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***IN UTERO* PROGRAMMING OF CHILDHOOD ASTHMA**

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IN UTERO PROGRAMMING OF CHILDHOOD ASTHMA

By

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ABSTRACT

IN UTERO PROGRAMMING OF CHILDHOOD ASTHMA

By

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Asthma, the most common chronic illness of childhood, is a public health problem and its prevalence has increased in the last decades. The purpose of this dissertation was to investigate whether maternal exposures during pregnancy can increase the risk of childhood asthma in offspring, a process known as *in utero* programming. To address this concept, a theoretical model with three components was proposed:

1- Gene-environment interactions during pregnancy affect a marker (susceptibility marker) that is involved in asthma pathogenesis.

2- The susceptibility marker, detected at birth, has a long-lasting effect on asthma.

3- The combination of genetic predisposition and exposures during pregnancy affect asthma later in life.

Information on maternal smoking during pregnancy, the interleukin 13 gene (*IL13*), cord serum immunoglobulin E (CS-IgE, a susceptibility marker), and asthma phenotypes were used to build the theoretical model for the *in utero* programming process. The data used in the analyses is from the Isle of Wight (United Kingdom) birth cohort, established in 1989, and comprised of 1,456 children who have been thoroughly characterized for asthma phenotypes and other allergic diseases as well as allergic markers at birth, 1,

2, 4 and 10 years of age.

The results suggests first, a modifying effect of maternal smoking on the association between the common variant of the *IL13* gene and levels of cord serum immunoglobulin E (CS-IgE); second, an association between CS-IgE and childhood asthma; third, a combined effect of the common *IL13* variant and maternal smoking during pregnancy on *early onset persistent* wheeze phenotype. The three pieces of this work suggest that *in utero* exposure to tobacco smoke may alter the expression of the *IL13* gene at birth that lasts as late as age 10. The interaction of *IL13* polymorphism was not observed in those who were exposed to tobacco smoke after birth unless they had exposure during pregnancy. This suggests that the *in utero* environment has a critical effect on the pathogenesis of asthma and that the prenatal period is of utmost importance.

It is of public health importance to consider the prenatal period as a critical time in the pathogenesis of diseases and to start education for exposure prevention before a pregnancy happens. Future studies may explore the molecular basis for the interaction between prenatal exposure to tobacco smoke (and other pollutants) and genes. If epigenetic changes play a role in the pathogenesis of asthma, it may even be possible to reverse the effect of pollutants through intervention therapies.

DEDICATION

This work is dedicated to my parents, Negin and Eilia.

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LIST OF ABBREVIATIONS

CI ..... CONFIDENCE INTERVAL

IgE ..... IMMUNOGLOBULIN E

IL ..... INTERLEUKIN

DNA ..... DEOXYRIBONUCLEIC ACID

OR ..... ODDS RATIOS

SAS ..... STATISTICAL ANALYSIS SYSTEM

SNP ..... SINGLE NUCLEOTIDE POLYMORPHISM

SPT ..... SKIN PRICK TEST

χ^2 ..... CHI-SQUARE

INTRODUCTION

1.1. Overview

Asthma is a public health problem, the most common chronic illness of childhood, with a prevalence that has increased in the last decades. The purpose of this dissertation is to investigate whether maternal exposures during pregnancy can increase the risk of childhood asthma in offspring, a process known as *in utero* programming. To address this concept, a theoretical model is proposed. The three components of this model are:

1- Gene-environment interactions during pregnancy affect a marker (susceptibility marker) that is involved in asthma pathogenesis.

2- The susceptibility marker, detected at birth, has a long-lasting effect on asthma.

3- The combination of genetic predisposition and exposures during pregnancy affect asthma later in life.

Information on maternal smoking during pregnancy, the interleukin 13 gene (*IL13*), cord serum immunoglobulin E (CS-IgE, a susceptibility marker), and asthma phenotypes were used to build the theoretical model for the *in utero* programming process. The data used in the analyses are from the Isle of Wight (United Kingdom) birth cohort, established in 1989, and comprised of 1,456 children who have been thoroughly characterized for asthma phenotypes and other allergic diseases as well as allergic markers at birth, 1, 2, 4 and 10 years of age. The Institutional Review Board at Michigan State University approved this work.

In this first chapter, I give the definition and pathophysiology of asthma; its public health significance; and the effects of exposure to tobacco smoke, genetic predisposition, and their interactions on asthma. Next, the importance of time windows of exposures and the main concepts that try to explain the increase in the prevalence of asthma are introduced. Then the theoretical model, and its three components (see above) and their backgrounds are presented. In 'Methods' I describe the methods that are not discussed thoroughly in the manuscripts that are available in the 'Results' section. More details on methods can be found in the manuscripts. The 'Results' section is composed of three manuscripts, one published and two submitted for publication, and provides findings related to the following questions:

1- Is there an interaction between interleukin 13 gene (*IL13*) and maternal smoking during pregnancy on CS-IgE?

2- Is CS-IgE related to childhood asthma?

3- Is there a combined effect of *IL13* polymorphism and maternal smoking during pregnancy on childhood asthma phenotypes?

In the last section, 'Discussion and Conclusion' the results are discussed, conclusions are presented, practical issues regarding public health are addressed, and future scientific steps toward the early life prevention of childhood asthma are offered. Appendices comprise a glossary and logs from the statistical programs SAS and PHASE used in analyses.

1.2. Definition and pathophysiology of asthma

Asthma is a chronic inflammatory disorder of the lungs that is characterized by a reversible obstruction of the airways. (1, 2) Allergy, an immune response to allergens, is characterized by an increased level of IgE, and can be one of the elements of asthma (typical allergen sources include house dust mite, grass pollens and animal dander). (3) Classically two different pathologic entities have been described, allergic and non-allergic asthma, although the differences are not yet clearly understood. (4, 5) From pathophysiologic aspect, asthma is characterized by a variable airway obstruction due to airway inflammation and increased bronchial responsiveness to different external and internal stimuli. The inflammation results in mucosal edema, cellular infiltrate, and glandular hyperplasia, whereas bronchial hyper-responsiveness (BHR) causes excessive tone and contraction of the bronchial smooth muscles. Both of these processes lead to an intermittent narrowing of the airways. This narrowing produces "wheeze", which is considered to be the clinical hallmark of asthma. The immune response that produces inflammation in asthma is driven by a predominance of cytokines: interleukin (IL)-4, IL-5, and IL-13. It has been suggested that immune dysregulation causes inflammation with resultant airway structural damage and repair (remodeling). (6) The structural changes observed include thickening of the reticular basement membrane, an increase in the underlying collagen, and hypertrophy of the airway smooth muscle. Abnormally thickened airways may be the mechanism underlying both BHR and fixed airway obstruction. (7) These changes begin in childhood and underscore the importance of early detection

and the appropriate intervention for persistent wheeze and asthma. (8) Thus, it is important to study the natural history of childhood asthma and identify risk factors associated with its development.

One challenge in studying asthma is the presence of enormous variability in the features of asthma (including symptoms and lung function) both between and within patients over time. For genetic studies, the phenotype associated with asthma can be defined by subjective measures (e.g., symptoms), objective measures (e.g., airway hyper-responsiveness or serum IgE level), or both. Because of the complex clinical presentation of asthma, the genetic basis of the disease is often studied through intermediate phenotypes that can be measured objectively, such as the presence of sensitization or airway hyper-responsiveness. However, the latter phenotypes are not specific to asthma, and they may lead to a false positive diagnosis of asthma. (9, 10) This lack of a clear definition of the asthma phenotype is an obstacle for studies of the genetic basis of asthma. (11-13)

Epidemiological definitions of asthma are based on symptoms, tests for airway hyper-responsiveness, and an evaluation of allergy. Questionnaire information has formed the basis of many epidemiological studies and has been used to define asthma in terms of symptoms alone. Asthma and some wheeze phenotypes, like '*persistent wheeze*', have been used interchangeably. Commonly used definitions of asthma are based on "wheeze ever" and "wheeze in the past 12 months". (2) The Tucson Children's Respiratory Cohort Study has identified childhood wheezing phenotypes with differing characteristics and

prognoses, such as early transient wheeze, non-atopic wheeze, IgE-mediated wheeze, and asthma. (14) The Isle of Wight birth cohort study has extended these observations, describing characteristics of childhood wheezing phenotypes and identifying risk factors associated with their development. (15, 16)

1.3. Public health significance of asthma

Asthma is a serious global public health problem and it is the most common chronic illness of childhood. The prevalence of asthma symptoms in children is different in different populations and can be as high as 30 percent. The highest prevalences are found in Australia, New Zealand, and England. Childhood asthma is more common in boys than in girls. Asthma is a disease that places a huge burden on society. It is estimated that asthma affects more than 15 million persons in the U.S., leading to more than 500,000 hospitalizations and over 5,000 deaths annually. (17) The annual direct cost of asthma was recently estimated to be 8 billion dollars. (18)

There is evidence that the prevalence of asthma and other allergic diseases has been rising at a dramatic rate in the last three decades. It is also becoming clear that this rise is largely restricted to developed countries, and is widely believed to be linked to a Western lifestyle. In the United States, the prevalence of asthma has increased by nearly 75% in the past 2 decades. (19) This recent increase has been attributed to environmental factors and their interactions with genes. (5, 20-24) Exposure to tobacco smoke is one of the major risk factors for asthma that has been reported in a large number of studies and it is a focus of this dissertation.

1.4. Exposure to tobacco smoke

Among environmental exposures, the association of tobacco smoke and asthma has been investigated to some extent, and there is evidence that both maternal smoking during pregnancy and passive smoke exposure after pregnancy affect asthma later in life. (25-30) Using data from a large cohort study (n~60,000), Jaakkola et al. demonstrated that maternal smoking in pregnancy increases the childhood risk of asthma. (22)

Using the information from more than 20,000 children (6 to 12 years of age) from nine countries in Europe and North America, Moshammer et al. reported that smoking during pregnancy decreases lung function parameters. (31) The effects of past and current passive exposure to tobacco smoke were smaller than the effect of smoking during pregnancy. This study strongly suggests that maternal smoking during pregnancy subsequently affects the lung function of children.

Despite the evidence for the effect of prenatal exposure to tobacco smoke on asthma phenotypes, there is very limited knowledge about the mechanism of this effect. (22) This dissertation investigates whether the effect of prenatal exposure to tobacco smoke on asthma phenotypes later in life is related to a genetic predisposition (*IL13* gene polymorphism).

1.5. Genetic predisposition

There is evidence that asthma has genetic components. (2) A number of studies have shown an increased prevalence of asthma phenotypes among the offspring of subjects with asthma compared to the offspring of subjects without

asthma. (11, 12) Family studies have convincingly indicated that atopy (as measured by allergen skin tests, total IgE, and/or specific IgE), airway hyper-responsiveness, and asthma as diagnosed by questionnaire are at least partly under genetic control. (11, 32) Numerous studies of twins have demonstrated that concordance rates for asthma phenotypes are all substantially higher for monozygotic than for dizygotic twins, suggesting a strong genetic contribution. In population-based studies of twins, the estimated effect of genetic factors is about 35 to 70 percent, depending on the population and the design of the study. (11, 12) However, despite extensive effort and advances in molecular biology and genetics, no gene for asthma has been identified with any certainty. (11, 12) The results of several studies provide an indication that multiple genes may be involved in the pathogenesis of asthma, and chromosomal regions likely to harbor asthma susceptibility genes have been identified. (12, 33, 34) Several genes on chromosome 5q may be important in the development or progression of inflammation associated with asthma and atopy, including genes encoding the cytokines interleukin (IL)-3, IL-4, IL-5, IL-9, IL-12, and IL-13. (4,5) In particular, IL-13 plays an essential role in the allergic immune response, both by causing the differentiation of T-helper cells toward an allergic response and by inducing IgE production by B cells. (35, 36) IL-13 also affects both ciliated and secretory cell differentiation, leading to airway damage and obstruction. (37) The other effect of IL-13 that is very important in the pathogenesis of asthma is increased mucus production in the respiratory tract. (38) Therefore, *IL13* is a strong candidate gene for having a role in asthma. In this work information on *IL13* gene

polymorphism (see 'methods' section) is used to investigate its combined effect (interaction) with maternal smoking during pregnancy on the outcomes of interest.

1.6. Gene-environment interaction

It has been suggested that common diseases, such as diabetes, cancer, and asthma, are the result of a complex interplay of genetic and environmental factors. The concept of gene-environment interaction implies that a combination of the two factors (a gene and an environmental exposure) has a different effect than their independent impacts. Statistically, a gene-environment interaction can be tested in several ways. One way is to stratify the sample by one factor and look at the effect of the other. Another way is to explore the statistical significance of a gene-environment interaction by including terms for the gene, the environmental factor, and their interaction in a model. (39)

The fact that no single gene has been identified for asthma, and that the recent increase in the prevalence of asthma cannot be explained by genes, has prompted researchers to investigate gene-environment interaction effects for asthma. In consideration of the objectives of this dissertation, I will address the following issues:

1- Evidence for the combined effect of genetic predisposition and exposure to tobacco smoke on asthma

2- The epigenetic explanation as one of the mechanisms for gene-environment interactions

3- The importance of a time window for a gene-environment interaction

4- The prenatal programming concept

1.6.1. Evidence for the combined effect of genetic predisposition and exposure to tobacco smoke on asthma

For asthma, several studies have shown a combined effect of genetic predisposition and exposure to tobacco smoke. (30, 40, 41) Wang et al. observed that the association of β2 adrenergic receptor genotypes and asthma is affected by exposure to tobacco smoke. Kabesch et al. reported that the effect of passive smoking on asthma was larger when children had glutathione S transferase deficiency. A genome-wide analysis has suggested more links between genetic markers and asthma when subjects were exposed to passive tobacco exposure. (30)

1.6.2. Epigenetic phenomena as one of the mechanisms for gene-environment interactions

One mechanism used to explain the effect of gene-environment interactions on asthma is known as epigenetics. (5) All cells of an individual have a unique composition of DNA and hence, genes. The process of a gene becoming active (turned on) is known as gene expression. The structural and functional heterogeneity of cells is the result of differential patterns of gene expression. A mutation is a change in the DNA sequence. A mutation of a gene can potentially alter gene expression, but mutation is a rare event. Usually alteration in gene expression happens without mutations in the DNA sequence

and rest on the potential ability of genetic information to change through developmental events and/or environmental exposures. These changes happen via the post synthetic modifications of either the structure of the double strand helix (by methylation) or of histones (by acetylation, methylation, or phosphorylation) (42). Based on animal studies, it is evident that the methylation status of DNA and the chromatin conformation are affected by environmental exposures (42, 43). These stable alterations in genetic information that are heritable in the short term and do not involve a sequence variation (mutation) in the DNA itself, are called epigenetic changes. If a modification of the genetic information occurs during early fetal life, it may be retained through cell division (mitosis) (5, 42, 43) and over generations. (44, 45) This possible life long impact highlights the importance of a time-window for environmental exposures in the pathogenesis of asthma.

1.6.3. The importance of a time window for gene-environment interaction

Regarding the effect of exposures on asthma and its recent increase in prevalence, there are two concepts: First, the *hygiene concept,* and second, the *prenatal priming concept*. The first *concept* emphasizes the time window after birth as a critical period of exposure and considers that a decline in the exposure to microbial antigens (*hygiene*) is responsible for the recent increase in allergic disorders. Supporters of this concept suggest that improved hygiene in industrialized societies and the use of vaccines and antibiotics have reduced the incidence of infections. The assumption is that cytokines involved in immune responses against infection can suppress immune responses that are involved in

allergic reactions. Therefore, a reduced exposure to infectious agents can result in an augmented allergic response and, hence, allergy. (46, 47) The studies that suggest this concept are epidemiological investigations that show the following: an inverse relationship between family size and the risk of developing asthma; early placement in day care settings, and presumed exposure to infectious agents, appears to protect against the development of asthma; and, observations demonstrating that exposure to farm animals and raw milk early in life reduces the likelihood of developing asthma, allergic rhinitis and atopic sensitization. (48-50). The studies of the *hygiene concept* are contradictory and also they fail to provide a causal relationship between decreased exposure to infectious agents and allergy. (51, 52) The second concept (*prenatal priming,* the focus of this dissertation) considers fetal life as the critical period of exposure and suggests that exposure to environmental factors during pregnancy are important for the rise in the prevalence of asthma and allergy. (53-55) A more detailed discussion of prenatal programming follows.

1.6.4. The prenatal programming concept

During organogenesis in fetal life, tissues face 'critical' periods of development. 'Programming' describes the process whereby a factor at a critical period of development has lasting or lifelong effects. (56) As an example, it has been shown that offspring of women who were pregnant when they experienced the Dutch Hunger Winter of 1944-1945 had more insulin resistance and Type II diabetes than those born before or conceived after the famine. (57) Although the concept of "*in utero* programming" has been investigated for many chronic

disorders, the previous studies mainly focused on fetal undernutrition, without considering a specific factor or exposure. For asthma and allergic diseases, the role of the uterine environment in programming the immune responses of the infant has not been widely studied and allergen exposure after birth has been considered as the major factor in whether a baby at high genetic risk of atopy develops disease. (58)

It is generally accepted that dramatic changes in maternal immune responses occur during pregnancy. (59) Fetal markers of an immune response are detected as early as 10-22 weeks gestation. (60) T-cells have essential roles in the ways that the immune system responds to infectious agents and antigens. Among the different types of T-cells, Th1 cells react to intracellular infectious agents and foreign cells; Th2 cells are involved in antibody production and allergic reactions. The type of T-cell reaction that is related to allergic outcomes (Th2) is also enhanced in the feto-maternal unit during gestation. (61) The reason for Th2 enhancement during pregnancy is to diminish reactions of maternal and fetal immune systems against each other that may terminate pregnancy. (62, 63) Although the above-mentioned dichotomy for T-cells has been challenged recently, immune reactions in allergy and in a normal pregnancy are considered to be similar. (64) The latter suggests that the prenatal period is a critical time window for the development of allergic diseases. There is also evidence that maternal immune status and exposures during pregnancy may change the susceptibility of the mother's fetus to childhood atopic disorders. (5) Furthermore, antigens that can be transferred to the fetus during pregnancy may

play a role in the future development of atopy. There is, in fact, evidence that exposure to antigens during pregnancy can affect the fetal immune response. (65, 66) For example, Van Duren-Schmidt et al. showed that proliferative responses of umbilical cord blood mononuclear cells (CBMC) to birch pollen and timothy grass pollen were higher when the amount of exposure to these pollens was higher during fetal life. (67) CBMC responses to specific allergens are indicators that *in utero* exposure to factors that have entered the maternal circulation during pregnancy results in the development of early infant responses (58, 66), which may be predictive of later allergic disease. Additionally, as discussed previously, gene-environment interactions through epigenetic mechanisms can have a profound effect during fetal life compared to the postnatal period.

CS-IgE has been suggested as a possible marker of exposure to environmental factors during pregnancy that may be used to predict allergy later in life. Several groups investigating the predictive capacity of elevated CS-IgE for the development of allergic disease have demonstrated that IgE specific for certain allergens can be detected at birth. (68, 69) Therefore, the *in utero* environment influences fetal responses in a way that may have major implications later in life.

Despite the importance of the prenatal period as the critical time window for the effects of environmental exposures, there is very limited information for the effect of interactions between genes and exposures during pregnancy on childhood asthma. Based on the prenatal programming concept, I conceptualized

a theoretical model. Using the interleukin 13 gene (*IL13*) and maternal smoking during pregnancy as examples for the theoretical model, this dissertation investigates whether there is an *in utero* programming process for the development of asthma in offspring.

1.7. Theoretical model

For an *in utero* programming process for childhood asthma to exist, I deem that three conditions need to be met: **First,** presence of evidence that a biologic mechanism can change the susceptibility to asthma. Cord serum immunoglobulin E was used as the marker of susceptibility for asthma (CS-IgE of course, would not be affected by after birth exposures; and could potentially be an indicator of *in utero* influences). The biologic mechanism for programming was hypothesized to be a gene-environment interaction between the *IL13* and maternal smoking during pregnancy; **Second,** existence of an association between the marker of susceptibility at birth, CS-IgE, and asthma phenotypes later in life. This association suggests that some stable risk of asthma can be attributed to fetal life events; **Third,** evidence is required that the biologic mechanism during fetal life (the *IL13* genotypes – maternal smoking during pregnancy interaction) can affect asthma phenotypes later in life. The requirement of a similar gene-environment interaction for both CS-IgE and asthma provides further support for the existence of a biological process rather than mere statistical associations. The three associations for the overarching theme of an *in utero* programming process are shown in figure 1 and discussed in the background section (below).

