278 ± 9	277 ± 10	276 ± 12	275 ± 9	280 ± 9	277 ± 12	276 ± 10
Birthweight (g)	3661 ± 311	3525 ± 505	3474 ± 467	3593 ± 563	3681 ± 192	3570 ± 461	3391 ± 368	3615 ± 571	3511 ± 291	3685 ± 498	3390 ± 447	3718 ± 542
Birth weight centile	56.8 ± 25.2	47.1 ± 32.9	50.4 ± 29.9	65.5 ± 31.7	60.0 ± 25.8	49.6 ± 28.4	49.6 ± 28.4	62.2 ± 32.5	48.4 ± 28.5	56.1 ± 29.8	46.0 ± 30.8	72.8 ± 25.1

Mean ± standard deviation

Table 2: Inflammatory genes selected for analysis in the placenta that were not altered in relation to childhood allergy.

Gene name	Description
FOXO1	forkhead box O1
CXCR1	chemokine (C-X-C motif) receptor 1
VEGFA	vascular endothelial growth factor A
MMP2	matrix metallopeptidase 2
ALOX15	15-lipoxygenase
KIT	c-kit
MAOB	monoamine oxidase B

not included as it was a preliminary assessment) in male (n = 28) and female (n = 26) placentae. Target genes are listed in table 2 and figure 1 and figure 2. qRT-PCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Mulgrave, VIC, Australia) according the manufacturer's instructions, and two technical replicates were included for each sample per assay. PCR data was analysed using the $2^{-\Delta\Delta Ct}$ method, with expression of each gene normalised to β-actin expression as used previously for gene expression analyses of placental tissue [18,19].

Statistical analysis

Statistical analyses were performed with SPSS Statistics software (version 20, Statistical Package for Social Sciences, SPSS Inc, IBM, Chicago, IL). All data are expressed as median unless otherwise indicated. Sex differences in gene expression between individuals with a specific allergy type relative to individuals without an allergy were tested using non-parametric Mann-Whitney U tests. Non-parametric Kruskal Wallace analysis of variance (KW-ANOVA) was used to compare all groups by sex and allergy phenotype. The impact of maternal asthma on placental gene expression was also examined using Mann-Whitney U tests to determine whether any changes observed in the placenta may be related to the presence of maternal disease.

Results

Characteristics of study populations

Maternal characteristics between "no allergy" and "allergy" study groups were not significantly different. The mean age and BMI of mothers in the "no allergy" group was 29.8 years of age and 25.7, while the mean age was 27.1 years and mean BMI was 28.3 in the "allergy" group. Neonatal characteristics are summarised in supplementary table 1. Maternal and neonatal characteristics of each study group are summarised in table 1.

Quantitative RT-PCR of selected genes

Some placental genes identified from the preliminary microarray work that were selected for analysis were not found to vary significantly in relation to allergic phenotype and so have been listed in table 2.

Placental genes altered in association with childhood eczema

Expression of matrix metallopeptidase 9 (MMP9) mRNA was reduced in placentae from males and females with eczema compared to placentae from males females without eczema (Mann Whitney U test, males: P = 0.039, females: P = 0.039) (Figure 1A and 1B). Overall, *MMP9* levels were significantly higher in males than females in subjects with eczema (KW-ANOVA, P = 0.048, data not shown) Levels of Toll-like receptor 7 (*TLR7*) did not differ in males with or without eczema (Figure 1C), but was lower in placentae of females with eczema compared to placentae of females without eczema (Figure 1D).

Placental genes altered in association with childhood rhinitis

Levels of kit ligand 2 (*KITL2*) mRNA were very low or absent with Cq values over 35, and thus was not included in statistical analyses. Kit ligand 1 (*KITL1*) expression was significantly higher in placentae from males with rhinitis compared to placentae from males without rhinitis (P = 0.041) (Figure 2A), while expression did not differ between placentae from females with or without rhinitis (Figure 2B).

Placental genes altered in association with childhood asthma

Expression of ORM (yeast)-like protein isoforms 3 (*ORMDL3*) mRNA did not differ between the placentae of males with or without asthma, but was reduced in the placentae of females with asthma compared to the placentae of females without asthma (P = 0.027) (Figure 2D).

Relationship of placental gene expression to maternal asthma during pregnancy

We assessed whether there was any relationship between maternal asthma and changes in gene expression, as asthma is a significant risk factor that increases the likelihood of offspring developing allergy [20]. As shown in supplementary table 2, the presence or absence of maternal asthma was not significantly associated with any of the placental genes identified to be associated with childhood allergy.

Discussion

In this study, we have shown that a number of genes expressed in the placenta at birth are differentially expressed in association with the subsequent development of childhood eczema, rhinitis and asthma. This data supports the current hypothesis that in utero events may pre-program the fetal immune system to an allergic phenotype [21]. Our results indicate that placentae of children who subsequently develop allergy have a bias towards a Th2 immune profile which varied sex specifically. These data suggest that both sex-specific and gene-specific mechanisms may underlie the development of each allergy.

