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Madeline Pike April 4, 2019

An Exploratory Study of Maternal Prenatal Stress, Immunogenetic Risk, and Pediatric Asthma: Identifying Temporal-and Sex-specific Associations

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Abstract

An Exploratory Study of Maternal Prenatal Stress, Immunogenetic Risk, and Pediatric Asthma:

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Pediatric asthma is among the most prevalent chronic illnesses in the world. Persisting prevalence rates and associated health burdens justify greater exploration of the genetic and environmental susceptibilities that may put some children at a heightened risk for future asthma development. The current study examined the role of psychological stress experienced by the mother during pregnancy in the development of asthma in offspring. Although prenatal stress and asthma associations have previously been found, the operationalization of prenatal stress is often inconsistent and warrants further study. The current study explored the potentially differential effects of objective versus subjective prenatal stress on asthma outcomes. In addition, it explored the moderation effects of biological sex and genetic risk on the relationships between prenatal stress exposure and asthma outcomes. The investigation utilized the archival data of the longitudinal Mater Misericordiae Mothers' Hospital-University of Queensland Study of Pregnancy (MUSP). Participants were 444 Australian young adults, ages 22–25 years, drawn from the MUSP birth cohort of 7,775 children for a genetic sub-study. Maternal prenatal stress was measured both during pregnancy and 3 to 5 days after birth, operationalized as objective life events and subjective stress. Asthma was measured using a maternal report at youth ages 5 and 15 years. Hypotheses were tested using logistic regression models. Results showed maternal reports of objective stressful events in the last six months of pregnancy were correlated with elevated asthma risk in offspring at age 5 years. Biological sex was found to moderate the association between maternal prenatal reports of subjective stress and offspring asthma at age 15 years. Specifically, and in line with our hypotheses, prenatal stress predicted post puberty asthma in girls, but not boys. Our genetic analyses resulted in unexpected findings, detecting a possible protective effect of the IL1β-511 TT genotype in the context of maternal objective stress during pregnancy and offspring asthma at age 15 years. Together, these results reinforce the importance of timing, perception and experience in the prenatal stress-asthma connection. Future directions and clinical implications are discussed.

Keywords: asthma, maternal prenatal stress, objective stressful life events, genotype, inflammation, biological sex.

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An Exploratory Study of Maternal Prenatal Stress, Immunogenetic Risk, and Pediatric Asthma: Identifying Temporal-and Sex-specific Associations

Pediatric asthma is emerging as one of the most prevalent chronic respiratory diseases worldwide, affecting up to 18 percent of the world population (GINA, 2018; Dietert, 2011). Ranking 23rd in the global scale of disability-adjusted life years (Soriano et al., 2017), asthma carries a particularly high burden in children (Nunes, Pereira & Morais-Almeida, 2017). Persisting prevalence rates and associated health burdens justify greater exploration of the genetic and environmental susceptibilities that may put some children at a heightened risk for future asthma development.

The *Developmental Origin of Health and Disease* (DoHaD) paradigm (Gillman, 2005) provides a foundation for the exploration of the transgenerational inheritance of asthma (Li et al., 2015). The DoHaD paradigm refers to the interplay of genetic vulnerability and early life environmental stressors on later health outcomes and, similarly, asthma susceptibility (Holgate, Arshad, Roberts, Howarth, Thurner, & Davies, 2009; Alvarez, Kubzansky, Campen & Slawisch, 2008). In congruence with the DOHaD theory and the field of behavioral immunology, asthma has become a standard model for health disorders largely influenced by prenatal and early life development (Dietert, 2011; Flanigan et al., 2018). Despite this interest, the underlying genotypes hypothesized to moderate these stress-health associations remain unclear (Kudsen, Rezwan, Jiang, Karmaus, Svanes & Holloway, 2018).

The primary focus of the current study is to examine the role of psychological stress experienced by the mother during pregnancy in the development of asthma in offspring. We will also explore whether biological sex and genetic risk moderate the relationships between prenatal stress exposure and asthma in a longitudinal study spanning from gestation to early adulthood.

Prenatal Stress and Pediatric Asthma Risk. Accumulating evidence exists regarding several prenatal environmental factors associated with subsequent asthma risk, ranging from maternal tobacco use, diet, prenatal infection and air quality (Kumar, 2008). While the relationships between these environmental stressors and asthma are well documented, the connection between maternal prenatal stress and asthma remains somewhat obscure. Maternal prenatal stress is considered to be a potent predictor of both maternal and fetal well-being. Moreover, maternal prenatal stress is a strong contributor to the dysregulation of the growing fetal immune system and Hypothalamic–Pituitary–Adrenal (HPA) Axis (Kapoor, Dunn, Kostaki, Andrews & Matthews, 2006), which are highly intertwined with asthma risk (Priftis, Papadimitriou, Nicolaidou & Chrousos, 2008). However, much as past researchers have stated, maternal prenatal stress is often a neglected area of research due to the complexities regarding measurement and the need for long term follow-up studies to accurately assess prospective associations with later health outcomes (Heinrich, 2015).

The optimal measurement of prenatal stress is an issue that remains in contention, particularly as it relates to later pediatric health outcomes such as asthma. In accordance with a review by Heinrich (2015), adverse life events and maternal self-reports of anxiety were reported as the most common operationalizations of prenatal maternal stress in the asthma field. Because maternal anxiety is often complicated by the array of confounds associated with psychiatric disorders, Heinrich argues that adverse life events measurements carry multiple methodological advantages. Additionally, Heinrich noted that maternal perception of stress resulting from the adverse life events has yet to be thoroughly explored (Heinrich, 2015).

Bandoli von Ehrenstein, Ghosh, Flores, Schetter and Ritza (2017) offer an intriguing solution to the concerns surrounding operationalization of prenatal maternal stress. In their work on prenatal maternal stress and the risk of lifetime wheezing—a primary symptom of asthma—in

young offspring, prenatal stress was operationalized as pregnancy anxiety, chronic stress, and acute stress due to negative life events, as well as a lack of paternal support. Risk of offspring lifetime wheezing was associated with maternal reports of high pregnancy anxiety and negative life events. Maternal chronic stress also increased with risk of offspring lifetime wheezing albeit minimal effects. The findings from Bandoli and colleagues (2017) suggest that an analysis of multiple dimensions of prenatal stress are ideal, as they allow for the assessment of risks associated with particular aspects of this construct.