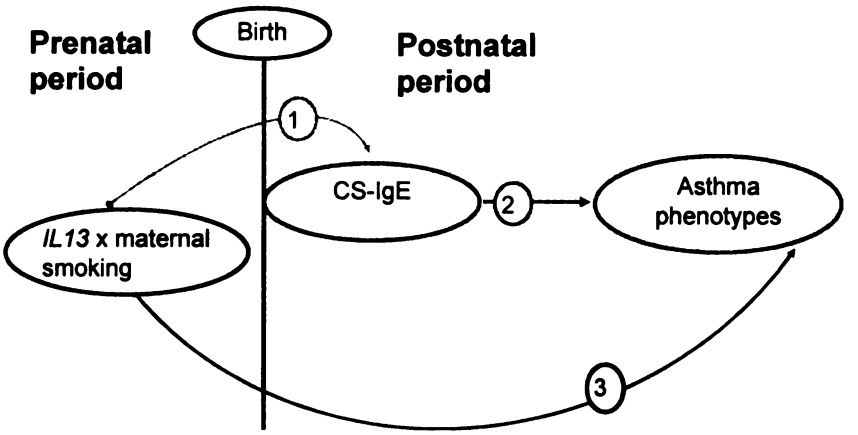


Figure 1.1. The three associations for the overarching theme of an in utero programming process.
Interleukin 13 gene (*IL13*), maternal smoking, cord serum immunoglobulin E (CS-IgE) and asthma phenotypes are examples used in this dissertation. 1- The interaction between *IL13* and maternal smoking affects CS-IgE (a susceptibility factor for asthma) 2- CS-IgE is related to asthma later in life 3- The interaction between *IL13* and maternal smoking is present for asthma phenotypes.

1.8. Background

1.8.1. The effect of maternal smoking during pregnancy and *IL13* gene polymorphism on cord serum immunoglobulin E.

Allergic reactions have long been recognized to be IgE-mediated. (70, 71) Evidence suggests that factors early in life have major effects on the later development of allergy, and cord serum IgE (CS-IgE) has been suggested to be a biomarker for allergic diseases later in life. Several reports have shown a relationship between *IL13* and serum IgE (72, 73), but to the best of my knowledge, there has been no investigation of the effect of *IL13* polymorphisms or their interaction with environmental exposures on CS-IgE. In fact there is very limited information on the relationship between genes and CS-IgE. (74) Chang et al. reported a gender-limited association of cytotoxic T-lymphocyte antigen-4 (CTLA-4) polymorphism with cord blood IgE levels. The results of studies of the effect of maternal smoking on CS-IgE have been inconsistent. (75-81). If we assume that the genetic background is different across populations, and if the effect of cigarette smoking on CS-IgE is dependent on the genetic background, then some of this inconsistency can be attributable to gene-smoking interactions.

1.8.2. Cord serum IgE is related to childhood asthma.

Orgel et al., 1975, was first to report a correlation between serum IgE during the first year of life and atopic diseases in the first 2 years of life. (82) As mentioned above, CS-IgE has been suggested to be a biomarker for atopic diseases later in life. (78, 82-88) However, there are studies that disagree with the use of CS-IgE as a biomarker for asthma and allergy. (68, 83, 88-93) A limitation of these studies is that only early childhood manifestations (up to 5 years) were investigated. (78, 82, 90, 94) Except Nambu et al., who investigated CS-IgE specific to a particular allergen, all of the previous studies used total CS-IgE. (83) Comparisons among previous studies are complicated by their use of different allergic outcomes. For this dissertation the association between CS-IgE levels and asthma up to age 10 was investigated.

1.8.3. The combined effect of maternal smoking during pregnancy and *IL13* gene polymorphism on childhood wheeze phenotypes.

It has been suggested that the increased prevalence of asthma-related phenotypes over the last three decades is due to exposure to environmental factors (95). Among such exposures, cigarette smoking is regarded as an important risk factor for asthma (22). In particular, an effect of maternal smoking during pregnancy on the development of asthma in offspring has been proposed (96-102). A number of studies in children have linked parental smoking with markers of allergy such as serum IgE levels (103, 104). Interleukin 13 (IL-13) is an important cytokine involved in the IgE pathway and pathogenesis of asthma (35, 105, 106). Noakes et al. noted that exposure to tobacco smoke during

pregnancy was associated with a significantly higher neonatal IL-13 response to both house dust mite and ovalbumin, indicating that a prenatal exposure to tobacco may have immunological effects (105). These observations suggest that part of the effect of exposure to tobacco on asthma might be through cytokines.

Researchers have speculated that individuals may vary in genetic susceptibility to cigarette smoke exposure (104, 107). A few studies have suggested a combined effect (interaction) of genes and exposure to tobacco for asthma (30, 40, 41). In spite of the importance of IL-13 in asthma (35, 105, 106), some studies have failed to show an association between the interleukin 13 gene (*IL13*) polymorphism and asthma phenotypes (108, 109) and there is no information as to whether the adverse effect of maternal smoking on asthma may result from an interaction between exposure to tobacco smoke and cytokine regulating genes. To the best of my knowledge, no study has investigated whether there is an interaction between tobacco smoke exposure and *IL13* polymorphism on wheezing. Because of the high prevalence (25-30 percent) of smoking among women of reproductive age (110) there is a need to investigate its possible role in the development of asthma and related phenotypes. Here, I investigate the presence of an interaction between exposure to tobacco smoke and *IL13* gene polymorphism on longitudinal assessments of wheeze phenotypes during the first decade of life in the Isle of Wight birth cohort.

Table 1.1. Studies which investigated the association between cord serum immunoglobulin E (CS-IgE) and allergy later in life

Author, year	Cut off point for CS-IgE (kU/L)	Sample size	Age (years) at follow up	Association with Asthma phenotypes
Nambu, 2003	0.07	10	3	No
Kaan, 2000	0.5	384	1	No
Bergmann, 1997	0.35	1314	2	No
Kjellman, 1994	0.9, 1.3	1654	11	Yes
Eiriksson, 1994	0.2, 0.7	180	1.5- 2	No
Ruiz, 1991	0.7	92	1	No
Magnusson, 1988	1.20	190	1.5	No
Strimas, 1988	0.5	83	1	No
Croner, 1982	1.3	1701	1.5	No

METHODS

In this chapter, I describe the Isle of Wight Birth Cohort Study and methods used for statistical and genetic analyses. Methods, specific to each research question are available in the three manuscripts in the 'Results' section.

2.1. The Isle of Wight Birth Cohort Study

The Isle of Wight Birth Cohort Study represents an unselected whole population birth cohort based on the Isle of Wight, U.K. The Isle of Wight is an island (21 x 37 km) off the southern coast of England with a resident population of 130,000. The majority of people live in small towns and villages. The population density is similar to that of the rest of England (340 per sq km). There is no major manufacturing or large industries on the island. In 2001 only 2.3% of residents were employed in agriculture trade. Thus, the Isle of Wight is primarily a semi-rural but not a farming community. The population is mainly Caucasian (99%) and it is neither geographically nor genetically isolated.

Figure 2.1. Isle of Wight is located in the south of United Kingdom

The Isle of Wight birth cohort study was initiated by Drs. David Hide and Hasan Arshad at the David Hide Asthma and Allergy Research Centre on the Isle

of Wight. The intention was to prospectively study a whole population cohort for the development of asthma and allergic diseases and identify genetic and environmental risk factors relevant to these conditions. Enrollment took place at birth. Of the 1,536 children born on the Isle of Wight between January 1, 1989 and February 28, 1990, informed consent was obtained from the parents of 1,456 children. These children have since been seen at the ages of 1, 2, 4 and 10 years. Detailed information on family history and environmental exposures have been collected at birth and updated at each follow-up.

At each follow-up, detailed questionnaires were completed with the parents for each child regarding asthma and allergy prevalence. Information on asthma morbidity and medication requirement was also obtained. Skin prick testing to 14 common food and aero-allergens was performed at 4 and 10 years. Further information was collected at 10 years using standardized International Study of Asthma and Allergy in Childhood (ISAAC) questionnaires. (111) Spirometry and bronchial provocation tests were performed, blood was collected for genetic studies, and serum was used for IgE measurements and stored for future analysis.

The Research Ethics Committees of the David Hide Asthma and Allergy Research Center and Michigan State University approved the study of children born and enrolled (n=1,456) between January 1, 1989, and February 28, 1990, on the Isle of Wight. Informed written consent was obtained from parents. Children were followed up at the ages of 1 (n = 1,167; 80.2%), 2 (n = 1,174; 80.6 percent), 4 (n = 1,218; 83.7%) and 10 years (n = 1,373; 94.3%).

2.2. Statistical analysis

SAS/STAT® program versions 8.2 and 9.1 were used to perform statistical analysis. (112) Statistical significance was defined as a *p* value of ≤ 0.05. The analyses include Chi-square (χ^2) tests, likelihood ratios, logistic regression, and assessment of statistical interactions.

2.2.1. χ^2 distribution and tests

The Chi-square (χ^2) test is a statistical procedure to assess association between two categorical variables. When the variables are arrayed in r-rows and c-columns, the test of the null hypothesis of no association has degrees of freedom (r-1)(c-1). The χ^2 test can be used when no more than 20% of the expected counts are less than 5 and all individual expected counts are 1 or greater. For a 2×2 table, all four expected counts should be 5 or greater. (113) Formula for getting the expected frequency of a particular cell

- 1^{st} column, 1^{st} row = [(row 1 total) × (column 1 total)]/ grand total
- 1^{st} column, 2nd row = [(row 1 total) × (column 2 total)]/ grand total
- In general, expected frequency of i^{th} column, j^{th} row=[(row i total) × (column j total)]/ grand total

In this dissertation χ^2 tests were used to compare the sample used in each analysis with the reference sample. Consider that Sample 1 is the original group and Sample 2 is the one used in the analysis and number of missing is M (table 2.1). Let us review the previous exercise that about χ^2 test for equality of proportion

Table 2.1. A scenario to investigate whether a missing process is related to the different values of a variable

Variable of interest	Value 1	Value 2	TOTAL
Sample 2	a	b	a + b
Sample 1- Sample 2	M1	M2	M
TOTAL (Sample 1)	A	B	A + B

The question is whether a significant difference between the proportions of missing by the value of the variable exists. We test:

H_0: $p_1=p_2$ vs H_1: $p_1 \neq p_2$

where, p_1= proportion of missing for Value 1 and

p_2= proportion of missing for Value 2

2.2.2. Likelihood ratios

The likelihood ratio is defined as: sensitivity / (1-specificity). These ratios are used to compare cut-off points of a marker, for example CS-IgE, and choose the cut-off point that provides the optimal sensitivity and specificity for the outcome of interest, for example asthma. This method is used in the second manuscript in the 'Results' section.

2.2.3. Logistic regression

Logistic regression is primarily used to assess the relationship to the binary outcome of a primary exposure, after adjusting for the effects of other variables (covariates) and their interaction with the exposure. When these variables are not known to be in the causal pathway of association between

exposure and the outcome they are known as confounders. Confounders that interact with the primary exposure are called effect-modifiers.

Let Y denote the dichotomous outcome of interest with Y =1 labeling the presence of the condition under study, and Y=0 labeling the absence of the condition. Covariates, including the primary exposure and interactions are denoted by X_1, $X_2 \ldots X_k$. Then the logistic model for P [Y=1 | X_1, $X_2 \ldots X_k$] is written simply as P($\underline{X}$) where $\underline{X}$ is abbreviation for X_1, $X_2 \ldots X_k$. The model formula is given by

$$P(\underline{X}) = 1/ (1 + e^{-(\beta 0 + \Sigma \beta i \, Xi)}) \text{ or logit } (P(\underline{X})) = \log \left(\frac{p}{1 - p} \right) = \beta_0 + \Sigma \beta_i \, X_i \text{ in}$$

which β_0 and β_i are representing unknown parameters to be estimated based on the observed variables X_1 through X_k and the outcome variable Y. The coefficients β_1 through β_k can be interpreted as adjusted log-odds ratios. For example, if X_1 is the primary exposure, coded X_1 = 1 for "exposed" and X_1 =0 for "not exposed", then assuming the variables X_2 through X_k do not include X_1, the odds ratio is given by exp(β_1).

2.2.4. Statistical interaction

2.2.4.1. The concept of an interaction.

An interaction evaluates whether a change in one variable affects the association between two other variables. In other words, it reveals whether or not the effect of exposure on the outcome changes by different levels of a third variable. For instance, consider two variants for *IL13* gene, major and minor; and

status of maternal smoking during pregnancy. When children had the minor *IL13* variant, they had about 25% chance of having *early onset persistent* wheeze, regardless of maternal smoking status. But when children had the major *IL13* variant, maternal smoking increased their chance of having *early onset persistent* wheeze by 52% (41.7+ 27.4, table 2.1). Graphically, interaction is present (Figure 2.2) when the slope for the effect of one factor (maternal smoking) changes by different levels of the other factor (major and minor variants of *IL13* gene).

Table 2.2. proportion of children who had early onset persistent wheeze by different condition of interleukin 13 (IL13) genotype and maternal smoking during pregnancy, Isle of Wight birth cohort study

		Maternal smoked	
		no	yes
IL13 genotype	minor	30/114(26.3)	10/40 (25.0)
	major	28/102 (27.4)	15/36 (41.7)

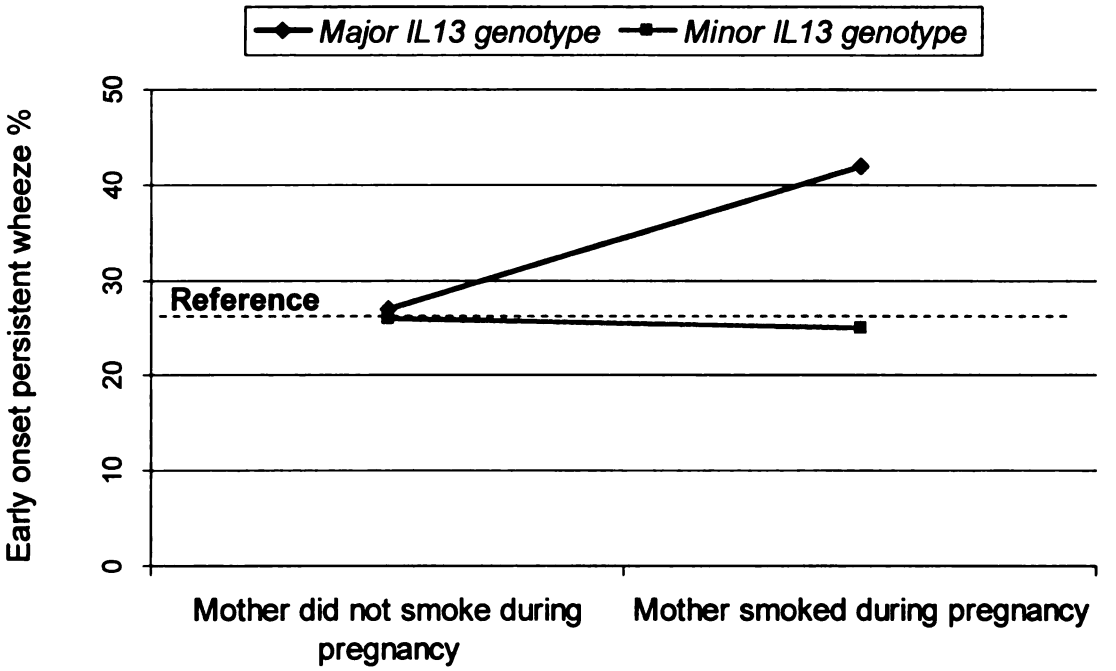


Figure 2.2. Graphical demonstration of a statistical interaction. The slope for the effect of maternal smoking changes by different variants (major and minor) of interleukin 13 gene (IL13).

2.2.4.2. Evaluation of an interaction (additive) by stratification.

One way to test whether the dichotomous variable X_1 (value '0' for the reference group and value '1' for the comparison group) influences the association between the dichotomous variable X_2 (value '0' for the reference group and value '1' for the comparison group) and the outcome of interest, Y, is to stratify the sample by values of X_1 and evaluate the association between the other two variables, X_2 and Y, in a logistic regression model. If the odds ratio of X_2 for Y is different when X_1=0 versus when X_1=1, it means that X_1 may modify the relationship of X_2 and Y. This kind of interaction is known as additive interaction.

It is also possible to test for the presence of a multiplicative interaction as follow.

2.2.4.3. Evaluation of an interaction (multiplicative) effect using logistic regression.

To test the hypothesis that the dichotomous variable X_1 (value '0' for the reference group and value '1' for the comparison group) influences the effect of the dichotomous variable X_2 (value '0' for the reference group and value '1' for the comparison group), and vice-versa, the model should include an interaction term that is the product of these two variables. If the effect of one variable is altered by the other, the coefficient on this interaction term (X_1X_2) will be statistically significant.

In a logistic model, one can present the interaction between X_1 and X_2 as:

$$\text{logit}\,(P(\underline{X})) = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_1X_2$$

To test whether a multiplicative interaction between X_1 and X_2 is present, one can perform a likelihood ratio test.

2.2.4.4. Likelihood ratio test

First, the model with the interaction term is run and its likelihood is recorded: Model 1: $\text{logit}\,(Y) = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_1X_2 \rightarrow$ Likelihood=a

Second, the model without the interaction term is run and its likelihood is recorded: Model 2: $\text{logit}\,(Y) = \beta'_0 + \beta'_1X_1 + \beta'_2X_2 \rightarrow$ Likelihood=b

Third, as the difference between –2log (likelihood) of two nested (Model 1 and Model 2) has a χ^2 distribution under the null hypothesis that $\beta_3=0$, one can test the significance of the above-mentioned interaction by calculating *p* value for the χ^2-test, in this case with one degree of freedom:

$$\chi^2 = -2\log(a) - (-2\log(b)) = -2\log(a/b)$$

It is also possible to determine the effect of an interaction:

For X_1, the change brought in logit (P($\underline{X}$)) by a change in X_1 from '0' to '1' while $X_2 = 1$ is $\beta_1 + \beta_2$ as follow:

$$\text{logit}(P_{11}) = \beta_0 + \beta_1(1) + \beta_2(1) + \beta_3(1)(1) = \beta_0 + \beta_1 + \beta_2 + \beta_3$$

$$\text{logit}(P_{01}) = \beta_0 + \beta_1(0) + \beta_2(1) + \beta_3(0)(1) = \beta_0 + \beta_1$$

$$\text{logit}(P_{11}) - \text{logit}(P_{01}) = \beta_1 + \beta_2$$

For X_1, the change brought in logit (P($\underline{X}$)) by a change in X_1 from '0' to '1' while $X_2 = 0$ is β_1 as follow:

$$\text{logit}(P_{10}) = \beta_0 + \beta_1(1) + \beta_2(0) + \beta_3(1)(0) = \beta_0 + \beta_1$$

$$\text{logit}(P_{00}) = \beta_0 + \beta_1(0) + \beta_2(0) + \beta_3(0)(0) = \beta_0$$

$$\text{logit}(P_{10}) - \text{logit}(P_{00}) = \beta_1$$

As presented, the effect of value 1 of X_1 on outcome is different when $X_2 = 0$ from when $X_2 = 1$.