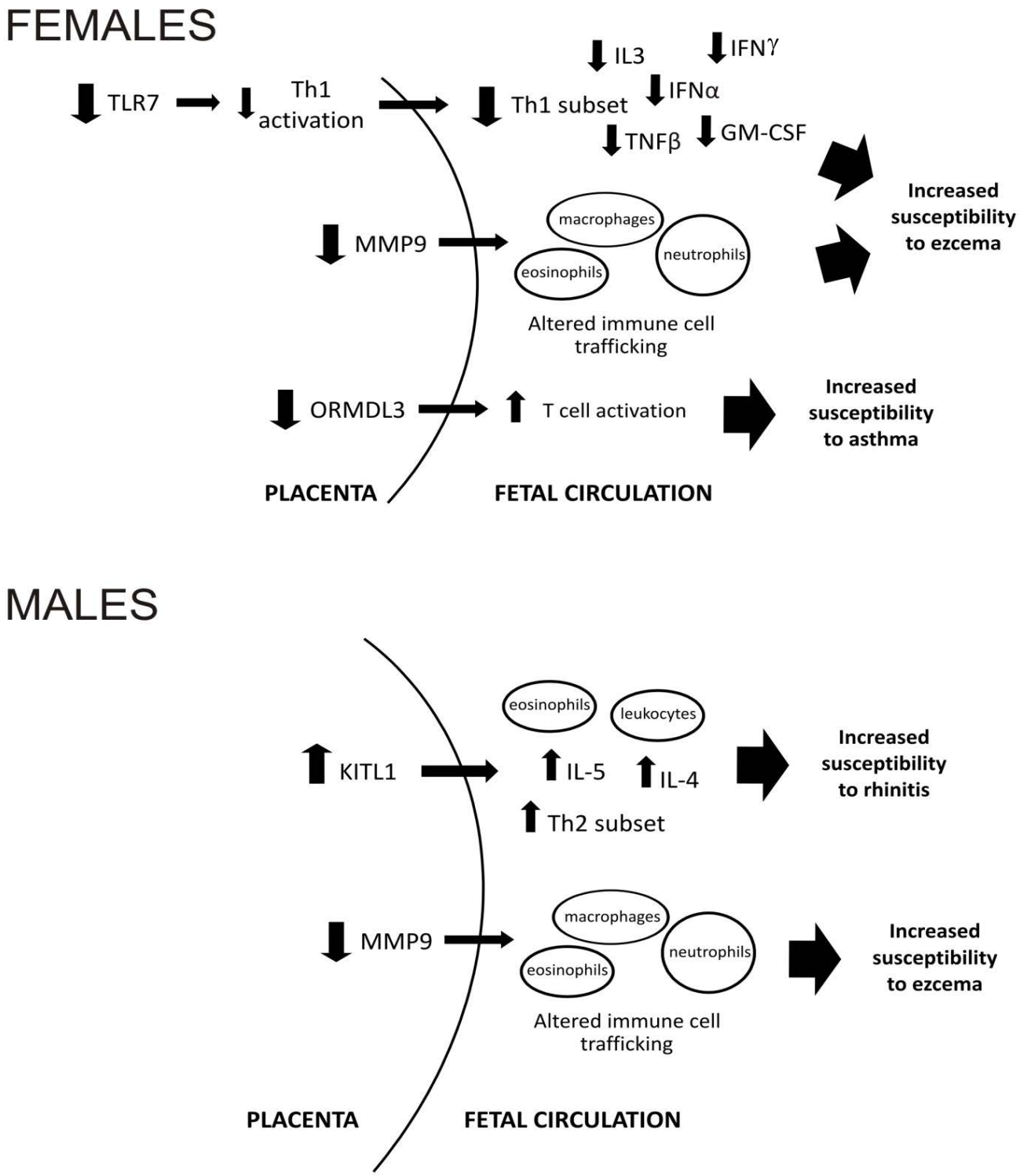

Figure 3: Proposed model of mechanisms underlying increased susceptibility to specific allergy types in females and males.

We identified two genes associated with the development of eczema, specifically MMP9 in males and TLR7 in females. TLR7 is an X chromosome gene which does not escape X inactivation; however, it has been suggested that epigenetic mechanisms may cause altered expression between males and females. TLR7 promotes a Th1-type immune response via differentiation of Th1 cells and production of cytokines, and strong evidence indicates that it is protective against the development and severity of allergic disorders [22-25]. Decreased mRNA in placentae from females who subsequently developed eczema may drive decreased placental Th1 cytokine activation including IFNγ, IFNα, TNFβ, IL-3 and GM-CSF [22-25] (summary in Figure 3). A reduction in these pro-inflammatory cytokines may influence T cell differentiation including CD8+ cells [26], T regulatory cells [27] and dendritic cells in the fetus [28], thereby increasing susceptibility to developing eczema.