A Model of Moderation. The research of Bousquet, Yssel, Vignola and Humbert (2004) provides one of the many foundational theories by which prenatal stress is thought to contribute to pediatric asthma risk. Studying the methylation of fetal genes associated with allergic asthma, Bousquet, Yssel, Vignola and Humbert (2004) hypothesized that fetal programming may lead to gene expression associated with the epigenetic inheritance phenomena. The team specifically explored genes of the embryologic differentiation of ectodermic and endodermic tissues, such as those encoding Th-2 cytokines. Their research suggests asthma is associated with the persistence and conservation of fetal genes through which alterations of the prenatal environment could maintain long-lasting consequences on the growing lungs and immune system. Although these epigenetic findings are intriguing and suggest a potentially important role for genetic influences on asthma outcomes, relatively less research has examined genotype specific risk for asthma outcomes. The current study aims to do so by examining the moderation of the relationship between prenatal stress and asthma by genotypes related to inflammatory processes.

Biological Sex and Asthma Risk: The Puberty "Switch"

Prior to puberty, males are at a higher risk than their female counterparts for onset of asthmatic symptomology, symptom severity, and hospitalization (Balzano, Fuschillo, Melillo & Bonini, 2008). Thoroughly reviewed by Almqvist, Worm and Leynaert (2008), there is a general

consensus amongst researchers that early pediatric asthma is more prevalent among males. Although the literature supports a male bias in pediatric asthma, the gap between males and females appears to reverse following puberty (Balzano, Fuschillo, Melillo & Bonini, 2008). Results of the International Study of Asthma and Allergies in Childhood on the largest asthmatic cohort to date, with 463,801 children ages 13–14 and 257,800 children ages 6–7 years, strongly validate the prevalence puberty "switch". The team concluded that asthma was greater in males in the 6–7 age group and greater in females in the 13–14 age group (ISAAC, 1998).

Sex hormones are suspected to play a large role in the pubescent "switch" in asthma prevalence. Researchers have long known that abnormal serum levels of the sex hormones, estradiol, progesterone, and cortisol are more prevalent in asthmatic females (Rubio, Rodriguez, Collazzo, Heredia & Fernandez, 1988). For example, in 1985, Danazol—an androgenic hormone treatment for endometriosis—was found to not only benefit endometriosis symptoms, but also unexpectedly improve asthma symptoms (Gorrel, 1985). Various models have been proposed to explain the biological sex differences in asthma development; however, fetal programming appears most promising due to its strong empirical evidence.

Biological Sex, Prenatal Stress and Asthma Risk. Sexual dimorphisms in disease trajectory are leading to a reconceptualization of the interaction of hormones and environmental stressors in the prediction of asthma outcomes (Postma, 2007). Several studies, including the seminal Trivers–Willard (TW) sex ratio hypothesis (Trivers & Willard, 1973) document the significant male bias in fetal mortality, birth complications and future developmental impairments. Male viability is said to be significantly more resource-sensitive and vulnerable to environmental influences than females due to a hypothesized evolutionary bias towards female offspring in adverse conditions. Specifically, an unfavorable maternal environment, such as that marked by high maternal stress, will favor female births to initiate a faster reproductive cycling (Trivers &

Willard, 1973). The TW hypothesis predicts that male fetuses will consistently respond to adversity more severely than females (Sandman, Glynn, & Davis, 2013), placing females at an evolutionary advantage. The literary review of Sandman, Glynn & Davis (2013) introduces overwhelming evidence of the sex-dependent viability–vulnerability trade-off in prenatal stress research, documenting multiple developmental sex differences as early as meiosis and conception. Unlike females, male meiosis is interrupted during adverse circumstances resulting in infertility or elevated risk of early sperm mortality (Sandman, Glynn, & Davis, 2013). As a result, male fetuses exposed to early adversity are less likely to survive pregnancy than females. However, Sandman, Glynn & Davis (2013) argue that because of this low male survival rate, there is instead a greater stress variability in females later in development. The sex-dependent viability–vulnerability trade-off hypothesis thus provides a basis for a conflicting female-dominated vulnerability to stress.

Acknowledging the disconnect between the two main theoretical orientations of biological sex-specific stress research, evidence appears to support a male-dominated stress vulnerability. Emerging as early as the first trimester, both male fetuses and their placentas are larger than their female counterparts (Clifton, 2010). The fetus-to-placental-weight discrepancy provides a convincing argument for greater risk of placental supply disturbances in growing male fetuses (Gabory et al., 2013). A vast resource of nutrients and cytokine production, the placenta could predispose males to the prenatal stress influences on asthma risk discussed above. In addition to the male heightened sensitivity to prenatal environmental pressures, fetal lung development also supports a fetal origin of male pediatric asthma risk. As early as 28 weeks of gestation, female fetuses' lungs are significantly more advanced than their male counterparts (Balzano, Fuschillo, Melillo & Bonini, 2008). The delay in male lung maturation occurs in conjunction with later development in surfactant components which are directly correlated to neonatal airflow capacity (Postma, 2007). The consequential discrepancies in both lung and surfactant maturity at birth

among males and females provides a strong argument for lower lung function in males and a greater risk of asthma development in the following years. Together, these unique fetal experiences suggest biological sex differences may provide insight into the pathways linking prenatal stress and asthma.

Empirical findings regarding the role of sex as a moderator in the prenatal stress and asthma relationship are mixed. For example, some studies have documented a stronger relationship between prenatal stress and asthma in males versus females (Bose et al., 2017; Hsu et al., 2015; Lee et al., 2016; Fang et al., 2011), while others have documented associations between prenatal maternal stress and pediatric asthma only in female children (Turcotte-Tremblay et al., 2014; Wright, Rodriguez & Cohen, 1998). Importantly, all three studies demonstrating that boys carried a greater susceptibility to prenatal stress studied children younger than age 6. Turcotte-Tremblay and colleagues (2014), on the other hand, studied children ranging from 11-12 years of age. These differences suggest that the pubertal switch may be relevant. In other words, the heightened male vulnerability to prenatal stress in asthma development may switch after puberty. With limited research of the post-pubertal effects of prenatal stress, study of the pubertal switch is needed. The current study will therefore explore biological sex as a moderator in the prenatal stress and asthma relationship, examining time points before and after puberty.