It is also possible to calculate the combined effect for X_1 and X_2 as follow:

$$\text{logit}(P_{11}) = \beta_0 + \beta_1(1) + \beta_2(1) + \beta_3(1)(1) = \beta_0 + \beta_1 + \beta_2 + \beta_3$$

$$\text{logit}(P_{00}) = \beta_0 + \beta_1(0) + \beta_2(0) + \beta_3(0)(0) = \beta_0$$

$$\text{logit}(P_{11}) - \text{logit}(P_{00}) = \beta_1 + \beta_2 + \beta_3$$

2.3. Genetic analysis

In this part three basic concepts are addressed:

1. Single nucleotide polymorphisms (SNPs)

2. Hardy-Weinberg equilibrium

3. Linkage Disequilibrium (LD) and tests for LD

2.3.1. Single nucleotide polymorphisms (SNPs)

Although more than 99% of human DNA sequences are the same across the population, variations in DNA sequence can have a major impact on human's genetic structure and function. Therefore, markers of variations in DNA are of great value for biomedical research. Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered. For example a SNP might change the DNA sequence AAGGCTAA to ATGGCTAA. For a variation to be considered a SNP, it must occur in at least 1% of the population. Usually a SNP needs a minor allele frequency (MAF) of at least 10% to be useful in genetic studies. SNPs, which could explain about 90% of all human genetic variation, occur every 100 to 300 bases along the 3-billion-base human genome. Two of every three SNPs involve the replacement of cytosine (C) with thymine (T). SNPs can occur in both coding (gene) and non-coding regions of the genome. Many SNPs have no obvious effect (for example, they do not change an amino acid in the protein made by a gene), but they still may change gene function (for example its expression). (114, 115)

Compared to other genetic markers, SNPs are evolutionarily stable, not changing much from generation to generation, so they are easier to follow in population studies. Evolution is simply a change in frequencies of alleles in the gene pool of a population. For instance, let us assume that there is a trait that is

determined by the inheritance of a gene with two alleles, B and b. If the parent generation has 92% B and 8% b and their offspring collectively have 90% B and 10% b, evolution has occurred between the generations. The entire population's gene pool has evolved in the direction of a higher frequency of the b allele--it was not just those individuals who inherited the b allele who evolved. This definition of evolution was developed largely as a result of independent work in the early 20th century by Godfrey Hardy, an English mathematician, and Wilhelm Weinberg, a German physician. (114, 115)

2.3.2. Hardy-Weinberg equilibrium

Hardy and Weinberg discovered an equation that can be used to demonstrate the probable genotype frequencies in a population and to track their changes from one generation to another. This is known as the **Hardy-Weinberg equilibrium** equation: $p^2+2pq+q^2=1$

In this equation, p is defined as the frequency of the common (major) allele and q as the frequency of the minor allele for a pair of alleles (A and a). In other words, p equals all of the alleles in individuals who are major allele homozygous (AA) and half of the alleles in people who are heterozygous (Aa) in a population. In mathematical terms, this is

$$p = AA + \frac{1}{2}Aa$$

Likewise, q equals all of the alleles in individuals who are homozygous for minor allele (aa) and the other half of the alleles in people who are heterozygous (Aa).

$$q = aa + \frac{1}{2}Aa$$

Because there are only two alleles in this case, the frequency of one plus the frequency of the other must equal 100%, which is to say:

$$p + q = 1 \text{ so, } (p + q)(p + q) = (p + q)^2 = 1$$

Therefore, the chance of all possible combinations ($p^2 + 2pq + q^2$) of alleles occurring randomly is 1. If the frequency of two alleles of one SNP do not violate this equation (insignificant *p* value), the SNP is considered to be in Hardy-Weinberg equilibrium. In other words the distribution of its alleles is not different from the whole population. (114, 115)

2.3.3. Haplotype blocks

If SNPs are close together in the genome, the alleles of SNPs will tend to be inherited together during meioses. The combinations of alleles of SNPs that tend to stay together during meioses make haplotypes. The reason is that there is less chance that recombination will separate the alleles of SNPs that are close together. Regions corresponding to blocks have a few common haplotypes that can explain most of the variability of a gene. As an example, for 2 SNPs, there are four possible haplotypes as demonstrated in Figure 2.3.

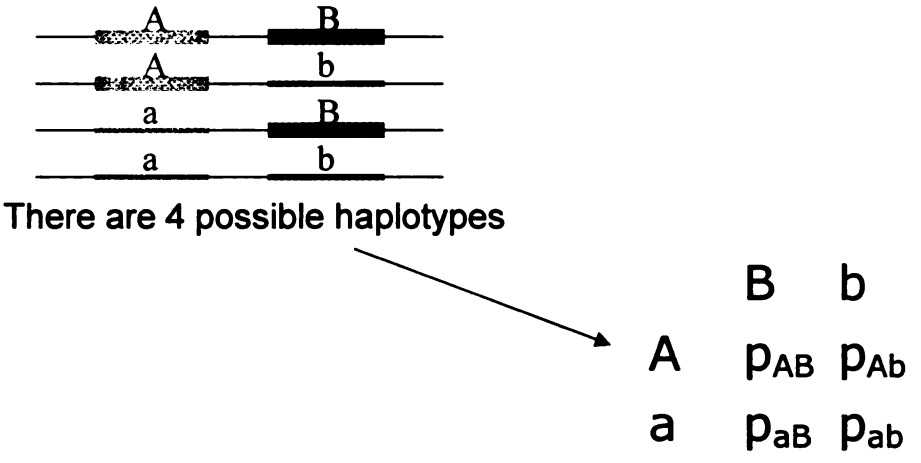


Figure 2.3.For two SNPs there are four possible haplotypes (AB, Ab, aB and ab) with their corresponding probabilities (lower right).

When the genetic information of parents is not available (unknown phase) there are several algorithms and programs to estimate haplotypes and their probabilities based on genotype data. One of the determinants to estimate haplotypes is 'linkage disequilibrium' as follow.

2.3.4. Linkage Disequilibrium (LD) and tests for LD

LD is defined as the non-random association of alleles at two adjacent SNPs. A pair of SNPs is considered to be in complete LD when their alleles are fully associated. For example, consider two SNPs, X and Y with C→T and A→G variation, respectively. These SNPs are in complete LD when C at X appears always with A at Y and T at X appears always with G at Y. Two tests, R^2 and D', are frequently used to assess pair wise LD. For low allele frequencies R^2 has more reliable sample properties than D'. (116)

Suppose two bi-allelic loci, alleles A,a at locus 1, alleles B,b at locus 2. Consider the following haplotype probabilities.

	B	b	
A	p_{AB}	p_{Ab}	p_A
a	p_{aB}	p_{ab}	p_a
	p_B	p_b	

2.3.4.1. (Lewontin's) D'

The absolute value of the difference between observed and expected probabilities, under independence, is the same for all four cells, $|p_{AB}p_{ab}-p_{Ab}p_{aB}|$. Let $p_{AB}p_{ab}-p_{Ab}p_{aB} = \delta$, since no cell can be negative the range of possible values is bounded as a function of the allele frequencies by:

$\delta_{max} = \min[p_b p_A - p_B p_a]$ if $\delta>0$,

$\delta_{max} = \min[p_A p_B - p_a p_b]$ if $\delta<0$,

$|D'|=|\delta|/\delta_{max}$

A disadvantage of the measure is that it is upwardly biased in small samples. (116)

2.3.4.2. Statistical association using R^2

$R^2= (p_{AB}p_{ab}-p_{Ab}p_{aB})^2/(p_A p_a p_B p_b)$

For low allele frequencies R^2 has more reliable sample properties than $|D'|$. R^2 measures statistical association and there is a simple inverse relationship

between this measure and the sample size required to detect association between susceptibility loci and SNPs has a direct relationship. R^2 takes value 1 if only two haplotypes are present.

2.3.4.3. Estimation of linkage disequilibrium in practice

For designs where unrelated individuals are studied, when considering 2 or more loci at a time, it is not possible to determine which alleles are on one chromosome in case of heterozygous genotypes. In practice, parental information is missing (phase is unknown) so one first has to estimate haplotypes. The most useful information about LD comes from double homozygotes and one can estimate LD from these alone, but if minor alleles are uncommon then the estimate of LD may be unstable. It is thus better to get point and interval estimates of LD parameters by estimating haplotype frequencies. (116)

2.3.5. Haplotype estimation

When parental genetic information is not available, it is possible to estimate haplotypes based on the frequencies of alleles of SNPs and their co-occurrence in the population (*in silico* methods). Some of the methods used to estimate haplotypes are maximum likelihood based, implemented via the expectation-maximization (EM) algorithm. As the first step, these methods start from genotypes that their haplotypes are certain. In the next steps, haplotypes of heterozygous genotype are inferred based on the likelihood methods. For these genotypes, the most likely combination of haplotype is estimated based on the frequency of known haplotypes. Generally when there is more than one

heterozygous genotype (for example CT-AG, table 2.3) the haplotypes are ambiguous. In the current work I used PHASE 2.0.2 that utilizes an improved EM algorithm to estimate haplotypes and construct haplotype pairs. (117)

Table 2.3. An example for haplotype inference based on genotype data.

SNP1	SNP2	n	Genotype	n, Haplotype1	n, Haplotype 2
CC	AA	6	CA/CA	6, CA	6, CA
CT	AA	27	CA/TA	27, CA	27, TA
TT	AA	8	TA/TA	8, TA	8, TA
CC	AG	85	CA/CG	85, CA	85, CG
CT	AG	72	CA/TG	***72, CA***	***72, TG***
			or	***or***	***or***
			TA/CG	***72, TA***	***72, CG***
TT	AG	23	TA/TG	23, TA	23, TG
CC	GG	485	CG/CG	485, CG	485, CG
CT	GG	95	CG/TG	95, CG	95, TG
TT	GG	4	TG/TG	4, TG	4, TG
Therefore:					
Haplotype				p	
CA	6 + 6 + 27 + 85 = 124			0.084	
TA	27 + 8 + 8 + 23 = 66			0.045	
CG	85 + 485 + 485 + 95 = 1150			0.784	
TG	23 + 95 + 4 + 4 = 126			0.086	
Total	1466				

Now the probabilities for possible haplotype pairs of the one with more than one heterozygous SNP (CT-AG) is calculated:

p_1 (CA/TG)=0.084 ×0.086

p_2 (TA/CG)=0.045 ×0.784

p_2/p_1 = 4.88 → TA/CG is more likely to be the actual haplotype pair

n, frequency; p, probability

RESULTS

In this chapter three manuscripts, in the order presented, are as follows:

1. Maternal smoking during pregnancy modifies the effect of *IL13* gene polymorphism on cord serum immunoglobulin E (in preparation)
2. Raised cord serum immunoglobulin E increases the risk of allergic sensitization at ages 4 and 10 and asthma at age 10 (Published)
3. The combined effect of maternal smoking during pregnancy and *IL13* gene polymorphism on childhood wheeze phenotypes (submitted)

These manuscripts correspond to the three research questions:

1. Is there an interaction between *IL13* and maternal smoking during pregnancy on CS-IgE?
2. Is CS-IgE related to childhood asthma?
3. Is there a combined effect of interleukin 13 gene (*IL13*) polymorphism and maternal smoking during pregnancy on childhood asthma phenotypes?

3.1. Maternal smoking during pregnancy modifies the effect of *IL13* gene polymorphism on cord serum immunoglobulin E

Abstract

Interleukin 13 (IL13) has a pivotal role in the pathway of immunoglobulin E and cord serum immunoglobulin E (CS-IgE) has been suggested to be associated with allergy later in life. We investigated the association of the *IL13* gene polymorphisms on CS-IgE and also the modifying effect of maternal smoking on this association. In the Isle of Wight birth cohort (UK, 1989-1999), CS-IgE was measured using the ULTRA EIA® kit and was dichotomized at 0.5 kU/L (n=1358). Five single nucleotide polymorphisms in *IL13* were genotyped by pyrosequencing methods and haplotype analysis was performed. Logistic regression was used and gene association analysis included 798 children. Confounders were gender, household cat and dog during pregnancy, position of child in family and birth weight. Children with the common *IL13* haplotype pair had lower percentage of raised CS-IgE (adjusted odds ratio (AOR) = 0.63, 95% CI: 0.41, 0.99). Maternal smoking was not associated with CS-IgE (AOR=1.00, 95% CI: 0.69, 1.46). However, when mothers smoked, the common *IL13* haplotype pair was not protective for raised CS-IgE (AOR=0.95, 95% CI: 0.32, 2.81), but when mothers did not smoke, it was (AOR=0.59, 95% CI: 0.36, 0.96). This is the first study that shows an association between *IL13* and cord serum IgE and also a modifying effect of maternal smoking on this association. The results suggest that the adverse effect of smoking on the immune system may also be via an epigenetic mechanism.

Prevalence of asthma and allergy has increased significantly in the past three decades. (95, 118) Allergic mechanism has long been attributed to IgE-mediated reactions and cord serum IgE (CS-IgE) has been suggested to be a biomarker for atopic diseases later in life (78, 82-88, 119). Although some researchers disagree with the use of CS-IgE as a biomarker for atopic diseases (68, 88-93), the value of CS-IgE as a marker for increased susceptibility in later life on allergy seems important.

There is limited information with regard to the effect of genes and their interaction with other factors on CS-IgE. Chang et al. reported an association of cytotoxic T-lymphocyte antigen-4 (CTLA-4) polymorphism with raised CS-IgE levels that was limited to girls. (74) Interleukin 13 (IL-13) is an important cytokine involved in the IgE pathway (35, 72, 105, 106) and yet there is no prior report for the effect of *IL13* gene on CS-IgE.

An important factor on the development of asthma in offspring is maternal smoking during pregnancy (96-102). A number of studies in children have linked parental smoking with markers of atopy such as serum immunoglobulin E (IgE) levels (103, 104). Noakes and colleagues noted that exposure to tobacco smoke during pregnancy was associated with a significantly higher neonatal IL-13 response to both house dust mite and ovalbumin, indicating that a prenatal exposure to tobacco may have immunological effects (105). The few studies for the effect of maternal smoking on CS-IgE did not report consistent results (75-81). Part of this inconsistency may be due to a different genetic susceptibility of the studied populations.

In the current study we test the hypotheses that *IL13* gene polymorphisms are associated with CS-IgE levels and also explore whether this association is modified by maternal smoking during pregnancy. The data is from the Isle of Wight cohort, established in 1989 in order to study the risk factors for atopy and its development during childhood.

Population and Methods

Study population

The local Research Ethics Committee approved the study of children born and enrolled (n=1,456) between January 1, 1989, and February 28, 1990, on the Isle of Wight, United Kingdom. Informed consent was obtained and children were followed up at the ages of 1 (n = 1,167; 80.2 percent), 2 (n = 1,174; 80.6 percent), 4 (n = 1,218; 83.7 percent) and 10 years (n = 1,373; 94.3 percent). The island is close to the British mainland, semi-rural, with no heavy industry. The population is 99 percent Caucasian.

Birth records, exposures during pregnancy and family history

Data was obtained from hospital records on neonatal complications (preterm delivery, foetal distress, newborn infection, jaundice, low blood sugar, hypothermia, and congenital abnormalities), antenatal complications (pregnancy induced hypertension, gestational diabetes, infections needing antibiotic therapy, intrauterine growth retardation or "miscellaneous") and method of delivery (non-instrumental vaginal, instrumental vaginal delivery or caesarean section).

Parents were asked about their age, maternal smoking and pet keeping during pregnancy, history of asthma, hay fever, and atopic eczema.

DNA isolation and *IL13* genotyping

Anticoagulated whole blood samples were obtained at the 10-year interview and stored frozen (n=921). Genomic DNA was isolated from these samples using QIAamp DNA Blood Kits (Qiagen, Valencia, CA) or the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA). Polymorphisms in the *IL13* gene were examined using the SNPper (120) and Applied Biosystems (121) databases. Two methods were used for genotyping: 1) fluorogenic 5' nuclease chemistry PCR using Assays on Demands kits cycled on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), and 2) biotin-streptavidin-based pyrosequencing performed on PSQ-96 instrumentation (Biotage AB, Uppsala, Sweden).

Five SNPs from the *IL13* gene were used in this study, rs1800925 in the 5' promoter region, rs2066960 in intron 1, rs1295686 in intron 3, rs20541 in exon 4 and rs1295685 in the 3' untranslated region (3' UTR) of exon 4. Because *IL13* is a small gene (2.9 kb), only a few SNPs were needed for a reasonable assessment of genetic associations. Polymorphism selection was based on SNP location, minor allele frequency, and function. Selected SNPs had minor allele frequencies ≥ 19% and were distributed throughout the *IL13* gene. The rs20541 is a coding variant with the common allele (G) coding for arginine and the minor allele (A) encoding glutamine at amino acid 144. This SNP was in strong linkage disequilibrium (LD) with rs1295685 and rs1295686. The former (rs1295685) was selected as a representative SNP of this block due to the availability of a commercial assay.

Cord serum immunoglobulin E (IgE) determination

Duplicate measurements of cord IgE were made on serum from umbilical vein blood samples using the ULTRA EIA® kit (Pharmacia Diagnostics AB, Uppsala, Sweden), which is designed to measure IgE between 0.2 and 50 kU/L on 0.1 mL of serum or plasma. It is a solid phase enzyme immunoassay, using monoclonal anti-human IgE, with a high degree of correlation with the Phadebas IgE PRIST® method (r=0.99). (122) Cord IgA was measured on samples where IgE was higher than 0.3 kU/L. Any samples with IgA > 10mg/L were deemed contaminated by maternal blood and excluded. (122, 123)

Statistical analysis

Each SNP was tested for Hardy-Weinberg equilibrium using Haploview 3.2 software (124). Estimates of LD between SNPs were calculated using D' and r^2 (116). For SNPs that were in strong LD, the authors took one SNP as the representative marker for the block. PHASE 2.0.2 was employed to build the most likely pair of haplotypes (diplotypes) and their probability for each child (117, 125), using the three representative SNPs. In the analysis of individual SNPs and haplotype pairs, genotypes with low frequencies were combined (126, 127).

Using SAS/STAT® version 9.1, statistical analysis was performed on the data only from children who had complete information on *IL13* genotypes, exposure to tobacco smoke, and wheeze phenotypes. Chi-square tests were used to compare the sample used in the analysis with that which was followed up at age 10. Logistic regression analysis and stratification were conducted to test the hypotheses. Statistical significance was defined as a p value of ≤ 0.05.

Potential confounders (table 3.1.1) were: gender, household cat present during pregnancy (yes, no).

RESULTS

Data on *IL13* genotypes and cord serum immunoglobulin E (CS-IgE) were available for 798 children. Compared to the original sample, mothers of children who were included in the analysis were younger and less smoked during pregnancy (p values of 0.03 and 0.0001, respectively, table 3.1.1).

One out of five children was exposed to maternal smoking during pregnancy (20.5 percent, table 3.1.1). Among covariates, maternal atopy, being born in fall and first born were risk factors for raised CS-IgE (table 3.1.2).

For *IL13* genotypes, all five SNPs were in Hardy-Weinberg equilibrium (p = 0.82 for rs1800925, p = 0.99 for rs2066960, p = 0.58 for rs1295686, p = 0.09 for rs20541, and p = 0.36 for rs1295685). Three of the SNPs (rs1295686, rs20541, and rs1295685) were in strong LD (table 3.1.5). Therefore we used only one SNP of this block for haplotype analysis. Thus haplotypes were inferred using rs1800925, rs2066960 and rs1295685. Due to the limited number of children homozygous for minor alleles at all SNPs (less than 5 percent, table 3.1.3), minor allele homozygous and heterozygous genotypes were grouped together. Among haplotype pairs, CCG/CCG had the highest frequency (0.48, table 3.1.4). The estimated probability for CCG/CCG as the best pair was 1.0 in 99 percent of children and was never less than 0.89 (results not shown). All other haplotype pairs had frequencies between 0.001-0.151. In addition, there were probabilities as low as 0.5 to be identified as the best pair for haplotype pairs

other than CCG/CCG. Thus, haplotype pairs other than CCG/CCG were combined (126, 127).

Crude analysis showed that children with CCG/CCG haplotype pair had lower percentage of raised CS-IgE (figure 3.1.1). When the sample was stratified, in children whose mother did not smoke during pregnancy and also in boys, CCG/CCG haplotype pair was significantly associated with a lower percentage of raised CS-IgE (p values of 0.040 and 0.025, respectively, figure 3.1.1). Girls whose mothers smoked during pregnancy had higher percentage of raised CS-IgE.

Using logistic regression and controlling for the confounders, we tested the associations of *IL13* haplotype pairs and maternal smoking with CS-IgE (figure 3.1.2). Children with the common *IL13* haplotype pair (CCG/CCG) had lower relative risk of raised CS-IgE (adjusted odds ratio (AOR) = 0.63, 95% CI: 0.41, 0.99). Maternal smoking was not associated with CS-IgE (AOR=1.00, 95% CI: 0.69, 1.46).

To determine whether maternal smoking modifies the effect of the common *IL13* haplotype pair on CS-IgE, the sample was stratified by maternal smoking. When mothers smoked during pregnancy, CCG/CCG was not protective for raised CS-IgE (OR=0.95, 95% CI: 0.32, 2.81), but when mothers did not smoke, it was (AOR=0.59, 95% CI: 0.36, 0.96). We also tested the multiplicative interaction between CCG/CCG and maternal smoking on raised CS-IgE in a logistic regression model. This interaction was not significant; however, the interaction term between gender, maternal smoking during

pregnancy and CCG/CCG haplotype pair was significant ($p < 0.05$). To demonstrate this interaction we looked at the effect of gender when the other two factors were fixed. The odds ratio for raised CS-IgE in girls whose mothers smoked during pregnancy and had CCG/CCG haplotype pair compared to boys whose mothers smoked during pregnancy and had CCG/CCG haplotype pair was 7.92 (results are not shown).