There may also be a bias towards a Th2 response in male eczema via different mechanisms. *MMP9* plays a significant role in inflammation by facilitating cellular traffic, including neutrophils and eosinophils, via degradation of extracellular matrix and establishment of chemokine gradients [29]. Importantly, upregulation of *MMP9* mRNA has been demonstrated to be concomitant with the recruitment of Th2 cells in mice [29], while *MMP9*-null mice showed a marked attenuation of the Th2 inflammatory response [30]. Altered expression of *MMP9* in placentae may result in altered immune cell trafficking and increased Th2 activation, including elevated numbers of CD4+ cells and release of inflammatory cytokines leading to increased susceptibility to developing eczema in male offspring (Figure 3).

This study has shown an association between increased *KITL1*

expression in male placentae and subsequent development of rhinitis, supporting mouse studies demonstrating *KITL* to be involved in the development of allergic inflammation [31-33]. We found no differences in expression of the KITL receptor, *c-kit*, suggesting that any influences of the KITL/c-kit signalling pathway on the programming of the fetal immune system are exerted through elevated *KITL* expression alone. Abnormal *KITL1* gene expression may contribute towards a skewed Th2 immune profile by promoting infiltration of inflammatory cells including eosinophils and leukocytes, and increased production of Th2 cytokines IL-4 and IL-5 [33].

Interestingly, this study identified decreased expression of *ORMDL3* to be associated with the development of asthma in females. To our knowledge, no study has characterised the association of *ORMDL3* with asthma by sex; thus, this is the first study to demonstrate a sex-specific association between *ORMDL3* and asthma. Increased *ORMDL3* has recently been identified as being strongly associated with both childhood and adult asthma [34-42]. Therefore, it is somewhat surprising that our study has observed decreased expression in association with asthma. The mechanisms underlying ORMDL3 function and its effects on asthma are unclear and studies have reported conflicting results. Notably, an *in vitro* study demonstrated that ORMDL3 reduced T-cell activation via an important immune activation mechanism [43]. Overexpression of *ORMDL3* in a T-cell line inhibited store-operated Ca^{2+} influx, thereby reducing nuclear translocation of the nuclear factor of activated T-cells (NFAT) [43]. It is clear that the contribution of ORMDL3 to the pathogenesis of asthma is quite complex and requires further study.

The presence of maternal allergy is a significant risk factor that increases the likelihood of offspring developing allergy [20]. Pregnancies complicated by asthma can have a significant effect on placental gene expression and potentially contribute to an atopic phenotype in offspring. We have previously shown that pregnancies complicated by maternal asthma are associated with reduced female fetal growth, accompanied by alterations in placental function such as decreased placental *11β-HSD2* [44]. However, the placental immune genes identified that were altered in the presence of maternal asthma including TNFα, IL-1β, IL-6, IL-8, IL10 and IL-5 [13] were not altered in the placentae of children who developed allergy. Furthermore, we did not find any association between maternal asthma and altered gene expression in placentae of children that developed an allergy. This suggests that certain immune mechanisms that are altered in the fetal-placental unit by the presence of maternal asthma are not associated with gene alterations associated with allergy susceptibility in early childhood.

In conclusion, this study has demonstrated a number of placental genes that are altered in association with the development of specific childhood allergic phenotypes and suggests the *in utero* environment may play a role in programming the immune system. The potential mechanisms driving increased susceptibility to allergy via these altered genes are illustrated in our proposed model in figure 3. Based on this model we propose that abnormal expression of these genes in the placenta may cause a bias towards a Th2 immune profile in the fetal circulation including increased levels of CD4+ cells, increased secretion of anti-inflammatory cytokines and decreased Treg activity as previously indicated by reduced placental *FOXP3* expression [10]. As a result, altered programming of fetal immune function may result in increased susceptibility allergic disease.

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Ethical Statement

This work was approved by the Government of South Australia SA Health Human Research Ethics Committee (TQEH/LMH/MH) and the University of Adelaide Human Research Ethics Committee (2009045).

Author Contributions

ART: study design, coordination, sample collection, experimental work, data analysis, manuscript writing. SME: data analysis, manuscript revision. LEG: data collection, data analysis, manuscript drafting and revision. AO: data collection, sample collection, manuscript revision. ZS: data collection, sample collection, manuscript revision. AT: study design, data collection, study advice and manuscript drafting and revision. SLP: sample collection, data collection and manuscript drafting and revision. MT: study advice and manuscript drafting and revision. RS: study advice and manuscript drafting and revision. VLC: study design, coordination, supervision and manuscript drafting and revision.

Conflict of Interest

The authors do not have any conflicts of interest to declare.

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