The Genetics of Asthma

Asthma susceptibility is largely attributed to an interplay of genetic and environmental factors related to the immune system (Murdoch & Lloyd, 2010). To fully grasp the importance of the genetic basis of asthma, we must explore the disease's inherent connection to inflammation. The majority of asthma symptomology—including wheezing, difficulty breathing, coughing and chest pains—derives from chronic pulmonary inflammation (McCallister & Mastronarde, 2009). Upon introduction of a benign airborne allergen, irritant or acute bodily stress, a cascade of

abnormal lymphocyte immune responses is triggered in the bronchial tubes, narrowing the airway. If left untreated, progressive narrowing of the airway may advance to complete airflow obstruction and ultimately death (Murdoch & Lloyd, 2010).

Evidence of the inflammation-asthma connection can be traced back to as far as the 19th century (Salter, 1859), yet attempts to document the exact immune mechanism underlying asthma attacks and development are not fully understood. Supporters of the Th-1/Th-2 model regard asthmatic symptoms as a byproduct of Th-2 T cell imbalance (Berger, 2000). A "cornerstone of immunity" (van Oosterhout & Motta, 2005), the Th-1/Th-2 paradigm gained its popularity in the early 1980's, attributing multiple immune-related health disorders to characteristic levels of Th-1 and Th-2 helper T-cell classes. Strong evidence documents the role of Th-2 cells and their secreted cytokines in the progression of airway hyperresponsiveness in allergic asthma (Shi et al., 2011). Due to its popularity, the Th-1/Th-2 paradigm has highly influenced the direction of asthma research, with the most commonly studied cytokines being Th-2 related: IL-4, IL-5, IL-13, and IL-9 (Baos et al., 2017).

Although the Th-1/Th-2 paradigm remains highly respected, it fails to account for the totality of current asthma findings. Specifically, Th-1/Th-2 reversal treatments cannot fully control asthmatic symptoms (Shi et al., 2011). As a result, researchers are expanding upon the Th-2 paradigm to explore the genotypes contributing to Th-1, Th-17 and other regulatory T cell-moderated responses in the context of asthma risk (Shi et al., 2011; Holtzman, 2012).

A number of candidate genes for asthma risk have been identified through genome-wide studies. In his paper on genetic susceptibility to asthma and allergy, Vercelli (2008) outlined the four standard categories these genes fall into: 1) innate immunity and immunoregulation; 2) Th-2 T cells pertaining to the Th-1/Th-2 paradigm (Holtzman, 2012); 3) epithelial biology and mucosal immunity and 4) genes associated with lung function, airway remodeling and disease severity. Of

these, the most prominent and relevant to the study of stress research are those associated with innate immunity and immunoregulation, specifically the genotypes regulating the production of Interlukin-6 (IL-6), Interlukin-1β (IL-1β) and Tumor-Necrosis-Factor-α (TNF-α) (Tartter, Hammen, Bower, Brennan & Cole, 2015).

Prenatal Stress, IL6-174G>C, TNF-308G>A and IL1β-511C>T and Asthma Development. Preoccupation with epigenetic models dominates the vast majority of maternal prenatal stress research with limited knowledge on specific fetal genetic risk alleles. The research team of Heim, Entringer and Buss (2018) calls into question this mismanagement of resources. With no documented fetal biomarkers of susceptibility, we cannot effectively begin to prevent the adverse health outcomes associated with asthma development. Their work highlights the utility of studying moderation of risk by genotype variation. In the context of asthma, this research suggests the impacts of prenatal stress on abnormal lung development, placental changes or fetal IL-6, IL-1β, and TNF-α cytokine production levels could be moderated by IL-6, IL-1β, and TNF-α genotypes.

The trio of functional SNPs, IL6-174G>C, TNF-308G>A and IL1β-511C>T, found in the promoter regions of cytokine encoding genes have not yet been explored in interaction with prenatal maternal stress in the prediction of asthma (Tartter et al., 2015). However, scientific rationale exists for the potential role of each of these SNPs as IL6-174G>C has been shown to be related to asthma risk (Li et al., 2015), TNF-308G>A has been significantly associated with asthma susceptibility (Berry, Brightling, Pavord & Wardlaw, 2007), and IL1β-511C>T has been associated with asthma severity (Padrón-Morales et al., 2013) and has also been shown to interact with early life stress exposure in the prediction of depression (Tartter et al., 2015). The current study will explore whether these immune related genotypes are associated with a particular vulnerability to the impact of prenatal stress on asthma outcomes.

The Current Study

The primary aim of this study is to examine associations between prenatal stress and offspring asthma outcomes before and after puberty. We will examine both maternal reports of prenatal objective exposures to stressful life events (objective stress) reported during pregnancy and after birth and prenatal subjective feelings of stress reported during pregnancy and explore biological sex as a moderator. The proposed study builds upon Alvarez, Kubzansky, Campen & Slawisch (2008) hypothesis that the fetal immune system may be programmed by maternal prenatal stress, inducing permanent immune maladaptation and a consequential elevated risk of asthma onset. To our knowledge, no study to date has examined the risk alleles, IL6-174G>C, TNF-308G>A, IL1 β-511C>T, as moderators of the association between the different measures of maternal prenatal stress and asthma before and after puberty. Because chronic inflammation is hypothesized to be a chief mechanism by which early stress influences physical health, our third aim is to examine the potential moderating influence of inflammatory gene alleles on susceptibility to asthma from prenatal stress.

Aims and Hypotheses

- **Aim 1: Examining the relationship between maternal prenatal stress measures and risk of offspring asthma.**
    - *Hypothesis 1a:* Different measures of maternal prenatal stress— (a) maternal subjective prenatal stress, (b) prenatal objective stressful life events reported during pregnancy (c) and prenatal objective stressful life events reported shortly after birth—will be positively associated with presence of offspring asthma before (age 5) and after puberty (age 15).
- **Aim 2: Analyze the role of biological sex as a moderator in the relationship between maternal prenatal stress and risk of offspring asthma.**
    - *Hypothesis 2a:* Biological sex will moderate the relationships between (a) maternal subjective prenatal stress, (b) prenatal objective stressful life events reported during

pregnancy and (c) prenatal objective stressful life events reported shortly after birth and presence of offspring asthma.