DISCUSSION

Using the data from the Isle of Wight birth cohort, this study investigated the effect of haplotype pairs of the *IL13* gene, maternal smoking during pregnancy and gender on cord serum immunoglobulin E (CS-IgE). *IL13* gene polymorphisms were associated with CS-IgE levels. Maternal smoking during pregnancy was not related to CS-IgE levels, but its combination with the common *IL13* haplotype pair was associated with raised CS-IgE, especially in girls. To the best of our knowledge this is the first investigation for the association between *IL13* and CS-IgE.

For this study, the information was available from a subset of children who had a CS-IgE measurement and who agreed to provide blood for genotyping. These children appeared to have younger mothers and also less exposure to maternal smoking in comparison to all children who had information on CS-IgE. As the distribution of the outcome variable, CS-IgE, was not different in the original sample and the sample used in the analysis, estimates were not affected by selection process. (128) With regard to genotypes, presence of a selection bias could result in a violation of the Hardy-Weinberg equilibrium. The genotypes

of the five SNPs used in the analysis were in Hardy-Weinberg equilibrium and their allele frequencies were comparable to the equivalent SNPs in other Caucasian populations (72, 73). Hence, with regard to the SNPs a selection bias is unlikely.

Contamination of cord blood with maternal blood was assessed using IgA as a biomarker. This led to the exclusion of 86 infants from further analysis. (129) (130) Hence, a potential misclassification of cord blood IgE is unlikely. To dichotomize CS-IgE levels, cut-off points between 0.5-1.3 kU/L have been used in prior studies. (77, 84, 86, 131) We chose to use the cut off point of 0.5 kU/L CS-IgE as it has been shown in previous analyses of this birth cohort as an optimal level. (129) (119).

With regard to genetic analysis, information on individual SNPs was used and the most likely pairs of haplotypes were estimated from genotype data. It would have been possible to use information on each individual SNP to investigate gene-environment interactions. However using haplotype pairs has some advantages over individual SNP analysis. The issue of "multiple testing" is avoided as all genetic markers were compiled in haplotype pairs. Additionally, it has been suggested, specifically for *IL13* (132), that haplotype analysis could confer more information than individual marker analysis (132-134). Haplotype pair analysis may misclassify genotypes when parents' genetic information is not available (ambiguous phase). However, the probability of having CCG/CCG, the major haplotype pair, was 1.00 in 430 out of 435 children with this genotype (the probability for the other 5 children was 0.89). Thus, CCG/CCG haplotype pair

was inferred with high certainty. The distribution of the data, with respect to minor haplotypes, did not allow testing for their interactions with tobacco smoke exposure. However, when children with a minor haplotype at both loci (frequency = 0.082, table 3.1.4) were removed from the analysis, we obtained similar results.

Previous studies have shown an association between *IL13* polymorphisms and serum IgE. (72, 108, 135, 136) However, there is no report for the effect of *IL13* polymorphisms on cord serum IgE. With regard to the effect of maternal smoking on CS-IgE, results from previous studies are not consistent. While some reported maternal smoking as a risk for higher levels of CS-IgE;(76, 77, 81, 137), others did not find such an association (75, 78, 80).

A different prevalence of allergic disorders in boys and girls has long been recognized. (138) In the only publication on the association of genes and cord serum IgE, Chang et al reported a link between cytotoxic T-lymphocyte antigen-4 (CTLA-4) polymorphism and cord serum IgE levels that was limited to girls. (74) This gender difference has been also shown for the effect of maternal smoking on pulmonary function tests. (139, 140) Some researchers have explained the gender discrepancy based on the effects of sex hormones. (141, 142) There is evidence that sex steroids and substances that mimic their physiological effect can increase allergic manifestations. (20, 143) There is evidence that fetal gender has an influence on hormone profiles. For example, placenta produces more hCG levels when the fetus is female. (144-147) The latter findings suggest a difference in placental function in boys and girls. The effect of gender on the relationship of *IL13* polymorphisms and their interaction with maternal smoking

on CS-IgE may result from a different activity of placenta in girls and boys and also various amounts of exposures due to placental activity. It is plausible to consider that maternal smoking, independent of fetal gender may also interplay with *IL13* polymorphisms to increase CS-IgE. Liu et al. reported that the association of *IL13* polymorphisms and serum IgE is modified by exposure to tobacco smoke. (148)

It has been shown that exposure to tobacco smoke can activate cytokine genes. (149) As mentioned, there is a disagreement between previous studies that investigated the effects of maternal smoking on CS-IgE. (75-78, 80, 81, 137) If we assume that the genetic make up of children studied in previous reports were different, the observed disagreement for the effect of maternal smoking on CS-IgE may be due to an interaction between exposure to tobacco smoke and genes.

In summary, in a sub-sample of the Isle of Wight cohort, the combined effect of exposure to tobacco smoke during pregnancy and the common haplotype pair of the *IL13* gene resulted in an increased relative risk of raised CS-IgE, especially in girls. These results highlight the importance of the interplay of prenatal exposures and genetic predisposition as a mechanism for allergy. Given that the genetic make up is different across studies, the current report suggests that negative reports for the effect of exposure to tobacco smoke on CS-IgE (75, 78, 80) may be dependent on genetic variations. Additionally the observed role of gender for the effect of *IL13* makes us propose future studies to investigate the expression of *IL13* in boys and girls and by various exposures.

These studies may shed light on possible mechanisms of gender discrepancy in allergic disorders.

Table 3.1.1 Comparison of children with a follow-up at age 10 and the subset used in the analysis

		Numbers at birth (%)	Numbers used in the analysis (%)	χ^2 *p* value
Variable	Total	1,358	798	
Maternal age (year)	≥ 34	389 (28.6)	211 (26.4)	0.03
	≥24, <34	801 (59.0)	488 (61.1)	
	<24	145 (10.7)	93 (11.6)	
	Missing	23 (1.7)	6 (0.7)	
Maternal atopy	1	438 (32.2)	256 (32.1)	0.95
	0	905 (66.6)	538 (67.4)	
	Missing	15 (1.1)	4 (0.5)	
Paternal atopy	1	352 (25.9)	215 (26.9)	0.51
	0	983 (72.4)	575 (72.1)	
	Missing	23 (1.7)	8 (1.0)	
Maternal smoking	1	334 (24.6)	164 (20.5)	0.0001
	0	1012 (74.5)	631 (79.1)	
	Missing	12 (0.9)	3 (0.4)	
Household dog	Yes	406 (29.9)	253 (31.7)	0.41
	No	935 (68.8)	540 (67.7)	
	Missing	17 (1.2)	5 (0.6)	
Household cat	Yes	434 (32.0)	267 (33.5)	0.45
	No	907 (66.8)	526 (65.9)	
	Missing	17 (1.2)	5 (0.6)	
Season of birth	Fall	296 (21.8)	171 (21.4)	0.76
	Spring	315 (23.2)	187 (23.4)	
	Summer	305 (22.5)	173 (21.7)	
	Winter	442 (32.5)	267 (33.5)	

Table 3.1.1 (cont'd)

Gender	Girl	661 (48. 7)	394 (49.4)	
	Boy	696 (51.2)	404 (50.6)	0.56
	Missing	1 (0.1)	0 (0.0)	
Position of child in family	2	636 (46.8)	432 (54.1)	
	1	446 (32.8)	289 (36.2)	0.26
	Missing	276 (20.3)	77 (9.6)	
Antenatal complications	Yes	132 (9.7)	81 (10.1)	0.53
	No	1226 (90.3)	717 (89.8)	
Non-instrumental delivery	No	174 (12.8)	100 (12.5)	
	Yes	924 (68.0)	543 (68.0)	0.69
	Missing	260 (19.1)	155 (19.4)	
neonatal complications	Yes	187 (13.8)	110 (13.8)	0.98
	No	1171 (86.2)	688 (86.2)	
Cord IgE	≥ 0.5 kU/dl	174 (12.8)	96 (12.0)	0.30
	< 0.5 kU/dl	1184 (87.2)	702 (88.0)	

Table 3.1.2. Unadjusted odds ratios, logistic regression, for covariates on cord serum immunoglobulin E

Covariate	Cord serum IgE		OR	95 % CI	P value
	< 0.5	≥ 0.5			
Mother's age[1] ≥ 34	67.6% (700/1035)	65.2% (101/155)	0.72	0.39, 1.31	0.28
Mother's age[1] ≥24, <34	28.0% (130/465)	21.7% (15/69)	0.89	0.63, 1.28	0.54
Maternal atopy	31.4% (368/1170)	40.5% (70/173)	1.48	1.07, 2.06	0.02
Paternal atopy	25.6% (299/1166)	31.4% (53/169)	1.32	0.93, 1.88	0.12
Maternal smoking	24.8% (291/1173)	24.9% (43/173)	1.00	0.69, 1.45	0.99
Household dog	32.2% (376/1168)	33.5% (58/173)	1.06	0.76, 1.49	0.73
Household cat	29.7% (347/1168)	34.1% (59/173)	1.22	0.87, 1.72	0.24
Born in fall[2]	38.6% (246/637)	49.5% (50/101)	1.56	1.02, 2.37	0.04
Born in spring[2]	41.5% (277/668)	42.7% (38/89)	1.05	0.67 ,1.64	0.82
Born in summer[2]	40.8% (270/661)	40.7% (35/86)	0.99	0.63, 1.57	0.98
Boy	51.0% (603/1183)	53.4% (93/174)	1.10	0.80, 1.52	0.54
First versus higher born	39.8% (374/939)	50.3% (72/143)	1.54	1.07, 2.17	0.02
Antenatal complications†	10.1% (120/1184)	6.9% (12/174)	0.66	0.35, 1.22	0.18
Non-instrumental delivery[3]	15.8% (150/950)	16.2% (24/148)	1.03	0.64, 1.65	0.89
neonatal complications‡	13.5% (160/1184)	15.5% (27/174)	1.18	0.75, 1.83	0.47

Reference groups:
1, Maternal age ≥34; 2, Born in winter; 3, Instrumental vaginal delivery and caesarean section
†Consist of: pregnancy induced hypertension, gestational diabetes, infections needing antibiotic therapy and intrauterine growth retardation
‡Consist of: preterm delivery, foetal distress, newborn infection, jaundice, low blood sugar, hypothermia, congenital abnormalities)

Table 3.1.3. Single nucleotide polymorphisms (SNPs) in the *IL13* gene for this analysis

SNP	Position (bp)	Location	Genotype	Frequency (%)
rs1800925	132,020,708	Promoter	CC	577 (63.6)
			CT	295 (32.5)
			TT	35 (3.9)
			Total	907 (100.0)
rs2066960	132,022,334	Intron 1	CC	729 (81.5)
			AC	157 (17.6)
			AA	8 (0.9)
			Total	894 (100.0)
rs1295686	132,023,742	Intron 3	CC	483 (64.6)
			CT	240 (32.1)
			TT	25 (3.3)
			Total	748 (100.0)
rs20541	132,023,863	Exon 4	GG	73 (53.7)
			AG	59 (43.4)
			AA	4 (2.9)
			Total	136 (100.0)
rs1295685	132,024,344	Exon 4	GG	584 (64.5)
			AG	280 (30.9)
			AA	41 (4.5)
			Total	905 (100.0)

Gene annotation is based on SNPper (120).
IL13: interleukin 13 gene; n, number of samples used in genetic studies; bp, basepairs.

Table 3.1.4. Frequency of haplotypes and haplotype pairs for three single nucleotide polymorphisms (rs1800925, rs2066960, rs1295685) of the interleukin 13 gene, inferred from genotype data of 907 children

		Frequency (SE)
Haplotype	CCG	0.683 (0.004)
	CCA	0.045 (0.002)
	CTG	0.067 (0.003)
	CTA	0.108 (0.003)
	ACG	0.041 (0.003)
	ACA	0.029 (0.002)
	ATG	0.008 (0.002)
	ATA	0.017 (0.003)
Haplotype pair(s)	CCG/CCG	0.480
	CCG/minor haplotypes*	0.438
	minor haplotypes/minor haplotypes†	0.082

PHASE 2.0.2. was used for this analysis (117, 125).
* This group consists of, with a frequency of:
CCG/ACA, 0.036; CCG/ACG, 0.055; CCG/ATA, 0.037; CCG/ATG, 0.019; CCG/CCA, 0.054; CCG/CTA, 0.151; CCG/CTG, 0.086.
† This group consists of, with a frequency of:
ACA/ATA, 0.001; ACG/ACA, 0.003; ACG/ACG, 0.001; ACG/ATA, 0.002; ATG/ATA, 0.001; CCA/ACA, 0.004; CCA/CCA, 0.002; CCA/CTA, 0.014; CTA/ACA, 0.014; CTA/ATA, 0.002; CTA/CTA, 0.007; CTG/ATA, 0.004; CTG/CTA, 0.020; CTG/CTG, 0.004.

Table 3.1.5. Using the Haploview program (124), three single nucleotide polymorphisms of the interleukin 13 gene appeared to be in a block.

Pair of SNPs	r^2	D' (95% CI)
rs1800925 – rs2066960:	0.01	0.14 (0.04, 0.24)
rs1800925 – rs1295686:	0.31	0.56 (0.50, 0.62)
rs1800925 – rs20541:	0.28	0.63 (0.46, 0.75)
rs1800925 – rs1295685:	0.28	0.54 (0.48, 0.59)
rs2066960 – rs1295686:	0.06	0.37 (0.27, 0.47)
rs2066960 – rs20541:	0.07	0.37 (0.04, 0.71)
rs2066960 – rs1295685:	0.06	0.38 (0.28, 0.47)
rs1295686 – rs20541:	0.78	1.00 (0.83, 1.00)
rs1295686 – rs1295685:	0.90	0.96 (0.93, 0.98)
rs20541 – rs1295685:	0.85	1.00 (0.94, 1.00)

D' and r^2 are pair wise linkage disequilibrium determinants (116); CI, confidence interval.

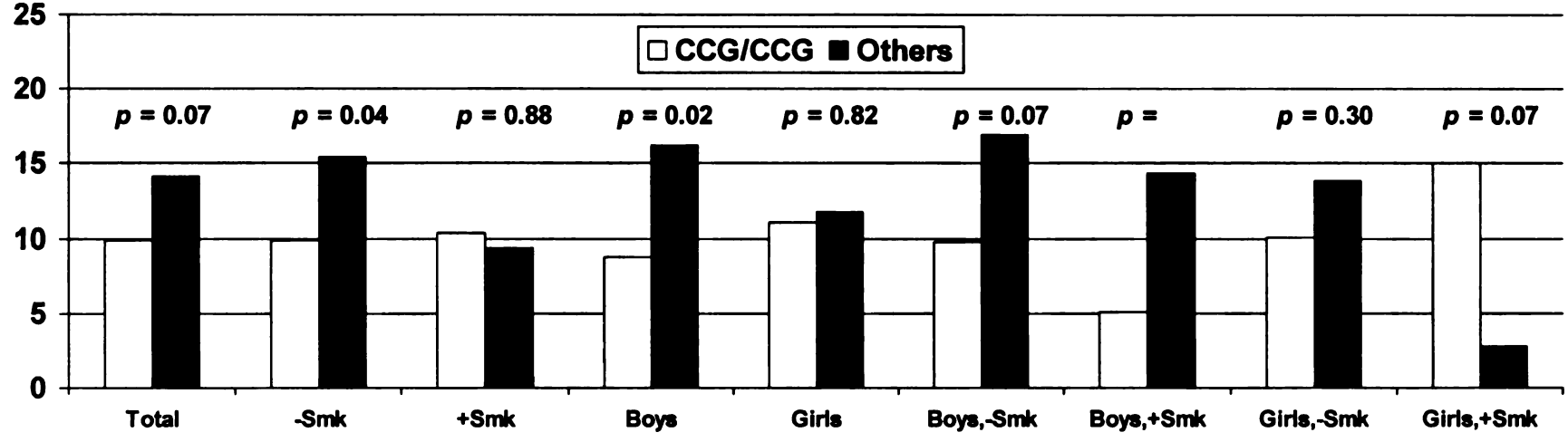


Figure 3.1.1. Percentage of raised cord serum immunoglobulin E (>0.5 kU/L) for the two groups of *IL13* genotypes† ('CCG/CCG' and 'Others') in total sample (n= 787) and stratified by gender, exposure to maternal smoking during pregnancy (Smk) and their combinations.
-Smk, not exposed; +Smk, exposed
† Inferred from genotype data of three single nucleotide polymorphisms of interleukin 13 gene, rs1800925, rs2066960 and rs1295685, using PHASE 2.0.2 software (117, 125).

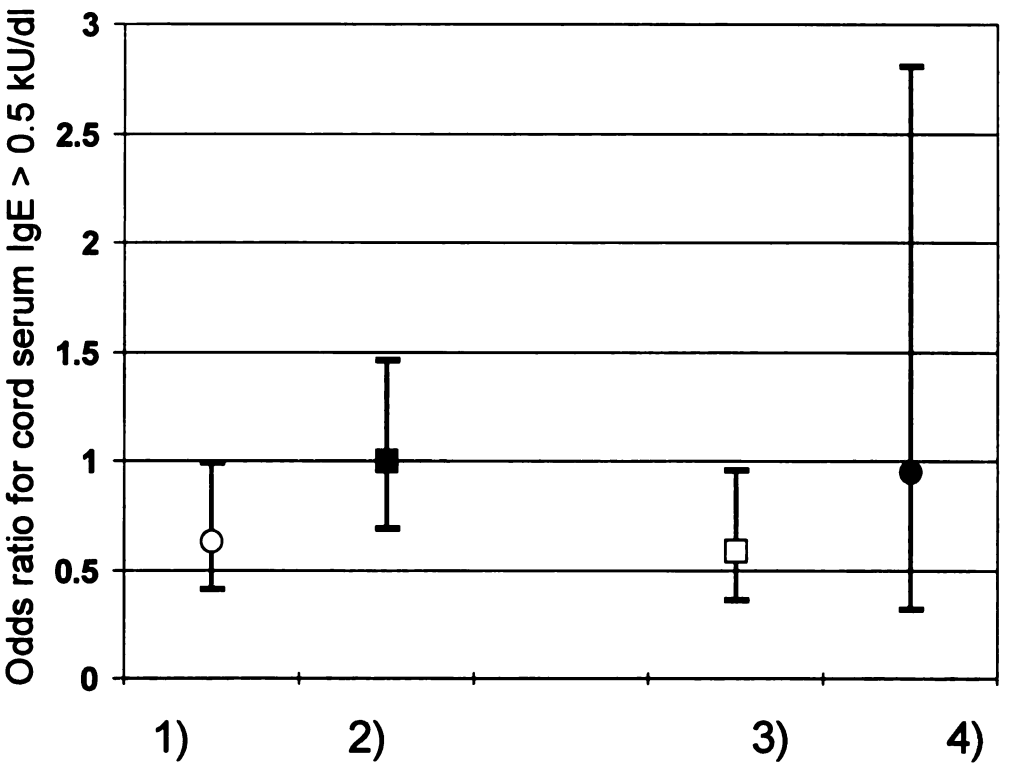


Figure 3.1.2. The association between CCG/CCG haplotype pair and smoking with raised cord serum immunoglobulin E (CS-IgE >0.5 kU/dl).

1) CCG/CCG versus other haplotype pairs
2) Mother smoked during pregnancy
3) CCG/CCG, mother did not smoke during pregnancy
4) CCG/CCG, mother smoked during pregnancy

3.2. Raised cord serum immunoglobulin E increases the risk of allergic sensitisation at age 4 and 10 and asthma at age 10

Background: Evidence suggests that elevated cord serum IgE (CS-IgE) is a risk factor for allergic sensitisation. However, whether CS-IgE is a risk for asthma is controversial.

Objective: To investigate the association between CS-IgE levels and allergic sensitisation at 4 and 10 years and asthma at 1-2, 4 and 10.

Methods: CS-IgE was available for 1358 of 1456 children born between 1989 and 1990. The cohort was evaluated for allergic diseases at ages 1, 2, 4 and 10 years. Skin prick tests for six aero-allergens were performed on 981 children at age 4 and 1036 at age 10. Asthma was defined based on physician's diagnosis. Using logistic regression analysis we estimated the risk of asthma and allergic sensitisation for elevated levels of CS-IgE (≥0.5 kU/L).

Results: At ages 4 and 10 years, 16.5% and 27.0% of children, respectively, showed allergic sensitisation. The risk of allergic sensitisation was significantly associated with elevated CS-IgE at ages 4 (OR: 2.29) and 10 years (OR: 1.73). The prevalence of asthma was 10.3% at age 1-2, 15.2% at age 4 years and 12.8% at age 10. CS-IgE was not associated with asthma at age 1-2 and 4, but showed an increased relative risk at age 10 (OR: 1.66, 95%CI 1.05-2.62). The association was stronger in children who did not develop allergic sensitisation at age 4 or 10 (OR: 3.35, 95%CI 1.41-7.93).

Conclusions: Elevated cord serum IgE is a risk factor for allergic sensitisation at ages 4 and 10 years. This is the second study suggesting that CS-IgE is also a risk factor for asthma at age 10, probably related to late onset of asthma. This association is not necessarily mediated by allergic sensitisation.

Introduction

There is evidence that factors early in life have major effects on the later development of allergy (atopy). (78, 82-88) A correlation between serum IgE during the first year of life and atopic diseases in the first 2 years of life was first reported in 1975 by Orgel et al., which led them to propose that cord serum IgE (CS-IgE) might be used as a predictor for allergic diseases. (82) Subsequent studies examined the use of CS-IgE as a biomarker for atopic diseases, but results were conflicting. (68, 83, 88-93) A limitation of these studies is that only early childhood manifestations (up to 5 years) were investigated,(78, 82, 90, 94) with the exception of a Swedish study that followed newborns to the age of 11. (88) In addition, almost all of the previous studies used total CS-IgE (Nambu et al. investigated CS-IgE specific to a particular allergen (83)). Comparisons among studies are further complicated by their use of different allergic outcomes.

The Isle of Wight cohort was established in 1989 in order to study the risk factors for atopy and its development during childhood. The first examination of CS-IgE in this cohort revealed it to be an insensitive predictor of asthma, eczema, and hay fever in the first year of life. (122) A subsequent study of 4 year olds by Tariq et al. showed that elevated CS-IgE increases the risk of aeroallergen sensitisation without increasing respiratory allergic symptoms. (129) The purpose of the current study is to investigate the association between CS-IgE levels and both allergic sensitisation and asthma in the Isle of Wight cohort up to the age of 10 years.

POPULATION AND METHODS

Study population

After approval by the Local Research Ethics Committee, 1456 of children born between January 1989 and February 1990 on the Isle of Wight, England, were recruited for the cohort. Informed written parental consent was obtained for all participants. To measure cord serum IgE, samples were obtained from 1358 newborns.

Questionnaire and examination

In the first survey, at the time of the child's birth, parents were asked about their age, maternal smoking during pregnancy, and history of asthma, hay fever, and atopic eczema. At 1 and 2 years of age, a questionnaire was administered seeking information on symptoms that are suggestive of allergic disease in children.

At 4 years of age, all children and their parents were invited to attend the allergy clinic. (10) A total of 981 children (67% of the original cohort) attended the clinic, a questionnaire was completed, and a physical examination was conducted. A follow up questionnaire (postal or telephone) was used to gather information for an additional 237 children (n=1218; 84% of the original cohort) who had not attended the clinic. Some families did not participate in the study because they moved from the Isle of Wight, while others declined to have their child skin prick tested. Questionnaire information was collected on the presence of allergic diseases. Birth order data and details of environmental factors such as parental smoking, exposure to pets and housing conditions, were updated.

At age 10 years, information was updated and physical examination was conducted during a visit to the Research Center (n=1043). Detailed interviewer-administered questionnaires were completed with a parent of each child regarding asthma and allergy development. ISAAC written questionnaires were used to assess respiratory, nasal, and dermatological symptoms. (16) When a visit was not possible, a telephone questionnaire was completed with a parent, or a modified postal questionnaire was sent for the parent to complete and return (n=330).

Extract of birth records

Data was obtained from hospital records on gestational age, birth weight, and neonatal complications (preterm delivery, foetal distress, newborn infection, jaundice, low blood sugar, hypothermia, congenital abnormalities, etc.).

Cord serum IgE determination

Duplicate measurements of cord IgE were made on serum from umbilical vein blood samples using the ULTRA EIA® kit (Pharmacia Diagnostics AB, Uppsala, Sweden), which is designed to measure IgE between 0.2 and 50 kU/L on 0.1 mL of serum or plasma. It is a solid phase enzyme immunoassay, using monoclonal anti-human IgE, with a high degree of correlation with the Phadebas IgE PRIST® method (r=0.99). (122) Cord IgA was measured on samples where IgE was higher than 0.3 kU/L. Any samples with IgA>10mg/L were deemed contaminated by maternal blood and excluded. The method of cord IgE estimation was described in greater detail in previous reports. (122, 123)

Diagnostic criteria for asthma

The study physicians obtained information regarding the presence of allergic symptoms. For asthma, information included the presence and frequency of cough and wheezing, physician-diagnosed asthma, and treatment given. At ages 1, 2 and 4 the minimum criteria for the diagnosis of asthma included three or more separate episodes of wheeze in the previous 12 months, each lasting at least three days. At age 10, asthma was defined as ever having a diagnosis of asthma, in addition to wheeze in the last 12 months.

Skin prick tests

Skin prick tests (SPT) were carried out on children at age 4 years (n=981) and 10 years (n=1036) using a standard battery of aeroallergens (house dust mite [Dermatophagoides pteronyssinus], grass pollen mix, cat and dog epithelia, Alternaria alternata, and Cladosporium herbarum). Histamine (0.1%) in phosphate-buffered saline and physiological saline were used as positive and negative controls, respectively. Extracts were from Biodiagnostics, Hamburg, Germany at age 4 and ALK-Abello, Denmark at age 10. Children were advised not to take antihistamines for 72 hours before the clinic appointment. Commercially available lancets (Medipoint, Inc., Mineola, NY) were used to prick the epidermis through the allergen extract drops. The tests were read after 15 minutes and a mean wheal diameter (the sum of the longest diameter and the diameter diagonal to it divided by two) of at least 3 mm greater than the negative control was taken as positive. Surrounding erythema was ignored. A child was

regarded as sensitised when the reaction to at least one allergen was positive and non-sensitized when all recorded reactions were negative.

Statistical analysis

We used likelihood ratios (=sensitivity / (1-specificity)) to compare cut-off points for CS-IgE. CS-IgE levels were then dichotomised into less than 0.5 kU/L and greater or equal to 0.5 kU/L. Asthma at age 1-2 years was regarded as positive when at least one positive record was available at either age 1 or 2 years, and negative when there was at least one negative record and no positive record. SPT and asthma at different ages were treated as dichotomous outcome variables and separate logistic regression models were run. Potential confounders were: seasons of birth (spring: March-May; summer: June-August; fall: September-November; winter: December-February); gender; birth weight (categorized as: <1500g, 1500-2500g, 2500-4000g, ≥4000g); neonatal complications (yes, no); maternal age (categorized as: 16-23, 24-33, 34 years and older); history of maternal and paternal atopy (asthma, hay fever, atopic eczema); maternal smoking during pregnancy (yes, no); and having a pet at birth (yes, no).

To test the effect of CS-IgE levels on sensitisation and asthma we used logistic regression, adjusting for the above-mentioned potential confounding factors (LOGISTIC procedure). We estimated adjusted odds ratios of CS-IgE for SPT and asthma at different ages. For asthma, we additionally stratified data for positive SPT. We estimated adjusted odds ratios of SPT and asthma at different ages for the two levels of CS-IgE. All statistical analyses were performed using

the SAS/STAT® program. (112)

RESULTS

Cord serum IgE (CS-IgE) was determined for 1358 children (93.3% of the original cohort). The proportion of newborns with increased CS-IgE (≥0.5 kU/L) for various characteristics is shown in Table 3.2.1. A maternal history of atopic disorders was a significant risk factor for increased CS-IgE. The number of children who had complete information on CS-IgE, SPT and all confounders was 862 at age 4 and 900 at age 10 (63.5% and 66.3% of the sample with available CS-IgE; table 3.2.2). Regarding asthma, children who had complete information on this outcome, as well as on CS-IgE and confounders, were 1194 at age 1-2, 1068 at age 4, and 1191 at age 10 (ranging between 86.9% and 87.3% of the sample of 1358; table 3.2.2). The proportion of children with elevated CS-IgE levels (≥0.5 kU/L) in the samples for different outcomes at different ages did not vary substantially (12.3-13.3%, table 3.2.2).

Likelihood ratios for different cut-off points of CS-IgE indicated the level of risk for allergic sensitisation and asthma at age 4 and 10 (table 3.2.3). The Likelihood Ratio (LR) is the likelihood that a specific level of a test result would be expected in a child with a disorder compared to the likelihood that that same result would be expected in a child without the disorder. (150) CS-IgE levels above the detection limit increased the likelihood of the development of allergic sensitisation (LR>1), but not of asthma. CS-IgE levels of 0.5 kU/L showed higher likelihoods ratios in three of the four outcomes and thus provided more confidence in abnormal test results.

A positive SPT was evident in 16.5% (142/862) of the children at age 4 and in 27.0% (243/900) at age 10 (table 3.2.4). CS-IgE was a risk factor for a positive SPT both at age 4 and at age 10, however, the association was stronger at age 4 years (table 3.2.4, figure 3.2.1). Regarding specific SPTs, the prevalence increased for most allergens. However, the association (OR) between CS-IgE and allergic sensitisation decreased between 4 and 10 years (table 3.2.4).

At age 1-2, physician diagnosed asthma was found in 10.3% of the children (123/1194, table 3.2.5). The prevalence of asthma at age 4 was 15.2% (162/1068) and at age 10 was 12.8% (152/1191). Logistic regressions were used to test the associations of CS-IgE with asthma at age 4 and 10. In addition, to assess whether these relationships were mediated by sensitisation, we stratified asthma at age 4 by SPT positivity at age 4 and asthma at age 10 by SPT positivity at age 4 or 10. Table 3.2.5 shows that cord serum IgE is not associated with asthma at age 4, irrespective of atopic status. The association of CS-IgE with asthma at age 10 showed an OR of 1.66 (95% CI 1.05 to 2.62), which is statistically significant. When stratifying by SPT results, the association lost its significance in children who had a positive SPT at age 4 or 10 (OR=0.83). However, the majority of children had a negative SPT at both ages. In these children the association between CS-IgE and asthma at age 10 became stronger (OR=3.35, 95% CI 1.41 to 7.93). Figure 3.2.1 shows that with increasing age of diagnosis the relative risk (OR) of CS-IgE for asthma increases.

The dynamics of the development of asthma are shown in figure 3.2.2. Of

the 138 children who were diagnosed with asthma at age 10, only 44 had asthma at age 1-2. Fifty-one children were first observed to have asthma at age 10 (37% of the children with asthma at age 10). For each subgroup in figure 3.2.2, the percentage of children with elevated cord serum IgE is shown. In children with late onset of asthma (developing after 4 and before 10 years), the proportion is substantially higher (20%), compared, for instance, to the subgroup with early onset of asthma at age 1-2 years (11.2%, figure 3.2.2).

DISCUSSION

Our analysis revealed that elevated cord serum IgE (≥0.5 kU/L) is a risk factor for allergic sensitisation at ages 4 and 10 years. Elevated cord serum IgE (CS-IgE) was also shown to be a risk factor for asthma at age 10 years, but not for asthma at age 1-2 or at 4 years. To the best of our knowledge this is the second study of the association between CS-IgE and asthma at a later age in childhood. (77, 82, 131) Studies over the past three decades conducted on the association between CS-IgE and asthma in childhood are not in agreement. (78, 84, 129, 151, 152) Most of these studies involved a small number of children and followed them to early childhood (up to 5 years). (77, 151, 152) Additionally, some studies were based only on a clinical assessment of atopy, not on a determination of allergic sensitisation. (77, 82, 131) Our results corroborate prior studies which found that CS-IgE is a risk factor for allergic sensitisation in early childhood and extend this observation, for the first time, to the age of 10 years. Our findings also support the report of Croner et al. (153) that elevated CS-IgE increases the incidence of asthma, cumulated during childhood up to the age of

11 years (four investigations at 1.5, 3, 7, and 11 years). (86, 88, 153)

The analyses are based on samples of those children with complete information on the outcome (sensitisation or asthma), exposure (CS-IgE) and potential confounders. Comparisons of the initial sample that had CS-IgE measurements (n=1358) and the samples used in the analyses indicate that selection bias is unlikely (table 3.2.2).

Contamination of cord blood with maternal blood was assessed using IgA as a biomarker. This led to the exclusion of 86 infants from further analysis. (129) (130) Hence, a potential misclassification of cord blood IgE is unlikely. To dichotomize CS-IgE levels, cut-off points between 0.5-1.3 kU/L have been used in prior studies. (77, 84, 86, 131) We chose to use the cut off point of 0.5 kU/L CS-IgE for two reasons. First, it has been shown in previous analyses of this birth cohort as an optimal level. (129) Second, likelihood ratios indicated that the certainty of atopic development is increased for CS-IgE of >0.5 kU/L (LR > 1.58 for three of four outcomes). Higher levels of CS-IgE were less reliable because of the small number of children (e.g. ≥1.0 kU/L and asthma at age 10: n=9). To assess allergic sensitisation, we used SPT, accepted as an objective marker for evaluating allergic sensitisation. (154, 155) The use of both negative and positive controls diminished the possibility of false results in this study.

IgE production is downstream in a pathway that starts with the presentation of antigens (allergens) to the immune system. Antigen presentation to the immune system may result in the preferential activation of Th2 cells which are programmed to secrete IL-4 and IL-13 and to induce IgE production. (156-

158) An important question is whether the association of cord IgE with asthma is due to the relation of cord IgE with early sensitisation. Our results indicate that the direct effect of cord serum IgE disappears in children who developed a sensitisation (SPT at age 4 or 10 years, table 3.2.5). Since CS-IgE is also a significant predictor of SPT (table 3.2.4), CS-IgE is likely to have an indirect effect on asthma: CS-IgE → SPT positivity → asthma. However, sensitisation is not a necessary link in the chain of reactions. The reason is that in children who did not develop a sensitisation, representing the majority, a strong direct effect between CS-IgE and asthma became obvious (OR=3.35, table 3.2.5). In addition, we investigated whether considering only early sensitisation (at age 4) changed the direct and indirect effect of CS-IgE on asthma. The results did not differ substantially from those when SPT at age 4 and 10 were combined.

Pet exposure at birth was not associated with cord serum IgE levels (table 3.2.1) and did not explain the occurrence of allergic sensitisation and asthma. In addition, we examined whether additional control of pet exposure at age four changed the results. This was not the case. We also controlled for the number of children in the home which did not change our findings. Lastly, we also stratified for pet at birth and at age four as well as for the number of children at home. We did not identify any combined effect of these factors with CS-IgE.

It is widely accepted that dramatic changes in immune responses occur during pregnancy and foetal markers of immune response are detected as early as 10-22 weeks gestation. (60) To prevent the termination of pregnancy, the foeto-maternal immune system is shifted in a Th2 direction. (62, 63) This Th2

shift begins with the secretion of human chorionic gonadotropin, which in turn stimulates progesterone production. Progesterone then promotes the secretion of Th2 cytokines over the secretion of Th1 cytokines. (60) Although cytokine production may be variable in the early stage of development, the Th1 and Th2 subsets become stable after repeated stimulation. (156-158) There are two alternative concepts regarding the time when sensitisation and T-cell stability occurs: the *prenatal priming hypothesis* considers that foetal life immunologic events are more important in the development of allergy in childhood, and the *hygiene hypothesis,* which emphasises that the insults happen after birth.

Given that CS-IgE is a biomarker of prenatal immunologic conditions, our findings favour the *prenatal priming hypothesis.* CS-IgE was associated with overall SPT at ages four and 10 (table 3.2.4). The presence of a significant adjusted OR for the overall SPT and specific SPTs both at age 4 and 6 years later, supports this idea. There is a non-significant decline in ORs and percentage of elevated CS-IgE at age 10, which may be a reflection of the weaker effect of postnatal life events as opposed to prenatal life events (table 3.2.4, figure 3.2.1).

For elevated CS-IgE levels, we observed a stronger association between maternal history of atopy in comparison to paternal history of atopy (table 3.2.1). This observation is in agreement with previous findings and other evidence that maternal immune responses may influence allergic responses in offspring. (159-162)

For asthma at age 10, CS-IgE gained a prognostic value (OR=1.66). In the

one prior investigation with a comparable follow-up time after birth, Croner et al. showed a 5-fold increase in the cumulated incidence of asthma in children at 11 years who had cord serum IgE levels of 0.9 kU/L and higher. (153)

At ages 1-2 and 4 years the minimum criteria for the diagnosis of asthma included three or more separate episodes of wheeze in the previous 12 months, each lasting at least three days. At age 10, the definition of asthma was at least one episode of wheezing in the last 12 months in addition to being diagnosed with asthma by a physician at least once. Different criteria for asthma diagnosis at age 4 and 10 may have led to misclassification. This misclassification, should it exist, would be non-differential. A previous diagnosis of asthma was less likely to have biased asthma diagnosis at age 10 because fewer children were diagnosed as asthmatics at age 10 (12.8%) in comparison to age four (15.2%). It is unlikely that the level of CS-IgE may have affected asthma diagnosis at age 10 but not at age 4, as this information was not available to the field workers who collected information on the presence of wheeze and asthma diagnosis.

As mentioned, there is evidence that early life events may influence atopy and asthma later in life. But how could CS-IgE information predict asthma at age 10 years but not at an earlier stage of life? One possible explanation is that in early childhood diagnosis of asthma is based on transient symptoms that tend to disappear later in life. Kurukulaaratchy et al. showed that approximately 2/3 of children who had wheezing in the first four years of life lost their wheezing by age 10. (163) Additionally, it has been reported that most infants who wheeze are not necessarily atopic and that many lose their wheezing as they grow older. (164)

Assuming this kind of sorting process, the diagnosis of asthma becomes more reliable for children with persistent wheezing. However, our data does not support this explanation, as the percentage of elevated CS-IgE in those who persistently had asthma at ages 1-2, 4 and 10 years (12.5%) was not different from those who never had asthma (12.3%, figure 3.2.2).

Another possible explanation is that the late onset of asthma is related to CS-IgE. In this case, we would expect a higher proportion of children with elevated CS-IgE in the late onset group. In our study, 51 children (figure 3.2.2) had asthma at age 10 but not earlier; of this group, only a few had wheezing before age 10 (6% at age 1-2 and10% at age 4). However, 20% of these children had elevated CS-IgE, which is the highest proportion of any of the groups (figure 3.2.2). The next highest proportion of CS-IgE (16.6%) was found in children who had asthma at age 4 and again at age 10 years. Hence, our results support the late-onset explanation over the sorting process explanation.

As seen in figure 3.2.1, the trend for ORs for asthma increases until it reaches significance at age 10. Additionally, the percentage of asthmatics among those who had elevated CS-IgE was 9.0% at age 1-2, 14.8% at age 4 and 18.8% at age 10 years. These observations could be regarded as the gradual development of asthma in those who had elevated CS-IgE and hence support the importance of CS-IgE as a biomarker of feotal life events in the development of asthma later in life.

In conclusion, elevated cord IgE measured at birth increases sensitisation to aeroallergens by the ages of 4 and 10 years as well as asthma at 10 years, but

not asthma at younger ages. These findings support the importance of prenatal conditioning in the development of allergy later in life. The lack of significance of CS-IgE as a risk factor for asthma early in life may be due to the gradual development of asthma as the cohort aged and the existence of a late-onset variety of asthma. Our findings encourage investigations which would lead to an increased understanding of the probable immunologic mechanisms that are responsible for the effect of foetal life events on childhood allergy. Additionally, we recommend long-term assessments because of the possibility of late-onset childhood allergy.