- *Hypothesis 2b:* The relationship between (a) maternal perceived prenatal stress and presence of offspring asthma, (b) objective stressful life events reported during pregnancy and presence of offspring asthma and (c) objective stressful life events reported shortly after birth and presence of offspring asthma will be stronger for boys in the younger ages (age 5) and girls following puberty (age 15).

- **Aim 3: Explore moderation of the IL6-174G>C, TNF-308G>A, and IL1β-511C>T genotypes in the associations between maternal prenatal stress and risk of offspring asthma.**
  - *Hypothesis 3a:* IL6-174G>C, TNF-308G>A, and IL1β-511C>T genotypes will moderate the relationship between different measures of maternal prenatal stress measures, including (a) maternal subjective prenatal stress (b) prenatal objective stressful life events reported during pregnancy (c) and prenatal objective stressful life events reported after birth, and risk of offspring asthma before (age 5) and after puberty (age 15) .

Method

Participants

A total of 444 Australian young adults, ages 22–25 years (M= 20.12), were recruited from the longitudinal Mater Misericordiae Mothers' Hospital-University of Queensland Study of Pregnancy (MUSP) for completion of a genetic follow-up study. Born between 1981 and 1984, the original birth cohort of 7,775 children and their mothers were assessed during pregnancy, 3 to 5 days after birth, and at child ages 6 months and 5 years for physical, social and mental health (Keeping et al., 1989). Of current interest, maternal perceived stress level and objective life events data were collected during pregnancy with a self-report questionnaire. At youth age 15 years, 815 families were invited to participate in a MUSP depression sub-study, oversampling for maternal depression (Hammen and Brennan, 2001). From this sample, participants were also re-contacted

at youth ages 23-25 for further psychological assessments and genetic testing via blood samples. Participants without genetic data collected and assayed were excluded from this study.

Sample selection criteria are further detailed in Hammen and Brennan (2001) and Tartter et al. (2015). This sample identified as primarily low to lower-middle class Caucasian (92.1%), with 4.1% Asian, 1.1% Maori/Pacific Islander, 2% Aboriginal and 0.2% other. Although there was a minor female bias towards completion of the genetic study ($\chi 2(1) = 33.66$, $p < 0.001$), there was no difference in maternal depression history for the youth that did or did not complete the genetic study and biological sex was evenly divided within the sample (49.4% female).

Procedure

The MUSP genetic sub-cohort of 444 young adults and their mothers were assessed at pregnancy, 3 to 5 days after birth, and at youth ages 5, 15, 20 and 25 with various self-report questionnaires regarding mental, physical and emotional wellbeing. Interviews were conducted by Postgraduate psychology students either in the family homes or locations convenient to the family and children. Interviewer reliability was assessed and monitored throughout the study.

We previously described our genetic data collection procedure for this analysis in Tartter et al., 2015. Participants (the youth in the cohort) were contacted in 2006 for the MUSP genetic sub-cohort requiring a blood draw for genetic analysis. Upon indicating their intent to participate, participants were mailed the consent forms, genetic kit for the phlebotomist and questionnaires. Instructions were given to visit a local phlebotomist to perform the blood draw. Samples were then transported by our laboratory staff to the Genetic Epidemiological Laboratory of the Queensland Institute of Medical Research (QIMR) for DNA extraction and storage until assay. DNA assays occurred at the UCLA Social Genomics Core Laboratory.

All participants were consented or given assent in the case of minors. The research protocol was approved by the institutional review boards of the University of Queensland, University of California, Los Angeles, and Emory University.

Prenatal Stress Measures

For the purposes of the current study, maternal prenatal stress was operationalized by reports of both subjective stress and objective stressful life events during pregnancy (captured at 2 timepoints). Subjective stress was assessed from the questionnaire given to mothers during their first prenatal appointment at Mater Hospital**:** the Reeder Stress Inventory (RSI) (Reeder, Schrama, & Dirken, 1973). The RSI is a self-report measure comprised of four questions that assess physiological and psychological reactions to daily life. At the prenatal study visit, mothers were asked to report the degree to which the following four statements applied to them on a 5-point Likert scale from Never to All of the Time: 1) "In general, I am usually tense or nervous"; 2) "There is a great amount of nervous strain connected with my daily activities"; 3) "At the end of the day I am completely exhausted mentally and physically"; and 4) "My daily activities are extremely trying and stressful." The RSI is a well-established measure of subjective stress, carrying strong reliability and validity ((Betts, Williams, Najman, & Alati, 2014; Metcalfe, Smith, Wadsworth, Sterne, Heslop, Macleod, & Smith, 2003). In the present sample, the RSI had good reliability with a Cronbach's alpha of 0.804. Scores on the prenatal RSI ranged from 5 to 25 with an average of 11.90 (SD = 4.53).

Objective Stressful Life Events – Prenatal and Newborn Study Visits. A series of 9 questions regarding life events during the last six months were asked during the prenatal study visit and the 3-5-day post-birth timepoint. Questions pertained to death or sickness of a loved one, personal health problems, disagreements with partner and/or loved one, financial problems, major changes in work situation for self and/or partner, serious problems with housing or accommodation

and personal and/or partner having problems with the law. Scores on the objective stressful life events questionnaire ranged from 0 to 7 with an average of 1.70 (SD =1.62) for the prenatal study visit and an average of 1.37 (SD =1.45) for the newborn study visit. Objective stressful life events scores during pregnancy were significantly correlated with objective life events scores reported shortly after birth ($r = 0.59$, $p< 0.001$). The Reeder Stress Inventory (RSI) measure was also correlated with the 2 objective stress measures. See Table 3 for more detail.