Table 3.2.1 Characteristics of children with an available CS-IgE measurement

		Number at birth (%)	percentage by levels of CS-IgE		
			<0.5	≥0.5	χ^2 p-value
Variable	Total	1358	87.2	12.8	
Gender	Male	696 (48.7)	86.6	13.4	
	Female	661 (51.2)	87.8	12.2	0.54
	Missing	1 (0.1)	100	0.0	
Birth weight	<1500g	27 (2.0)	88.9	11.1	
	1500-2500g	44 (3.3)	87.2	12.8	0.76
	2500-4000g	1124 (82.8)	90.9	9.1	
	4000g<	163 (12.0)	85.3	14.7	
Neonatal complications	Yes	188 (13.8)	85.6	14.4	0.76
	No	1170 (86.2)	87.4	12.6	
Having a pet at recruitment	Yes	779 (56.6)	87.0	13.0	0.81
	No	589 (43.4)	87.4	12.6	
Season of birth	Spring	315 (56.3)	87.2	12.8	
	Summer	305 (56.3)	87.9	12.1	0.13
	Fall	296 (56.3)	83.1	16.9	
	Winter	442 (56.3)	88.5	11.5	
Age of mother at delivery	Up to 23	389 (28.6)	86.1	13.9	
	24-33 years	801 (59.0)	87.4	12.6	0.54
	34 and older	145 (10.7)	89.7	10.3	
	Missing	23 (1.7)	82.6	17.4	
Maternal smoking during pregnancy	Yes	332 (24.6)	87.2	12.8	
	No	1012 (74.5)	87.1	12.9	0.99
	Missing	12 (0.9)	91.7	8.3	
Maternal history of atopy	Yes	438 (32.3)	84.0	16.0	
	No	905 (66.6)	88.6	11.4	0.02
	Missing	15 (1.1)	93.3	6.7	
Paternal history of atopy	Yes	352 (25.9)	85.0	15.0	
	No	984 (72.4)	88.2	11.8	0.11
	Missing	23 (1.7)	78.3	21.7	

CS-IgE=cord serum immunoglobulin

Table 3.2.2 Number of children who had a measurement of cord serum IgE level at birth and at different analyses, by cord serum IgE levels

Age		Birth	1-2 years	4 years		10 years	
Outcome of interest		—	Asthma	Asthma	SPT	Asthma	SPT
Total number of sample		1358	1194	1068	862	1191	900
Number (%) by different levels of Cord serum IgE	<0.5	1184 (87.2)	1044 (87.4)	928 (86.9)	748 (86.8)	1040 (87.3)	789 (87.7)
	≥0.5	174 (12.8)	150 (12.4)	140 (13.1)	114 (13.2)	151 (12.7)	111 (12.3)

SPT=skin prick test

Table 3.2.3 Likelihood ratios (LR) of allergic sensitisation (SPT $^{\Psi}$) and asthma for different levels of cord serum IgE

	SPT at age 4			SPT at age 10			Asthma at age 4			Asthma at age 10		
Cord IgE (kU/L)	+	-	LR	+	-	LR	+	-	LR	+	-	LR
> detection limit (0.06)	76	297	1.27	124	273	1.23	69	396	1.04	73	447	1.12
≥0.25	67	228	1.46	102	209	1.32	58	308	1.05	59	351	1.15
≥0.50	31	83	1.86	43	68	1.71	21	119	.98	28	123	1.56
Total (n)	144	718	--	243	657	--	162	902	--	152	1039	--

$^{\Psi}$ skin prick test

Table 3.2.4 ORs and CI for the risk of sensitisation with elevated CS-IgE levels (Logistic Regression Analysis)

Age	SPT	Total sample (N)	Cord serum IgE levels		Unadjusted OR (95% CI)	Adjusted† OR (95% CI)
			<0.5 kU/L % (n/N)	≥0.5 kU/L % (n/N)		
4 years	Any		18.1% (135/748)	34.2% (39/114)	2.36 (1.54-3.63)*	2.29 (1.47-3.57)*
	House dust mite		11.8% (85/748)	16.7% (19/114)	1.56 (0.91/2.68)	1.47 (0.85-2.57)
	Grass pollen		7.1% (53/748)	14.9% (17/114)	2.30 (1.28-4.13)	2.29 (1.25-4.19)
	Cat	862	4.8% (36/748)	14.0% (16/114)	3.23 (1.73-6.04)	3.19 (1.67-6.08)
	Dog		2.1% (16/748)	5.3% (6/114)	2.54 (0.97-6.64)	2.34 (0.85-6.43)
	Alternaria		4.0% (30/748)	12.3% (14/114)	3.35 (1.72-6.53)	3.18 (1.59-6.35)
	Cladosporium		2.1% (16/748)	3.5% (4/114)	1.66 (0.55-5.07)	1.79 (0.55-5.86)
10 years	Any		25.3% (200/789)	38.7% (43/111)	1.86 (1.23-2.82)*	1.73 (1.13-2.65)*
	House dust mite		18.6% (147/789)	26.1% (29/111)	1.55 (0.98-2.45)	1.42 (0.89-2.28)
	Grass pollen		11.7% (92/789)	23.4% (26/111)	2.77 (1.66-4.63)	2.20 (1.32-3.67)
	Cat	900	7.7% (61/789)	9.9% (11/111)	1.31 (0.67-2.58)	1.14 (0.56-2.32)
	Dog		3.5% (28/789)	8.1% (9/111)	2.40 (1.10-5.23)	2.19 (0.97-4.95)
	Alternaria		1.9% (15/789)	2.7% (3/111)	1.43 (0.41-5.03)	1.22 (0.33-4.51)
	Cladosporium		100% (10/789)	0.0% (0/111)	Cannot be calculated	

† Controlling for gender, maternal smoking during pregnancy, age of mother at delivery, maternal and paternal atopy, season of birth, birth weight, and neonatal complications and having a pet at recruitment *$P<.05$; CI=confidence interval; OR=odds ratio; SPT=skin prick test

Table 3.2.5 ORs and CI for the risk of asthma with elevated CS-IgE levels (Logistic Regression Analysis)

Age	Strata	Total sample (N)	Cord serum IgE levels <0.5 kU/L %	(n/N)	≥0.5 kU/L %	(n/N)	Unadjusted OR (95% CI)	Adjusted† OR (95% CI)
1-2	*All*	1194	10.5%	(110/1044)	8.7%	(13/150)	0.80 (0.44-1.47)	0.72 (0.39-1.34)
4	*All*	1068	15.2%	(141/928)	15.0%	(21/140)	0.98 (0.60-1.62)	0.92 (0.55-1.52)
	Positive SPT	175	30.8%	(12/39)	38.5%	(52/136)	0.71 (0.33-1.52)	0.60 (0.27-1.36)
	Negative SPT	688	10.7%	(8/75)	11.9%	(73/613)	0. 88 (0.41-1.92)	0. 90 (0.41-1.97)
	No SPT conducted	205	8.9%	(16/179)	3.8%	(1/26)	0.41 (0.05-3.21)	0. 54 (0.11-2.60)
10	*All*	1191	11.9%	(124/1040)	18.5%	(28/151)	1.68 (1.07-2.64)*	1.66 (1.05-2.62)*
	Positive SPT	288	27.5%	(14/51)	30.5%	(72/237)	0.86 (0.44-1.69)	0.83 (0.41-1.67)
	Negative SPT	485	16.3%	(8/49)	5.5%	(24/436)	3.81 (1.54-9.39)*	3.35 (1.41-7.93)*
	Incomplete SPT information$^{\psi}$	418	7.6%	(28/367)	11.8%	(6/51)	1.56 (0.49-4.93)	1.77 (0.52-6.07)

† Controlling for gender, maternal smoking during pregnancy, age of mother at delivery, maternal and paternal atopy, season of birth, birth weight, and neonatal complications and having a pet at recruitment

ψ Includes missing observation at age 4 and 10 as well as negative SPT at one age and missing at the other

**P*<. 05; CI=confidence interval; OR=odds ratio; SPT=skin prick test

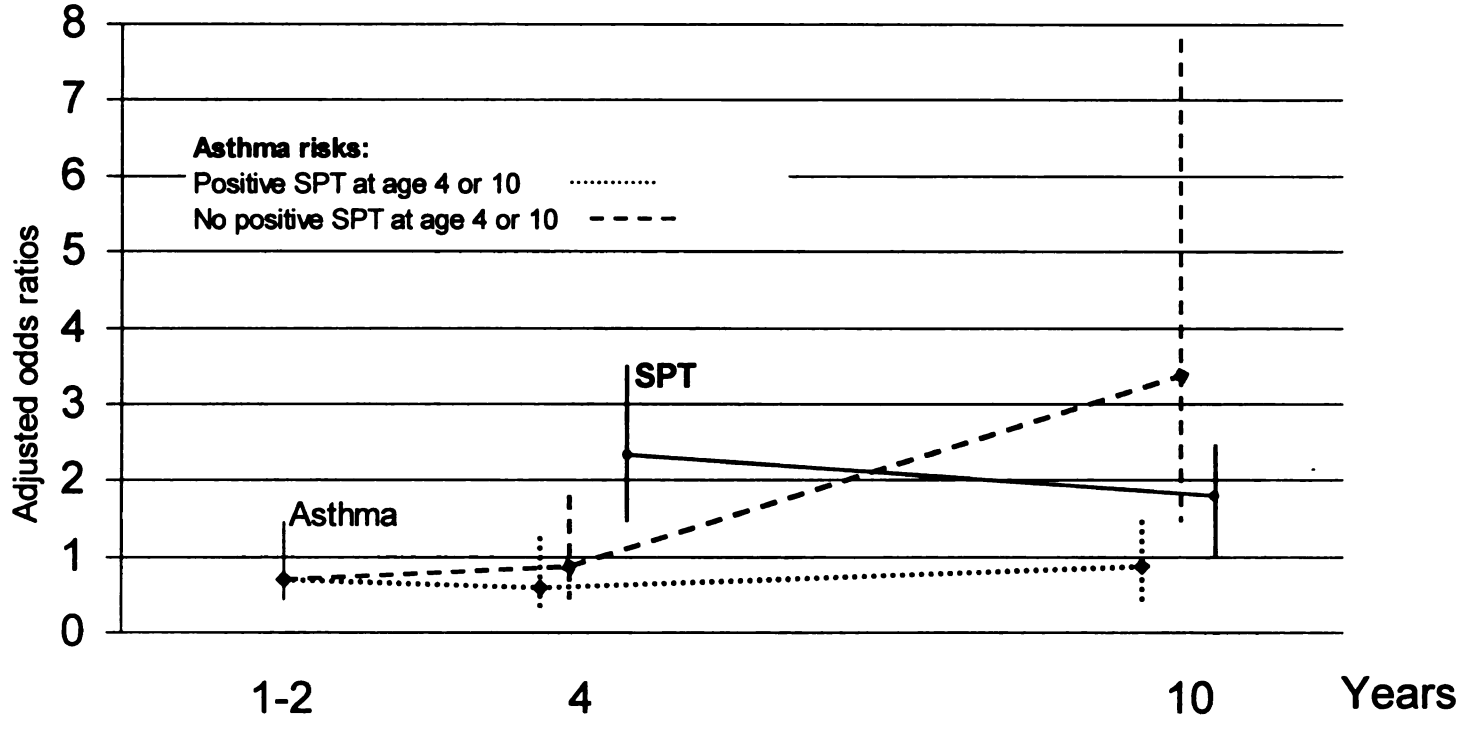


Figure 3.2.1 Adjusted odds ratios (OR) of skin prick test (SPT) and asthma at different ages for elevated levels of cord serum immunoglobulin E. The dots show the odds ratios and the vertical bars represent 95% confidence intervals.

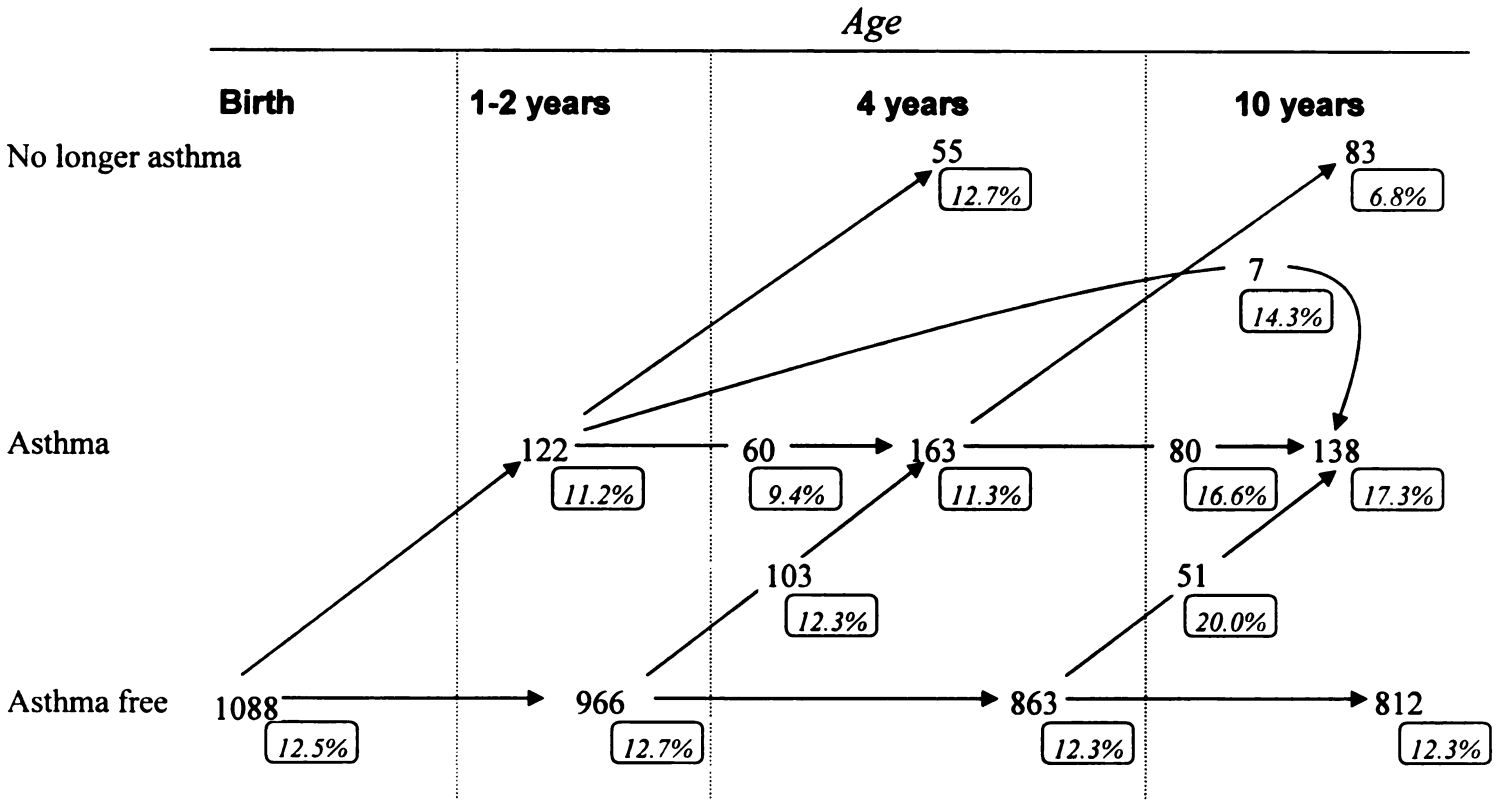


Figure 3.2.2 Dynamic for groups of asthmatics diagnosed in different ages and *percentage* of elevated (≥ 0.5 kU/l) cord serum immunoglobulin E

3.3. The combined effect of maternal smoking during pregnancy and *IL13* gene polymorphism on childhood wheeze phenotypes

Abstract

Tobacco smoke and genetic susceptibility are risk factors for asthma and wheezing. The aim of this study was to investigate whether there is a combined effect of tobacco smoke and the interleukin 13 gene (*IL13*) polymorphisms on wheezing. In the Isle of Wight birth cohort (UK, 1989-1999), five single nucleotide polymorphisms were genotyped. Wheeze phenotypes were *early-onset persistent* (onset in the first 4 years, present at age 10); *late-onset persistent* (onset after age 4 years, present at age10); *early transient* (only in the first 4 years); and *non-wheezers*. Logistic regression analyses included 787 children. Controlling for confounders, maternal smoking during pregnancy was associated with *early-onset persistent* wheeze (odds ratio (OR) = 3.05; $p < 0.001$); the common *IL13* haplotype pair was not (OR = 1.01; $p = 0.95$). However, the association of smoking was stronger in children with the common *IL13* haplotype pair (OR = 5.54) compared to those without it (OR = 1.67; p for interaction = 0.026). This is the first report that shows a combined effect of *in utero* exposure to smoking and *IL13* on wheezing in childhood. The results emphasize that genetic association studies need to take exposures into account, since they may explain contradictory findings.

It has been suggested that the increased prevalence of asthma-related phenotypes over the last three decades is due to exposure to environmental factors (95). Among such exposures, cigarette smoking is regarded as an important risk factor for asthma (22). In particular, an effect of maternal smoking during pregnancy on the development of asthma in offspring has been proposed (96-102). A number of studies in children have linked parental smoking with markers of atopy such as serum immunoglobulin E (IgE) levels (103, 104). Interleukin 13 (IL-13) is an important cytokine involved in the IgE pathway and pathogenesis of asthma (35, 105, 106). Noakes and colleagues noted that exposure to tobacco smoke during pregnancy was associated with a significantly higher neonatal IL-13 response to both house dust mite and ovalbumin, indicating that a prenatal exposure to tobacco may have immunological effects (105). These observations suggest that part of the effect of exposure to tobacco on asthma might be through cytokines.

Researchers have speculated that individuals may vary in genetic susceptibility to cigarette smoke exposure (104, 107) and a few studies have examined gene-smoking interactions for asthma (30, 40, 41). Wang et al. observed that an interaction between maternal smoking and the β_2 adrenergic receptor gene was associated with asthma in offspring (41). Kabesch et al. reported an effect for the interaction between smoking and glutathione S transferase deficiency on asthma (40). In spite of the importance of IL-13 in asthma (35, 105, 106), some studies failed to show an association between the interleukin 13 gene (*IL13*) polymorphism and asthma phenotypes (108, 109) and

there is no information as to whether the adverse effect of maternal smoking on asthma may result from an interaction between exposure to tobacco smoke and cytokine regulating genes. To the best of our knowledge, no study has investigated whether there is an interaction between tobacco smoke exposure and *IL13* polymorohism on wheezing. Because of the high prevalence (25-30 percent) of smoking among women of reproductive age (110) there is a need to investigate its possible role in the development of asthma and its related phenotypes. In this work the authors test the hypothesis that an interaction between exposure to tobacco smoke and *IL13* gene polymorphism affects longitudinal assessments of wheeze phenotypes during the first decade of life in the Isle of Wight birth cohort.

POPULATION AND METHODS

Study population

The local Research Ethics Committee approved the study of children born and enrolled (n=1,456) between January 1, 1989, and February 28, 1990, on the Isle of Wight, United Kingdom. Informed consent was obtained and children were followed up at the ages of one (n = 1,167; 80.2 percent), two (n = 1,174; 80.6 percent), four (n = 1,218; 83.7 percent) and 10 years (n = 1,373; 94.3 percent). The island is close to the British mainland, semi-rural, with no heavy industry. The population is 99 percent Caucasian.

Family history, birth data, and follow-ups

At birth, weight was obtained from hospital records and parents were asked about their age and exposure during pregnancy (maternal smoking and

household pets). At subsequent follow-ups, a questionnaire was administered seeking information on exposure to tobacco smoke, breast feeding, and symptoms that were suggestive of allergic diseases in the cohort children.

Exposure to tobacco smoke

Information on tobacco smoking by mother (during pregnancy and later), father, or any other individual inside the home was recorded at recruitment and updated at each follow-up. Exposures to environmental tobacco smoke (ETS) in the household and maternal smoking during pregnancy were combined and classified into three groups. When mothers did not smoke during pregnancy and there was no exposure to household ETS in children up to the age of 10, children were categorized as "ETS-0". When mothers did not smoke during pregnancy, but household members smoked within the home at some point up to the child's age of 10 years, the exposure status was categorized as "ETS-1". When mothers smoked during pregnancy and the children were also exposed to household ETS at some point up to the age of 10, the exposure was categorized as "ETS-2". None of the children had mothers who smoked during pregnancy but no exposure to household tobacco smoke after birth.

Defining of wheeze and wheeze phenotypes

At each of the four follow-ups at ages one, two, four, and 10 years, "current wheeze" was recorded if wheezing occurred on at least one occasion in the previous 12 months. Parents were also asked whether the child had any wheeze episode between ages four and 10. Children were categorized into wheezing phenotypes using the information on "current wheeze" at each follow-

up and also whether they had wheeze episode(s) between ages 4 and 10 years. *Non-wheezers* never wheezed in the first decade of life, *early-onset persistent* wheezers had wheezed in the first 4 years of life and still wheezed at age 10, while *late-onset persistent* wheezers had wheezing onset after 4 years of age and still wheezed at age 10. *Early transient* wheezers began wheezing in the first 4 years but stopped at least 12 months before age 10. Details on wheeze phenotypes from 1 to 10 years of this cohort have been reported previously (15).

DNA isolation and *IL13* genotyping

Anticoagulated whole blood samples were obtained at the 10-year interview and stored frozen (n=921). Genomic DNA was isolated from these samples using QIAamp DNA Blood Kits (Qiagen, Valencia, CA) or the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA). Polymorphisms in the *IL13* gene were examined using the SNPper (120) and Applied Biosystems (121) databases. Two methods were used for genotyping: 1) fluorogenic 5' nuclease chemistry PCR using Assays on Demands kits cycled on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), and 2) biotin-streptavidin-based pyrosequencing performed on PSQ-96 instrumentation (Biotage AB, Uppsala, Sweden).