Asthma

A measure of pediatric asthma over the lifespan of 15 years was created to document presence of the disease according to maternal report. Specifically, presence of asthma was queried at youth ages 5 (pre-puberty) and 15 (post-puberty) in a maternal questionnaire. Asthma data from ages 20 and 25 were thus excluded from analyses. **Pre-puberty:** Mothers were asked to report either presence or absence of offspring chronic asthma through a questionnaire administered at child age 5. Asthma was marked present if the mother answered yes to "has your child had any of these symptoms or conditions continuing longer than three months: Asthma". A total of 37 (8.3%) children were reported to have chronic asthma, with a high female prevalence (62.2% female). **Post-puberty:** Mother report of youth asthma at age 15 was measured with option choices "Never", "Sometimes" and "Always" over the past six months. Answer choices were recoded as 0 for "Never" and 1 for answers "Sometimes" and "Always". 109 children (24.6%) were reported to have asthma symptoms, 64 of whom were female (58.7%).

Asthma reports at age 5 and age 15 years were correlated ($N=430$, $r=0.317$, $p<0.001$). Of the 37 children reported to have chronic asthma at age 5, 24 of the children continued to show symptoms at age 15 (64.9%). 84 (21.2%) of the original 313 children who did not report chronic asthma at age 5 were reported to have asthma symptoms by age 15.

Immunogenetic Measures

DNA extraction was performed using the salting out method (Miller et al., 1988). DNA was eluted in 400 μl of 1× Tris–EDTA buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) with a concentration range of 100 ng/μl to 100 μg/μl and retained in freezer storage prior to shipment for assay.

DNA aliquots were later transported from the QIMR laboratory to the UCLA Social Genomics Core Laboratory for genotyping. IL6 (-174 G>C; rs1800795), IL1β (-511 C>T; rs16944) and TNF (-308 G>A; rs1800629) were assayed by a commercial TaqMan Genotyping Assay (Applied Biosystems, Foster City, CA) performed on an iCycler real-time PCR instrument (BioRad, Hercules, CA) following the manufacturer's specified protocol (Cole et al., 2010).

As discussed in Tartter et al. (2015), total genotyping error rate was below 1% following test-retest reliability. IL1β-511, IL6-174, and TNF-308 were in Hardy–Weinberg equilibrium ($\chi 2(1, 444)$ values: IL1β-511 = 0.26, IL6-174 = 0.13, TNF-308 = 0.08, all p-values > 0.05). Population level minor allele frequencies of the three SNPs were 18.5% at IL6-174, 46.5% at IL1β-511, and 9.6% at TNF-308, indicating that allelic variation at these loci is common, and could reasonably account for differences in prenatal stress-asthma outcomes (See Table 1).

Potential Confounds

Multiple demographic variables were assessed as potential confounds, including income during pregnancy, ethnicity, gestational age, birth weight, maternal depression, and mother's age.

Data Analytic Plan

All statistical tests were two-sided and conducted using SPSS statistical package, version 25.0 for Macintosh. All 3 prenatal stress variables were mean-centered to reduce multicollinearity. Univariate correlations of the asthma measures (age 5 and age 15), stress measures and genotypes were explored and documented above. Preliminary analyses examined bivariate correlations between covariates and asthma report. Ethnicity was recoded with dummy variables and assessed

separately for asthma at both age 5 and age 15. Due to the lack of correlation amongst covariates and our primary variable, no covariates were included in the secondary analyses. Missing cases were minimal and thus not accounted for. A series of 3 logistic regressions were then conducted for each asthma timepoint (age 5 and age 15) to assess the relationship for each prenatal stress measure –objective stressful life events at pregnancy, shortly after birth and subjective stress during pregnancy—with asthma.

Next moderation analysis of biological sex was conducted using logistic regression for each model of asthma and prenatal stress. Genetic moderation was analyzed in a similar logistic regression model. All genotypes were coded as three-level variables (0 = minor allele homozygotes, 1 = heterozygotes, 2 = major allele homozygotes). Post-hoc stratifications were planned to evaluate the simple effects of significant interactions between genotype and stress. Due to the exploratory nature of the analyses, correction for multiple testing was not planned and should be noted as a possible limitation of the design.

Results

Preliminary Analysis

Covariates. No significant correlation was found between presence asthma at age 5 or age 15 and the covariates of gestational age, birthweight, income during pregnancy, mother's age, number of cigarettes smoked per day and alcohol use during pregnancy. No statistically significant differences were found between reference group White and the Asian (B=0.17, SE=0.064, t=0.263, p=0.793) Aboriginal (B=0.042, SE=0.100, t=0.419, p=0.676) and Maori/Islander (B=0.017, SE=0.090, t=0.188, p=0.851) ethnicities with presence of asthma at age 5. Similarly, no statistically significant differences were found between reference group White and the Asian (B=-0.050, SE=0.099, t=-0.505, p=0.613) Aboriginal (B=0.083, SE=0.146, t=0.573, p=0.567) and Maori/Islander (B=-0.150, SE=0.138, t=-1.085, p=0.278) ethnicities with presence of asthma at age 15. See Table 2 for more details. Covariates were not factored into further analyses.

Prenatal Stress and Asthma. To test our first hypothesis that prenatal stress would be associated with presence of child asthma, and to explore whether objective stressful life events or subjective stress were stronger predictors, we completed 3 hierarchical logistic regression analyses. First, logistic regressions were conducted separately for each measure of prenatal stress predicting to maternal report of youth asthma at age 5 years. Prenatal reports of subjective stress and objective stressful life events were not significantly associated with asthma at age 5; however, a significant positive association was found for newborn reports of objective stressful life events that occurred in the previous six months and child asthma at 5 years (see Table 4).

Next, a similar set of 3 hierarchical logistic regression analyses was conducted with maternal report of youth asthma at age 15. None of the measures of prenatal stress were significantly associated with post puberty child asthma (see Table 4).

Biological Sex as a Moderator

To test our second set of hypotheses that biological sex would moderate the relationships between prenatal stress and child asthma (age 5 and age 15), we completed 3 hierarchical logistic regression analyses separately, one for each of the 3 prenatal stress measures and asthma before and after puberty. In total, 6 logistic regression combinations were performed. No biological sex interactions were found to predict child asthma at age 5 (see Table 5). In support of our hypothesis, a significant interaction was found between biological sex, prenatal subjective stress, and child asthma at age 15 (see Table 5). Sex stratified analysis revealed a significant association between prenatal subjective stress with asthma at age 15 in girls (Wald (N=261)=5.021, B=0.073, Exp(B)=1.076, p=0.025), but not boys (Wald (N=182)=2.781, B=-0.065, Exp(B)=0.937, p=0.095), matching our expectations for a post-puberty “switch.” No biological sex interactions were found at age 15 for objective stressful life events measures.