Five SNPs from the *IL13* gene were used in this study, rs1800925 in the 5' promoter region, rs2066960 in intron 1, rs1295686 in intron 3, rs20541 in exon 4 and rs1295685 in the 3' untranslated region (3' UTR) of exon 4. Because *IL13* is a small gene (2.9 kb), only a few SNPs were needed for a reasonable assessment of genetic associations. Polymorphism selection was based on SNP

location, minor allele frequency, and function. Selected SNPs had minor allele frequencies ≥ 19% and were distributed throughout the *IL13* gene. The rs20541 is a coding variant with the common allele (G) coding for arginine and the minor allele (A) encoding glutamine at amino acid 144. This SNP was in strong linkage disequilibrium (LD) with rs1295685 and rs1295686. The former (rs1295685) was selected as a representative SNP of this block due to the availability of a commercial assay.

Statistical analysis

Each SNP was tested for Hardy-Weinberg equilibrium using Haploview 3.2 software (124). Estimates of LD between SNPs were calculated using D' and r^2 (116). For SNPs that were in strong LD, the authors took one SNP as the representative marker for the block. PHASE 2.0.2 was employed to build the most likely pair of haplotypes (diplotypes) and their probability for each child (117, 125), using representative SNPs. In the analysis of individual SNPs and haplotype pairs, genotypes with low frequencies were combined (126, 127).

Using SAS/STAT® version 9.1, statistical analysis was performed on the data only from children who had complete information on *IL13* genotypes, exposure to tobacco smoke, and wheeze phenotypes. Chi-square tests were used to compare the sample used in the analysis with that which was followed up at age 10. Logistic regression analysis and Wald tests were conducted to investigate the statistical interaction between ETS and *IL13* haplotype pairs on wheeze up to age 10.

Statistical significance was defined as a p value of ≤ 0.05. As the four

wheeze phenotypes were not independent and we focused on only one haplotype pair, no adjustment for multiple testing in the haplotype pair analysis was performed (165).

For confounders, the authors considered covariates that have been suggested to be important in studying wheeze phenotypes in a prior report of the Isle of Wight cohort. (166) Potential confounders (table 3.3.1) were: gender, low birth weight (< 2,500 grams versus ≥ 2,500 grams), breast feeding (< three months and ≥ three months), household cat present during pregnancy (yes, no), and household dog present during pregnancy (yes, no).

RESULTS

Data on *IL13* genotypes, exposure to tobacco smoke, and wheeze phenotypes were available for 787 children. The percentages of children who wheezed and those who had ETS-0 were higher in the sample used in the analyses compared to all those who were followed up to age 10 (*p* values of 0.006 and 0.003, respectively, table 3.3.1).

More than half of the children were exposed to tobacco smoke up to age 10 (20.2 percent during and after pregnancy; 31.5 percent after pregnancy, table 3.3.1). In the sample used for the analysis, the odds ratio for ETS-2 (reference group: ETS-0) for *early-onset persistent* wheeze was 3.05, 95 percent confidence interval: 1.67, 5.57. To investigate whether there was a selection bias due to tobacco smoke exposure and wheeze, the authors calculated the odds ratio for their association in all children with a follow-up at age 10 (2.93, 95 percent confidence interval: 1.76, 4.89).

For *IL13* genotypes, all five SNPs were in Hardy-Weinberg equilibrium (p = 0.82 for rs1800925, p = 0.99 for rs2066960, p = 0.58 for rs1295686, p = 0.09 for rs20541, and p = 0.36 for rs1295685). Three of the SNPs (rs1295686, rs20541, and rs1295685) were in strong LD (figure 3.3.1). Therefore the authors used only one SNP of this block for haplotype analysis. Thus haplotypes were inferred using rs1800925, rs2066960 and rs1295685. Due to the limited number of children homozygous for minor alleles at all SNPs (less than 5 percent, table 3.3.2), minor allele homozygous and heterozygous genotypes were grouped together. Among haplotype pairs, CCG/CCG had the highest frequency (0.48, table 3.3.3). The estimated probability for CCG/CCG as the best pair was 1.0 in 99 percent of children and was never less than 0.89 (results not shown). All other haplotype pairs had frequencies between 0.001-0.151. In addition, there were probabilities as low as 0.5 that were identified as the best pair for haplotype pairs other than CCG/CCG. Thus, haplotype pairs other than CCG/ CCG were combined (126, 127).

Higher percentages of *early-onset persistent* wheeze were observed when children had exposure to maternal smoking during pregnancy (ETS-2) and the major genotype (major allele homozygous genotype of the SNPs or CCG/CCG of haplotype pairs, figure 3.3.2). In a logistic regression model, controlling for the confounders, ETS-2 was associated with *early-onset persistent* wheeze (odds ratio = 3.05, 95 percent confidence interval: 1.67, 5.56), but the CCG/CCG haplotype pair was not (odds ratio = 1.01, 95 percent confidence interval: 0.64, 1.61). References for ETS-2 and CCG/CCG haplotype pairs were ETS-0 and

other haplotype pairs, respectively. When the interaction term between ETS-2 and CCG/CCG was added to the model, the term showed statistical significance (odds ratio = 3.47; $p < 0.026$, table 3.3.4). To demonstrate the interaction between ETS-2 and the CCG/CCG haplotype pair on *early-onset persistent* wheeze, the authors stratified the sample by the *IL13* polymorphisms and calculated the odds ratios for ETS-2. Children who had the CCG/CCG genotype had an odds ratio = 5.54, 95 percent confidence interval: 2.28, 13.50 for ETS-2. The odds ratio in the stratum that had haplotype pairs other than CCG/CCG was 1.65, 95 percent confidence interval: 0.70, 3.91. To determine the effect of minor haplotype pairs (frequency= 0.082, table 3.3.3), the stratified analysis was repeated by excluding this group and results did not change significantly (data not shown).

Among confounders, the presence of a household cat during pregnancy was significantly associated with a lower risk of *early-onset persistent* wheeze (odds ratio = 0.51, 95 percent confidence interval: 0.30, 0.87), but not significantly associated with other wheezing phenotypes (odds ratio = 0.98 and 0.90 for *early transient* and *late-onset persistent* wheeze, respectively). Breast feeding for at least three months was protective for *early transient* wheeze (odds ratio = 0.68, 95 percent confidence interval: 0.46, 0.99) and for *early-onset persistent* wheeze (odds ratio = 0.67, 95 percent confidence interval: 0.41, 1.09), but not for *late-onset persistent* wheeze (odds ratio = 1.04, 95 percent confidence interval: 0.61, 1.78). Boys were at a higher relative risk of being among wheeze phenotypes, especially, of having *early-onset persistent* wheeze (odds ratio =

1.99, 95 percent confidence interval: 1.24, 3.21). The odds ratios for *early transient* wheeze and *late-onset persistent* wheeze were 1.56, 95 percent confidence interval: 1.08, 2.26 and 1.31, 95 percent confidence interval: 0.77, 2.24, respectively.

DISCUSSION

This study investigated the combined effect of exposure to tobacco smoke and haplotype pairs of the *IL13* gene on childhood wheeze phenotypes in the first decade of life using the data from the Isle of Wight birth cohort. No independent effect for the *IL13* gene was detected, but the common variant of the *IL13* gene polymorphism was shown to increase the adverse effect of maternal smoking during pregnancy (but not tobacco smoke exposure after pregnancy) on *early-onset persistent* wheeze. It was previously shown in this cohort that persistent wheezing with early childhood onset is associated with substantial morbidity in the first decade of life in association with high levels of atopy, bronchial hyper-responsiveness and impaired lung function at 10-years of age (15).

For this study, the information was available from a subset of children who were followed up at age 10 and who agreed to provide blood for genotyping. These children appeared to have more wheeze in comparison to all children who were followed up at age 10. In addition, the percentage of mothers who never smoked was higher in the group who provided a blood sample for genotyping. Therefore, a selection bias may exist. As presented in the results section, the odds ratios for the association between exposure to tobacco smoke during pregnancy and *early-onset persistent* wheeze in those who were used in the

analysis and all children (3.05 and 2.93, respectively) are nearly identical. This suggests that a selection bias for tobacco exposure pattern, if it exists, is negligible. In addition and with regard to genotypes, presence of a selection bias could result in a violation of the Hardy-Weinberg equilibrium. The genotypes of the five SNPs used in the analysis were in Hardy-Weinberg equilibrium and their allele frequencies were comparable to the equivalent SNPs in other Caucasian populations (72, 73). Hence, a selection bias is unlikely.

The wheeze phenotypes were specified in an assessment that was performed at age 10. A strength of this study is that the determination of wheeze phenotype is based on longitudinal records from ages one to 10 years. To avoid recall bias, as explained in more detail in a prior report of this cohort (15), the analysis was restricted to children who were seen prospectively with information at all study visits. Previously, the authors have shown that a prior diagnosis of asthma was less likely to produce biased reports (119).

With regard to genetic analysis, information on individual SNPs was used and the most likely pairs of haplotypes were estimated from genotype data. It was possible to use information on each individual SNP to investigate gene-environment interactions. The authors chose to perform tests of interaction for haplotype pairs, as this approach has some advantages over individual SNP analysis. The issue of "multiple testing" is avoided as all genetic markers were compiled in haplotype pairs. Additionally, it has been suggested, specifically for *IL13* (132), that haplotype analysis could confer more information than individual marker analysis (132-134). Haplotype pair analysis may misclassify genotypes

when parents' genetic information is not available (ambiguous phase). However, the probability of having CCG/CCG, the major haplotype pair, was 1.00 in 430 out of 435 children with this genotype (the probability for the other 5 children was 0.89). Also, the consistent pattern observed for the combined effect of ETS-2 and all markers (individual SNPs polymorphisms and haplotype pairs) on *early-onset persistent* wheeze (figure 3.3.2) suggests that misclassification of the genotypes was unlikely. The distribution of the data, with respect to minor haplotypes, did not allow testing for their interactions with tobacco smoke exposure or specifying a genetic model (additive, recessive or dominant). However, when children with a minor haplotype at both loci (frequency = 0.082, table 3.3.3) were removed from the analysis, the results did not change substantially.

Previous studies have shown an increased risk of asthma in children who were exposed to tobacco during pregnancy (22, 101, 107). In a prior examination of this cohort, a possible association between exposures during pregnancy and *early-onset persistent* wheeze was suggested (166). Additionally, several studies have suggested a gene-environment interaction for the effect of tobacco smoke exposure and asthma phenotypes (30, 40, 167). The current study demonstrates a scenario in which a gene modifies the effect of tobacco smoke exposure during pregnancy but not after (the time of the exposure may be of critical importance in gene-environment interaction studies). The finding of an interaction between *IL13* and ETS in the present study suggests that negative reports for the effect of a candidate gene (in this case, *IL13* on asthma phenotypes (108, 109)), could be explained by a failure to take into account environmental exposures. It is

therefore of utmost importance, for genetic association studies to describe environmental exposures in the target population.

Some authors have advised against publishing results without replication studies (168). In the present study there is no replication of the current findings in another population. However, the authors believe that the replication requirement is not a *conditio sine qua non* and should be considered in the context of external validity. Other studies have reported similar combined effects of *IL13* polymorphisms on asthma-related outcomes such as IgE, or smoking-related effects on the IL-13 cytokine (105, 148). Hence, there is external validity for these findings.

The mechanism behind the effect of tobacco exposure on childhood asthma is not clear. Most speculation has centered on the suggestion that tobacco smoke impairs lung development during infancy and early childhood and therefore leads to asthma (96-102). It is well known that in addition to structural defects, immune mechanisms and cytokines play prominent roles in asthma. IL-13 is one of the cytokines that has a pivotal function in the pathogenesis of asthma (6) and *IL13* genetic polymorphisms have been shown to be related to IgE and asthma (72, 73, 148). There is also evidence that tobacco smoke can alter immune markers that are involved in allergic immune reactions. Liu et al. reported a synergistic effect of smoking and *IL13* promoter polymorphism on the level of serum IgE (148), and a genome-wide analysis has suggested a gene-environment interaction for the effect of tobacco exposure on asthma (30). Noakes and colleagues demonstrated that cord blood cells produce significantly

higher levels of IL-13 in response to both house dust mite and ovalbumin when newborns are exposed to tobacco smoke during pregnancy (105). This suggests that prenatal exposure to tobacco elicits immunological effects. In our analysis, the interaction between tobacco exposure and *IL13* polymorphisms was present in those who were exposed during and after pregnancy, but was not evident for the group with the same polymorphism who were exposed only after pregnancy. As there was no group of children that was exposed only during pregnancy, it is not possible to distinguish definitively the effect of tobacco exposure before and after pregnancy. However, considering both the relatively short time of pregnancy and the large difference of risk between the two exposed groups, the authors assume that exposure to tobacco during pregnancy has more influence on asthma than tobacco exposure after pregnancy. There is evidence that remarkable changes in maternal immune responses occur during pregnancy. Also the fetal immune system is active as early as 10-22 weeks of gestation (62). Hence, the fetal period may be a critical time for the development of immune-related disorders later in life. It has been hypothesized that the dramatic increase in the prevalence of asthma in the last three decades in developed countries is because of environmental exposures. One possible explanation for the differential effects of environmental exposures in populations could be gene-environment interactions. There is a potential for genetic information to change without a sequence variation in DNA. These changes happen through post synthetic modifications of either DNA strands or histones and rest on developmental and/or environmental insults and are known as epigenetic

phenomena (5, 42). The results of the current study of the combined effect of *in utero* exposure to tobacco and *IL13* genotypes on wheeze could be explained by such epigenetic changes (5, 43).

In summary, in a sub-sample of the Isle of Wight cohort, the combined effect of exposure to tobacco smoke during pregnancy and the common haplotype pair of the *IL13* gene resulted in an increased relative risk of *early-onset persistent* wheeze. Tobacco smoke exposure later in childhood was less important. The *IL13* gene did not pose a risk in its own right. These results demonstrate that the association between exposures to environmental risk factors like tobacco smoke can be modified by gene polymorphisms. Given that there are various patterns and prevalences of exposure to tobacco smoke in different populations, this study suggests that negative reports of genetic association studies, for example Leung et al., 2001 (108) and Hakonarson et al., 2001 (109) for *IL13* and asthma, may be due to differences in environmental exposures. The authors propose that the next step in the investigation of the interaction between *IL13* and exposure to tobacco smoke is to examine the expression of *IL13*.

Table 3.3.1. Comparison of children with a follow-up at age 10 and the subset used in the analysis

		Numbers at age 10 (%)	Numbers used in the analysis (%)	χ^2 *p* value
Variable	Total	1,373	787	
Wheeze Phenotype	*Early-onset persistent**	139 (10.1)	105 (13.3)	0.006
	Late-onset persistent†	81 (5.9)	70 (8.9)	
	Early transient‡	259 (18.9)	188 (23.9)	
	Non-wheezer§	617 (44.9)	424 (53.9)	
	Missing	277 (20.2)	0 (0.0)	
Birth weight	≥2500 grams	1287 (93.7)	733 (93.1)	0.553
	<2500 grams	49 (3.6)	30 (3.8)	
	Missing	37 (2.7)	24 (3.1)	
Exposure to tobacco smoke	ETS-0	617 (44.9)	380 (48.3)	0.003
	ETS-1	430 (31.3)	248 (31.5)	
	ETS-2	319 (23.2)	159 (20.2)	
	Missing	7 (0.5)	0 (0)	
Gender	Girl	676 (49.2)	388 (49.3)	0.954
	Boy	697 (50.8)	399 (50.7)	
Breast fed	≥3 months	564 (41.1)	360 (45.7)	0.088
	<3 months	667 (48.6)	394 (50.1)	
	Missing	142 (10.3)	33 (4.2)	
Household cat during pregnancy	Yes	453 (33.0)	269 (34.2)	0.343
	No	912 (66.4)	517 (65.7)	
	Missing	8 (0.6)	1 (0.1)	
Household dog during pregnancy	Yes	395 (28.8)	239 (30.4)	0.163
	No	970 (70.6)	547 (69.5)	
	Missing	8 (0.6)	1 (0.1)	

* Onset of wheeze in the first 4 years, still present at age 10.
† Onset of wheeze after age 4 years, still present at age10.
‡ Wheeze only in the first 4 years.
§ No wheeze up to age 10.
ETS-0, mothers did not smoke during pregnancy and children not exposed to household ETS; ETS-1, mothers did not smoke during pregnancy, but children were exposed to household ETS; ETS-2, mothers smoked during pregnancy and children were exposed to household ETS

Table 3.3.2. Genotypes for *IL13* single nucleotide polymorphisms (SNPs)

SNP	Position (bp)	Location	Genotype	Frequency (%)
rs1800925	132,020,708	Promoter	CC	577 (63.6)
			CT	295 (32.5)
			TT	35 (3.9)
			Total	907 (100.0)
rs2066960	132,022,334	Intron 1	CC	729 (81.5)
			AC	157 (17.6)
			AA	8 (0.9)
			Total	894 (100.0)
rs1295686	132,023,742	Intron 3	CC	483 (64.6)
			CT	240 (32.1)
			TT	25 (3.3)
			Total	748 (100.0)
rs20541	132,023,863	Exon 4	GG	73 (53.7)
			AG	59 (43.4)
			AA	4 (2.9)
			Total	136 (100.0)
rs1295685	132,024,344	Exon 4	GG	584 (64.5)
			AG	280 (30.9)
			AA	41 (4.5)
			Total	905 (100.0)

Gene annotation is based on SNPper (120).
IL13: interleukin 13 gene; n, number of samples used in genetic studies; bp, basepairs.

Table 3.3.3. Frequency of haplotypes and haplotype pairs for three single nucleotide polymorphisms (rs1800925, rs2066960, rs1295685) of the interleukin 13 gene, inferred from genotype data of 907 children

		Frequency (SE)
Haplotype	CCG	0.683 (0.004)
	CCA	0.045 (0.002)
	CTG	0.067 (0.003)
	CTA	0.108 (0.003)
	ACG	0.041 (0.003)
	ACA	0.029 (0.002)
	ATG	0.008 (0.002)
	ATA	0.017 (0.003)
Haplotype pair(s)	CCG/CCG	0.480
	CCG/minor haplotypes*	0.438
	minor haplotypes/minor haplotypes†	0.082

PHASE 2.0.2. was used for this analysis (117, 125).
* This group consists of, with a frequency of:
CCG/ACA, 0.036; CCG/ACG, 0.055; CCG/ATA, 0.037; CCG/ATG, 0.019; CCG/CCA, 0.054; CCG/CTA, 0.151; CCG/CTG, 0.086.
† This group consists of, with a frequency of:
ACA/ATA, 0.001; ACG/ACA, 0.003; ACG/ACG, 0.001; ACG/ATA, 0.002; ATG/ATA, 0.001; CCA/ACA, 0.004; CCA/CCA, 0.002; CCA/CTA, 0.014; CTA/ACA, 0.014; CTA/ATA, 0.002; CTA/CTA, 0.007; CTG/ATA, 0.004; CTG/CTA, 0.020; CTG/CTG, 0.004.

Table 3.3.4. Logistic regression estimates* (odds ratio, *p* value) for the effect of smoke exposure and the major haplotype pair (CCG/CCG)† of the interleukin 13 gene on wheeze phenotypes

Wheeze phenotype‡	Odds ratio§	*p* value
Early-onset persistent, n=105		
ETS-1	1.64	0.186
ETS-2	1.57	0.294
CCG/CCG	0.58	0.168
ETS-1 × CCG/CCG§	1.58	0.370
ETS-2 × CCG/CCG§	3.47	0.026
Late-onset persistent, n=70		
ETS-1	0.78	0.559
ETS-2	0.39	0.159
CCG/CCG	0.90	0.766
ETS-1 × CCG/CCG§	0.67	0.929
ETS-2 × CCG/CCG§	0.65	0.499
Early transient, n=188		
ETS-1	1.42	0.248
ETS-2	1.84	0.067
CCG/CCG	0.95	0.854
ETS-1 × CCG/CCG§	1.69	0.609
ETS-2 × CCG/CCG§	1.33	0.462

* Adjusted for gender, birth weight, duration of breast feeding, having a cat during pregnancy, and having a dog during pregnancy.
† The most common haplotype pair, inferred from genotype data of three single nucleotide polymorphisms of interleukin 13 gene, rs1800925, rs2066960 and rs1295685, using PHASE 2.0.2 software (117, 125).
‡ The reference was the group who never report wheeze up to age 10 (n=424);
× Denotes an interaction.
§ Odds ratios for interactions were calculated by exponentiating the sum of the main effect terms and the interaction term; for example for ETS-2 × CCG/CCG in *early-onset persistent* phenotype group, odds ratio = exponential (0.56 – 0.45 + 1.34) = 3.47
ETS-0, mothers did not smoke during pregnancy and children not exposed to household ETS; ETS-1, mothers did not smoke during pregnancy, but children were exposed to household ETS; ETS-2, mothers smoked during pregnancy and children were exposed to household ETS

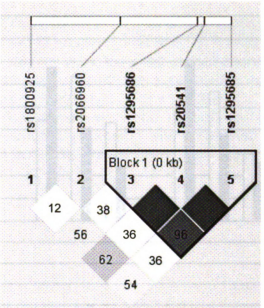


Figure 3.3.1. Using the Haploview program (124), three single nucleotide polymorphisms of the interleukin 13 gene appeared to be in a block.