Immunogenetic Moderation

To test our third hypothesis, hierarchical logistic regressions were performed assessing the moderation effect of the interactions of individual immune-related genes—IL6-174, IL1β-511 and TNF-308—with each of the three prenatal stress measures—objective stressful life events reported in pregnancy and shortly after birth and subjective stress reported during pregnancy. No significant associations were found with IL6-174, IL1β-511 and TNF-308 with objective stressful life events measured shortly after birth or subjective stress during pregnancy and asthma at age 5 (Table 6). At age 15, a significant two-way interaction was found between IL1β-511 and objective stressful life events during pregnancy in the prediction of asthma at youth age 15 (Exp(B)=1.235, Wald (N=443)=4.115, p=0.042). See Table 7 for more details. Stratifying the data by IL1β-511, the relationship was further probed, revealing a significant negative association between objective stressful life events measured during pregnancy and asthma at age 15 at IL1β-511 TT (Wald (N=443)=7.209, p=0.007), but not at IL1β-511 TC (Wald (N=443)=0.062, p=0.804) and IL1β-511 CC (Wald (N=443)=0.010, p=0.919). See Figure 1 for more details.

Discussion

To our knowledge, this is the first documented exploration of the moderation of immunogenetic risk in the relationship of prenatal stress and asthma development. We found that maternal reports of objective stressful life events occurring in the last six months of pregnancy were correlated with elevated asthma risk in offspring at age 5 years. In contrast, maternal subjective stress and reports of objective stressful life events early in pregnancy did not predict to offspring asthma at age 5 years. Biological sex was found to moderate the association between maternal prenatal reports of subjective stress and offspring asthma at age 15 years. Specifically, and in line with our hypotheses, prenatal stress predicted post puberty asthma in girls, but not boys. Our genetic analyses resulted in unexpected findings, detecting a possible protective effect of the IL1β-511 TT genotype in the context of maternal objective stressful life events during pregnancy

and offspring asthma at age 15 years. Together, these results reinforce the importance of timing, perception and experience in the prenatal stress-asthma connection.

Of note, few studies have conducted separate analyses of subjective and objective prenatal stress measures (Andersson, Hansen, Larsen, Hougaard, Kolstad & Schlünssen, 2016). Our results support the inherently intertwined, yet notably distinctive nature of perception and experience (Kingston, Heaman, Fell, Dzakpasu & Chalmers, 2012). Reactivity, frequency and duration of the stress response are diverse across individuals (Berens, Jensen & Nelson, 2017). Differences in personality, self-efficacy and experiences with the stressor ultimately contribute to variation in cognitive appraisal, emotinal distress and behavior (Thornton & Andersen, 2006). This is not to say that objective life events stressors are powerless. According to the research of Kingston and colleagues (2012), different factors (i.e., demographic characteristics), uniquely contribute to perceived stress and stressful life events during pregnancy and thus each measure represents distinctive aspects of the stress response. This may explain why objective stressful life events, but not subjective stress, correlated with asthma risk at age 5. However, it may be more plausible to attribute these differences to critical sensitive windows in fetal development. In our study, objective stressful life events reported during pregnancy and shortly after birth captured different time windows of gestational development, which may have contributed to the differences in stress-asthma associations. Indeed, our results are consistent with a recent meta-analysis that found that stress occurring in the third trimester pregnancy was more likely to be associated with offspring asthma. (Flanigan et al., 2018).

With regards to biological sex as a moderator, our results support an association between maternal subjective prenatal stress and asthma in girls, but not boys, at age 15 years. Parallel to the sex differences in the fetal programming model of Sandman, Glynn, & Davis (2013), these findings support a “puberty switch” from boys to girls in the risk for asthma (Balzano, Fuschillo,

Melillo & Bonini, 2008). Sandman and colleagues argue that male fetuses exposed to high levels of prenatal stress are more likely to succumb prior to birth, resulting in greater stress variability in females later in development. Puberty is a critical period for asthma development in girls throughout the prenatal stress literature (Turcotte-Tremblay et al., 2014; Wright, Rodriguez & Cohen, 1998). In addition, asthmatic airway hyperresponsiveness has been found to worsen during puberty in girls, yet improve in boys (Protudjer, Lundholm, Bergström, Kull & Almqvist, 2014). In part, this critical developmental period of risk may explain both the sleeper effect observed at age 15 and the higher susceptibility of girls to the long-term effects of prenatal stress on asthma.

Our genetic findings were unexpected and puzzling. The finding that the TT genotype of IL1β-511 carried a protective effect in the relationship between objective stressful life events during pregnancy and asthma development at age 15 is the opposite of the result that we hypothesized. Although no studies to date have explored IL1β-511 in the context of prenatal stress and asthma development, the TT genotype of IL1β-511 has been shown to elevate circulating plasma levels of interlukin-1β, a cytokine that is associated with inflammatory airway diseases and airway hyper-responsiveness (Joos et al., 2000). As the TT genotype of the IL1β-511C>T polymorphism is strongly associated with asthma severity (Padrón-Morales et al., 2013), our finding conflicts with current asthma literature.

At first glance, it appears the genetic protective effect could be related to our finding that prenatal stress predicts asthma post-puberty in girls, but not boys. In other words, perhaps the TT genotype of IL1β-511 plays a role in the lower post-puberty asthma rates in boys. Despite the intrigue of this hypothesis, our current understanding of the sex-differences in asthma development does not support this (Protudjer, Lundholm, Bergström, Kull & Almqvist, 2014). The fact that the protective effect occurs later, rather than earlier, in development also suggests that we should be cautious in our interpretations. Evolutionary adaptions that protect against prenatal stress are

designed to promote and favor survival early in life, not later in post pubertal development (Cole et al., 2010).

The combined evidence of the incongruent direction of the IL1β-511 TT allele finding as well as the lack of similar associations for IL6-174 and TNF-308 (which hold equal potency in the development of asthma), suggest that our genetic findings were more likely accounted for by Type I error. Nonetheless, replication is needed in larger samples, with greater levels of power to detect GxE effects. In addition, the current preferred design in genetic research is the large population GWAS study, that allows for simultaneous testing of associations across the genome. Candidate gene studies suffer from low power and Type I error on many occasions, and our study may not be an exception.

Limitations and Strengths

Limitations of the current study included the retrospective nature of the objective stressful life events reports and their overlap with pre-pregnancy and post-pregnancy time periods, the substantial timespan between pre-puberty and post-puberty follow-ups, and the sole reliance on maternal report for the operationalization of asthma. Subjective stress measures such as the one used in this project are often criticized for their inherent bias (Andersson, Hansen, Larsen, Hougaard, Kolstad & Schlünssen, 2016), yet they also carry great predictive strength as noted by Kingston and colleagues (2012). It is unclear whether more frequent follow-up visits throughout pre- and post- puberty would have revealed significant interactions missed with the prenatal stress measures, perhaps in support of the male pre-puberty relationship (Lee et al., 2016). Likewise, our operationalization of post-puberty asthma may have benefited from more frequent follow-up visits and specification of each individual's puberty onset. Due to our interest in the direct effects of prenatal stress, our study was not designed to account for the contributions of early childhood adversity, which has also been shown to contribute to the onset of pediatric asthma (Lee et al.,

2016). Additionally, although a wide range of data was collected on the mothers in the sample, we were unable to collect information on maternal history of asthma—one of the greatest predictors of child asthma risk (Oddy, Peat & Klerk, 2002). Asthma reports overall were narrow in scope as the questionnaires were reliant on maternal perception rather than official diagnosis. Severity, phenotype and time of diagnosis were not gathered at the time of the original data collection which weakened our ability to test hypotheses about quality of life and functional outcomes associated with asthma. The targeted sampling of our data, over-selecting for history of maternal depression at the youth age 15 visit should also be taken into consideration. The inherent associations between depression and stress-levels likely contributed to a restriction of range issue and thus our results should be replicated without such selection bias.

Despite its noted limitations, our study also had a number of methodological advantages. The longitudinal design, beginning in pregnancy and including pre- and post-puberty offspring follow-ups, allowed for a richer exploration of prospective prenatal stress effects, the pubertal switch in asthma development, and the test of genotypic moderation across development. As few studies have conducted separate analyses of their subjective and objective prenatal stress measures (Andersson, Hansen, Larsen, Hougaard, Kolstad & Schlünssen, 2016), our inclusion of both objective and subjective stress measures is also a notable strength of our study. Albeit the limitations of candidate gene studies, our study added to the field of risk allele research related to prenatal stress (Heim, Entringer and Buss, 2018).

Future Directions

To fully understand the potential impacts of prenatal stress on asthma development, further data is needed during the prenatal and post-natal period; specifically, future designs should increase the number of study visits across pregnancy and childhood, while also accounting for the

confounding nature of postnatal stress. It is suggested that future operationalizations of asthma include data from pediatric medical records in order to minimize self-report bias.

Additionally, the operationalization of stress continues to be a matter of great contention among researchers. A recent literature review of 12 studies examining maternal stress and asthma revealed that the operationalization of prenatal stress differs widely across investigations, with stress being defined as maternal depression, anxiety, bereavement, work-related stress or other life stressors (Flanigan et al., 2018). Further analysis of the 12 studies showed elevated risk of asthma onset (Hazard ratio (HR) 1.13, 95% CI 0.98-1.32; I2 = 91.5%) and current or ever asthma (RR 1.13, 95% CI 1.03-1.24; I2 = 83.5%) in the offspring with exposure to any one of the above prenatal stressors. When they parsed results by the type of stressor measure, they found maternal anxiety to be the strongest predictor of asthma outcomes. As our study found distinctive associations between asthma and objective stressful life events versus maternal reports of subjective stress, it appears there is a need to continue to explore the various aspects of prenatal stress in terms of how they impact the growing fetus, and potentially impair long term health outcomes in offspring.

Conclusion

The current study applied the Developmental Origins of Health and Disease framework to the growing literature on the prenatal stress and pediatric asthma. Specifically, we aimed to explore the moderating effects of biological sex and genetic risk on the association between prenatal stress exposure and asthma development. Our results highlight the potentially detrimental impacts of prenatal stress on the growing fetus. Despite inconclusive results surrounding our immunogenetic markers, a greater understanding the immune pathways by which prenatal stress contributes to asthma is of theoretical and clinical importance. In these future efforts, we may aid the International Society for Developmental Origins of Health and Disease in attempting to reduce

the burden of disease by promoting healthy development through prenatal interventions designed to reduce maternal stress (Anderson, Gupta, Strachan, & Limb, 2007).

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Table 1

Genetic characteristics and asthma prevalence of study participants

Gene	Genotype	Female	Male	Total
IL6 -174G>C	CC	98	65	163
	GC	118	91	209
	GG	46	26	72
TNF -308G>A	AA	9	4	13
	GA	76	45	121
	GG	177	133	310
IL1B-511C>T	TT	118	81	199
	TC	122	78	200
	CC	22	23	45
Age	Presence of Asthma	Female	Male	Total
Age 5	Yes	23	14	37
	No	233	165	398
Age 15	Yes	64	45	109
	No	197	137	334

Table 2

Correlations between Potential Covariates and Asthma

	Presence of Asthma			
	Age 5		**Age 15**	
Potential Covariates	r_{pb}	Sig. (2-tailed)	r_{pb}	Sig. (2-tailed)
Income in pregnancy	0.064	0.198	0.056	0.252
Parental education level	0.069	0.151	-0.790	0.096
Mother's lifetime depression	0.032	0.506	0.015	0.748
Mother's age at pregnancy	0.046	0.337	-0.005	0.915
Cigarettes/day during pregnancy	-0.017	0.719	0.008	0.873
Alcohol use in pregnancy	-0.005	0.915	0.042	0.373
Birthweight (grams)	-0.020	0.684	-0.023	0.624
Gestational age	-0.230	0.627	0.180	0.705

Note. ****Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed). Ethnicity assessed separately.**

Table 3

Correlations between Prenatal Stress Measures

Prenatal Stress Measure		Objective stressful life events: prenatal visit	Objective stressful life events: postnatal visit	Subjective stress: prenatal visit
Objective stressful life events: prenatal visit	Pearson Correlation	1	**0.588****	**0.286****
	Sig. (2-tailed)	-	0.000	0.000
	N	444	440	444
Objective stressful life events: postnatal visit	Pearson Correlation	**0.588****	1	**0.188****
	Sig. (2-tailed)	0.000	-	0.000
	N	440	440	440
Subjective stress: prenatal visit	Pearson Correlation	**0.286****	**0.188****	1
	Sig. (2-tailed)	0.000	0.000	-
	N	444	440	444

Note. ****Association is significant at the 0.01 level (2-tailed). *Association is significant at the 0.05 level (2-tailed).**

Table 4

Logistic Regression of Primary Variables: Prenatal Stress and Presence of Asthma

Presence of Asthma	Wald	Sig. (2-tailed)	Exp(B)	CI for Exp(B)	
Age 5				Lower	Upper
Objective stressful life events: prenatal visit	0.288	0.592	1.057	0.864	1.293
Objective stressful life events: postnatal visit	3.947	**0.047***	1.236	1.003	1.523
Subjective stress: prenatal visit	0.345	0.557	1.014	0.967	1.064
Presence of Asthma	Wald	Sig. (2-tailed)	Exp(B)	CI for Exp(B)	
Age 15				Lower	Upper
Objective stressful life events: prenatal visit	2.879	0.090	0.885	0.769	1.019
Objective stressful life events: postnatal visit	0.027	0.869	1.013	0.872	1.176
Subjective stress: prenatal visit	1.443	0.230	1.046	0.972	1.125

Note. ****Association is significant at the 0.01 level (2-tailed). *Association is significant at the 0.05 level (2-tailed).**

Table 5

Logistic Regression of Sex as a Moderator of Prenatal Stress and Presence of Asthma

Presence of Asthma Age 5	Wald	Sig. (2-tailed)	Exp(B)	CI for Exp(B)	
Interaction effects of sex with				Lower	Upper
Objective stressful life events: prenatal visit	1.850	0.174	1.356	0.874	2.101
Objective stressful life events: postnatal visit	1.436	0.231	1.330	0.834	2.121
Subjective stress: prenatal visit	0.197	0.657	1.034	0.891	1.201
Presence of Asthma Age 15	Wald	Sig. (2-tailed)	Exp(B)	CI for Exp(B)	
Interaction effects of sex with				Lower	Upper
Objective stressful life events: prenatal visit	2.799	0.094	1.288	0.958	1.733
Objective stressful life events: postnatal visit	0.558	0.455	1.127	0.824	1.540
Subjective stress: prenatal visit	7.391	**0.007***	1.148	1.039	1.268

Note. ****Association is significant at the 0.01 level (2-tailed). *Association is significant at the 0.05 level (2-tailed).**

Table 6
Interactions between genotypes and prenatal stress measures on asthma outcomes at age 5

Asthma Age 5	Wald	Sig. (2-tailed)	Exp(B)	CI for Exp(B)	
Interaction effects of IL6 -174 with				Lower	Upper
Objective stressful life events: prenatal visit	0.968	0.325	1.151	0.869	1.525
Objective stressful life events: postnatal visit	0.023	0.879	1.023	0.768	1.362
Subjective stress: prenatal visit	0.126	0.722	0.982	0.891	1.083
Asthma Age 5	Wald	Sig. (2-tailed)	Exp(B)	CI for Exp(B)	
Interaction effects of IL1β-511 with				Lower	Upper
Objective stressful life events: prenatal visit	0.228	0.633	1.075	0.798	1.450
Objective stressful life events: postnatal visit	0.906	0.341	1.156	0.858	1.557
Subjective stress: prenatal visit	2.751	0.097	0.909	0.812	1.018
Asthma Age 5	Wald	Sig. (2-tailed)	Exp(B)	CI for Exp(B)	
Interaction effects of TNF -308 with				Lower	Upper
Objective stressful life events: prenatal visit	0.119	0.730	1.067	0.739	1.542
Objective stressful life events: postnatal visit	0.195	0.659	1.084	0.757	1.552
Subjective stress: prenatal visit	0.008	0.927	0.993	0.862	1.144

Note. ****Association is significant at the 0.01 level (2-tailed). *Association is significant at the 0.05 level (2-tailed).**

Table 7
Interactions between genotypes and prenatal stress measures on asthma outcomes at age 15

Asthma Age 15	Wald	Sig. (2-tailed)	Exp(B)	CI for Exp(B)	
Interaction effects of IL6-174 with				Lower	Upper
Objective stressful life events: prenatal visit	0.648	0.421	0.917	0.744	1.132
Objective stressful life events: postnatal visit	0.122	0.727	0.963	0.777	1.192
Subjective stress: prenatal visit	1.332	0.248	0.961	0.899	1.028
Asthma Age 15	Wald	Sig. (2-tailed)	Exp(B)	CI for Exp(B)	
Interaction effects of IL1β-511 with				Lower	Upper
Objective stressful life events: prenatal visit	4.115	**0.042***	1.235	1.007	1.515
Objective stressful life events: postnatal visit	1.776	0.183	1.156	0.934	1.431
Subjective stress: prenatal visit	0.073	0.786	1.010	0.941	1.084
Asthma Age 15	Wald	Sig. (2-tailed)	Exp(B)	CI for Exp(B)	
Interaction effects of TNF-308 with				Lower	Upper
Objective stressful life events: prenatal visit	0.488	0.485	0.919	0.716	1.172
Objective stressful life events: postnatal visit	0.186	0.666	0.946	0.735	1.218
Subjective stress: prenatal visit	0.026	0.871	1.008	0.916	1.109

Note. ****Association is significant at the 0.01 level (2-tailed). *Association is significant at the 0.05 level (2-tailed).**

Figure 1
Analysis of IL1β-511 Allele Moderation of Prenatal Objective Stressful Life Events and Presence of Post-Puberty Asthma