Pair of SNPs	r^2	D' (95% CI)
rs1800925 – rs2066960:	0.01	0.14 (0.04, 0.24)
rs1800925 – rs1295686:	0.31	0.56 (0.50, 0.62)
rs1800925 – rs20541:	0.28	0.63 (0.46, 0.75)
rs1800925 – rs1295685:	0.28	0.54 (0.48, 0.59)
rs2066960 – rs1295686:	0.06	0.37 (0.27, 0.47)
rs2066960 – rs20541:	0.07	0.37 (0.04, 0.71)
rs2066960 – rs1295685:	0.06	0.38 (0.28, 0.47)
rs1295686 – rs20541:	0.78	1.00 (0.83, 1.00)
rs1295686 – rs1295685:	0.90	0.96 (0.93, 0.98)
rs20541 – rs1295685:	0.85	1.00 (0.94, 1.00)

D' and r^2 are pair wise linkage disequilibrium determinants (116); CI, confidence interval.

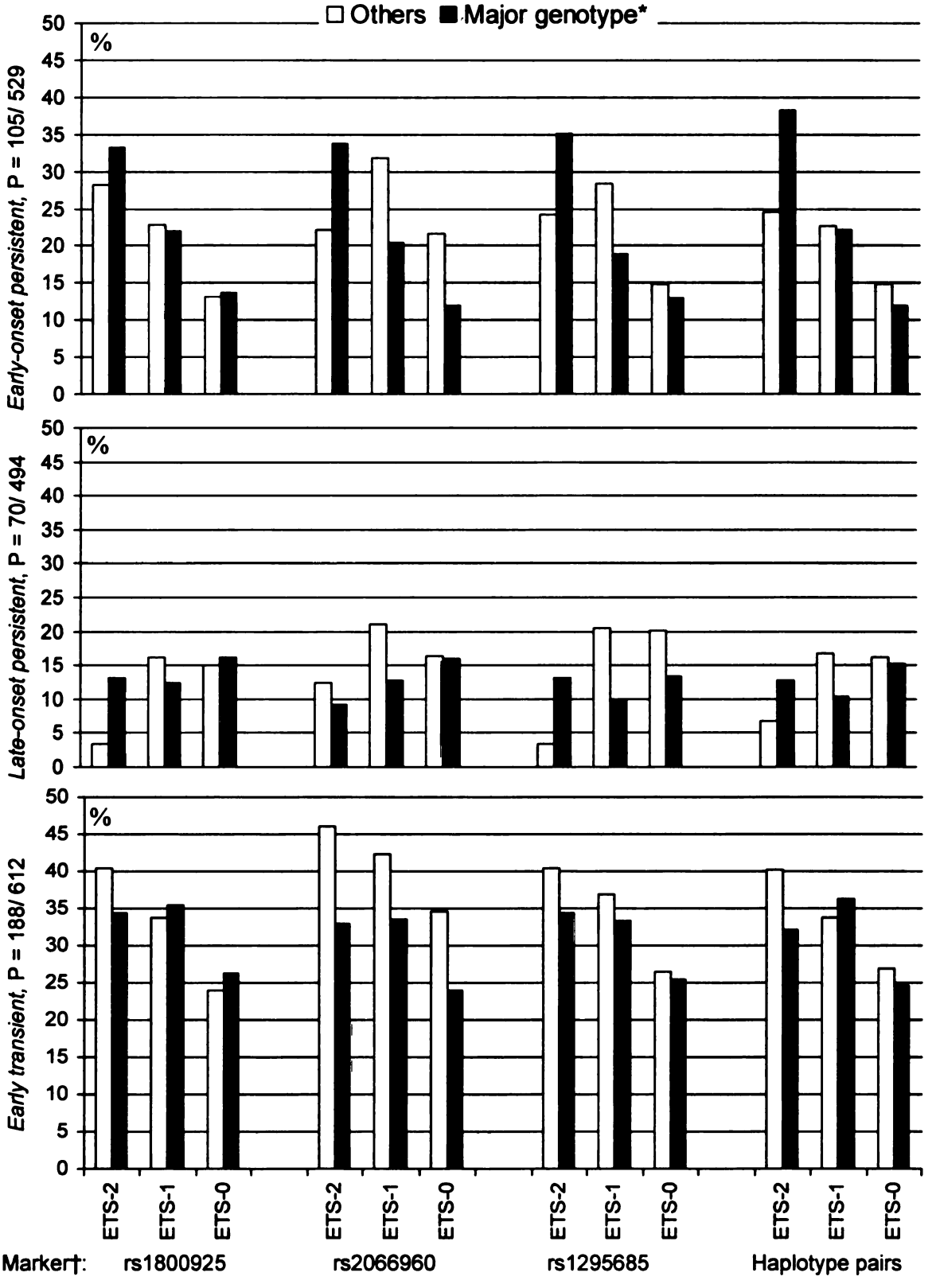


Figure 3.3.2. Proportions of children (P) with a specific wheeze phenotype: number of children with each wheeze phenotype divided by the sum of children with the respective phenotype plus 424 *non-wheezers*.

Figure 3.3.2. (cont'd) ETS-0, mothers did not smoke during pregnancy and children were not exposed to household ETS; ETS-1, mothers did not smoke during pregnancy, but children were exposed to household ETS; ETS-2, mothers smoked during pregnancy and children were exposed to household ETS. SNP, single nucleotide polymorphism; * For each marker, "major genotype" was the genotype with the highest frequency and "others" was a combination of all other genotypes; † rs1800925, rs2066960, and rs1295685 were three SNPs within the interleukin13 gene, "haplotype pairs" were constructed based on these SNPs, using PHASE 2.0.2 software (117, 125).

DISCUSSION

Using data from the Isle of Wight birth cohort study, I demonstrated first, a modifying effect of maternal smoking on the association between the common variant of the *IL13* gene and levels of cord serum immunoglobulin E (CS-IgE); second, an association between CS-IgE and childhood asthma; third, a combined effect of the common *IL13* variant and maternal smoking during pregnancy on the *early onset persistent* wheeze phenotype.

The motivation to conduct this research was to investigate whether exposures during pregnancy can have a long-term effect on health outcomes, the prenatal programming concept. The conceptual model for the long-lasting effect of exposures during pregnancy on diseases was exemplified by using tobacco smoke during pregnancy and *IL13* polymorphism as risk factors, CS-IgE as a marker of susceptibility for asthma, and asthma phenotypes as outcomes.

The mechanism for prenatal programming explored in the first and third papers was an interaction between *IL13* gene polymorphism, a predisposing factor, and exposure to tobacco smoke during pregnancy. In other words, the combined effect of the common *IL13* variant and maternal smoking during pregnancy was observed for both CS-IgE and persistent wheezing. The association between CS-IgE and asthma, the second manuscript, showed that the endpoints (CS-IgE and persistent wheeze) of the above-mentioned gene-environment interaction are related.

The three pieces of this work suggest that *in utero* exposure to tobacco smoke may alter the expression of the *IL13* gene at birth, a change that may last as late as age 10. The interaction of *IL13* polymorphism was not observed in

those who were exposed to tobacco smoke after birth unless they had exposure during pregnancy. This suggests that the *in utero* environment has a critical effect on the pathogenesis of asthma and that the prenatal period is of utmost importance.

To the best of my knowledge there is no investigation on the interactions between genes and prenatal exposures on childhood asthma. Barker was one of the first authors to raise the idea of prenatal priming of adulthood diseases. (169) His observations and their interpretations involve the effect of fetal malnutrition on the development of organs; they provide no explanation for effects at the molecular level. (169-171)

Regarding the puzzle of an effect of prenatal exposures on the development of asthma, several pieces of evidence exist, but they have not been explored together in one study. There is certain evidence that exposures during pregnancy can change newborns' immune markers that are involved in the biologic pathways that lead to asthma. (58, 66, 67, 172) Recently a large meta-analysis study has shown that the decrease in lung function parameters in children aged 6 to 12 years is greater when they had *in utero* exposure to maternal smoking compared to after birth exposure to tobacco smoke. (31) However, there is insufficient information on whether and how these changes may last until late childhood. One obstacle is that usually those children whose mother smoked during pregnancy also have exposure to tobacco smoke after pregnancy. For example, in the Isle of Wight cohort, there was no group of children who were exposed to maternal smoking during pregnancy but not to

tobacco smoke after birth. Therefore the evaluation for the effect of maternal smoking during pregnancy was only indirect by statistical modeling and controlling for the exposure to tobacco smoke after birth. Future studies may overcome this limitation by approaching a group of children that had exposure during pregnancy but not after, for example exposed children who were adopted by non-smoking parents shortly after birth.

With regard to the interaction between the *IL13* gene and environmental factors on serum IgE, the only report demonstrated a combined effect of one SNP in the *IL13* gene (rs20541) and exposure to tobacco smoke. (148) It has been suggested that smoking can change gene expression through chemical alteration, for example acetylation of chromatin (149, 173-175). There is no study that determines whether exposure to tobacco smoke can alter *IL13* expression, and if so, what mechanism is involved. Therefore, future studies are warranted to explore, in the first step, the *IL13* expression under different conditions of exposure to tobacco smoke; and, in the second step, the specific mechanism behind the effect of smoking on *IL13* gene expression.

The aim of this dissertation was to coalesce pieces of the prenatal programming concept of asthma. The longitudinal nature of the Isle of Wight study offered the opportunity to investigate the interaction between the *IL13* gene and maternal smoking during pregnancy on an asthma susceptibility factor (cord serum IgE) at birth and to track this gene-environment interaction effect up to 10 years of age, for which a similar interaction was observed for persistent wheeze. Therefore, this investigation provides internal validity for the studied gene-

environment interaction under consideration as a mechanism for prenatal programming.

In addition to the general advantages of a cohort study, such as the minimal amount of recall bias, longitudinal studies possess other strengths that are specific to the current study. The longitudinal nature of the data not only enabled me to connect the three pieces of the study mentioned above, but also provided the opportunity to obtain a longitudinal evaluation of exposure to tobacco smoke and wheezing phenotypes in the first decade of life. The latter benefit is an important aspect for asthma diagnosis because childhood asthma is a dynamic disease with considerable heterogeneity and can best be assessed through a cohort study.

Another strength of this study was a thorough assessment of the *IL13* gene polymorphism. The *IL13* gene is a relatively small gene and the five SNPs provided adequate representation of the *IL13* gene variability. The haplotype pair analysis approach empowered the analyses as the number of comparisons diminished.

The general limitation for gene-environment interaction studies is sample size. As the frequency for individual variants of a gene decreases or the number of these variants increases, the available sample size with those variants gets smaller. The sample size problem becomes more prominent when the research focuses on a specific time window for exposure, such as the prenatal period. In order to overcome the problems related to sample size, most of the genetic studies used a case control design rather than a cohort. Despite the advantage

of case control studies, larger sample size, this design usually suffers from recall bias. For example, in the context of the current study, for persistent wheezing and tobacco smoke exposure at different ages, it is unlikely to obtain valid information, not affected by recall bias, within a case control design. With regard to the *IL13* polymorphism in the current study, it was not possible to test the interaction effect for some of the variants in the gene. However, when the variants with small sample size were removed from the analyses, the change in the results was not considerable.

Epidemiology is the art and science of providing valid clues to the cause of a health-related problem. This dissertation met this definition by investigating an under-explored mechanism for the pathogenesis of childhood asthma. Although there is evidence for the adverse effect of smoking on childhood asthma, the mechanism behind this effect is unclear. The findings of the current work present a scenario in which a prenatal exposure (tobacco smoke) may induce a long-lasting change in a susceptibility factor (*IL13*) for asthma, the most common chronic illness of children. If this work is corroborated, it may then be found that the adverse effect of maternal smoking during pregnancy may last until adulthood and even transfer to offspring of the exposed individuals via epigenetic mechanisms. (44, 45)

It is generally believed that the recent increase of asthma and allergy is related to environmental pollutants. However, the idea of the *in utero* programming of asthma has not been explored either for exposure to tobacco or for any other environmental exposure. Some of these pollutants, for example

DDE, can mimic the effects of sex hormones, are known as endocrine disruptors and have been associated with asthma. (20) One of the findings in the first research paper of this dissertation was the augmenting effect of female sex on the interaction between maternal smoking and *IL13* gene on CS-IgE. This observation highlights the importance of hormones and the environmental pollutants that mimic their physiological effects (endocrine disruptors) in asthma. Therefore it is suggested that future studies investigate the gene-environment interaction for pollutants other than tobacco smoke.

In summary, it is of public health importance to consider the prenatal period as a critical time in the pathogenesis of diseases and to begin education regarding exposure prevention before a pregnancy occurs. Future studies may explore the molecular basis for the interaction between prenatal exposure to tobacco smoke (and other pollutants) and genes. If epigenetic changes play a role in the pathogenesis of asthma, it may even be possible to reverse the effect of pollutants through intervention therapies.

APPENDIX

The programs and procedures used to perform the statistical analysis are presented here. This appendix has three sections, HAPLOVIEW 3.2, PHASE 2.0.2 and SAS 9.0.1 programs used to perform statistical analysis.

HAPLOVIEW 3.2

To run HAPLOVIEW two datasets are needed:

First: A flat file data set that provides the program with the name of SNPs and their corresponding position, saved as *.info.

```
rs1800925 132069025
rs2066960 132070651
rs1295686 132072059
rs20541   132072180
rs1295685 132072661
```

Second: A flat file data set that provides the program with the identification number (ID) of each individual and genotypes for each SNP. The first two columns are IDs. Columns 3-6 have are constantly '0 0 1 1'. Each pair of subsequent columns (5 pairs here as there are 5 SNPs) are genotype information for the corresponding SNP. This file should be saved as *.ped and this is how it looks like:

```
1 1 0 0  1 1 0 0  0 0 0 0  0 0 0 0
2 2 0 0  1 1 2 2  2 2 2 2  0 0 3 3
3 3 0 0  1 1 2 2  2 2 2 2  0 0 3 3
.
.
.
1534 1534 0 0   1 1 0 0  0 0 0 0  0 0 0 0
1535 1535 0 0   1 1 2 4  2 2 2 2  0 0 3 3
1536 1536 0 0   1 1 2 4  0 0 0 0  0 0 3 3
```

For genotype information (columns 7-16) '0' stand for missing, '1' stands for 'A', '2' stands for 'C', '3' stands for 'G' and '4' stands for 'T'. To run the program the option 'Load genotype' should be chosen. The software browses the two data sets and runs.

PHASE 2.0.2

Step 1: A SAS program to get Phase input.

```
libname cohort89 'l:\IOW\data';
data  IL13_ALLELE1; set cohort89.il13_iow_v15;
keep id  C15862743_1  C8932056_1 C8932052_1;

if substr(C15862743_10,1,1) = 'A' then C15862743_1 = 'A';
if substr(C15862743_10,1,1) = 'C' then C15862743_1 = 'C';
if substr(C15862743_10,1,1) IN ('', '-') then C15862743_1 = 'X';

if substr(C8932056_10,1,1) = 'T' then C8932056_1 = 'T';
if substr(C8932056_10,1,1) = 'C' then C8932056_1 = 'C';
if substr(C8932056_10,1,1) IN ('', '-') then C8932056_1 = 'X';

if substr(C8932052_10,1,1) = 'A' then C8932052_1 = 'A';
if substr(C8932052_10,1,1) = 'G' then C8932052_1 = 'G';
if substr(C8932052_10,1,1) IN ('', '-') then C8932052_1 = 'X';

where C15862743_10 not in ('', '-') or
C8932052_10 not in ('', '-') or C8932056_10 not in ('', '-');
run;
data  IL13_ALLELE2; set cohort89.il13_iow_v15;
keep id  C15862743_2  C8932056_2 C8932052_2;

if substr(C15862743_10,2,1) = 'A' then C15862743_2 = 'A';
if substr(C15862743_10,2,1) = 'C' then C15862743_2 = 'C';
if substr(C15862743_10,2,1) IN ('', '-') then C15862743_2 = 'X';

if substr(C8932056_10,2,1) = 'T' then C8932056_2 = 'T';
if substr(C8932056_10,2,1) = 'C' then C8932056_2 = 'C';
if substr(C8932056_10,2,1) IN ('', '-') then C8932056_2 = 'X';

if substr(C8932052_10,2,1) = 'A' then C8932052_2 = 'A';
if substr(C8932052_10,2,1) = 'G' then C8932052_2 = 'G';
if substr(C8932052_10,2,1) IN ('', '-') then C8932052_2 = 'X';

where C15862743_10 not in ('', '-') or
C8932056_10 not in ('', '-') or C8932052_10 not in ('', '-');
RUN;
DATA IL13_ALLELE;MERGE IL13_ALLELE1 IL13_ALLELE2;BY ID;RUN;

DATA A;SET IL13_ALLELE;
FILE PRINT NOTITLES;
OPTIONS NODATE;
PUT
@1 ID @6 C15862743_1 @8 C8932056_1 @10 C8932052_1
/     @6 C15862743_2 @8 C8932056_2 @10 C8932052_2;
TITLE;
RUN;
```

Step 2: input file for PHASE 2.0.2

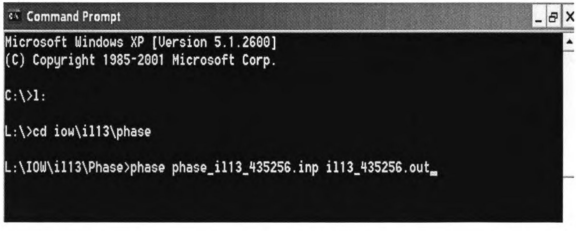
In order to have the input file for PHASE 2.0.2 the output of the program from step 1 was saved as a word file. Then missing values (X) were replaced by question marks (?). Next step was to type the number of observations (907), loci (3) and their type (SSS). The file should be saved as *.inp. This is the look for the PHASE input:

```
907
3
SSS

2     C C G
      C C G
3     C C G
      C C G
4     A C A
.
.
.

1535 C C G
     C T G
1536 ? C G
     ? T G
```

Step 3: Running PHASE 2.0.2. in DOS environment



This run produced several output files. I used 'il13_435256.out_freqs' to obtain the results for estimated haplotype frequencies.

Step 4:

The file 'il13_435256.out_pairs' from previous step is used to extract haplotype pairs with the highest probability for each individual and then merge this information into the original data.

```
data in3;
 infile 'L:\IOW\il13\phase\il13_435256.out_pairs' dsd truncover;
retain last ;
/* check to see what kind of line we're reading in
   and act accordingly */
 input dummy $1-3 @;
   if dummy='IND' then do;
     input @1 IND :$10. @;
     last=IND;
   end;
   else do;
    input @1 (vars1-vars3) (:$20.) ;
    var=trim(vars1)||"_"||vars2;
   end;
/* pull just the numeric off the IND value */
   IND=scan(last, 2, ':');
   if vars3=' ' then delete;
drop last dummy vars1 vars2;
run;

proc sort; by ind;

/* collapse our by groups so we have 1 line per
   IND value */
data cohort89.il13_435256;
  set in3;
  by ind;
  retain a1-a5 b1-b5;
  array a[5]  ;
  array b[5] $9. ;
  if first.ind then do;
    i=1;
    do j=1 to dim(a);
      a[j]=' ';
      b[j]=' ';
    end;
  end;
    a[i]=vars3;
    b[i]=var;
    if last.ind then output;
    if i<5 then i+1;
drop vars3 var i j;run;

proc print ; where b5 ne '';run;
data max;set cohort89.il13_435256;prob_best=max(a1,a2,a3,a4,a5);length
id 5.;run;
data cohort89.il13_435256snps(keep= id il13_diplo435256);
```

```
set max;
id=ind;
if a1=prob_best then il13_diplo435256=b1;
if a2=prob_best then il13_diplo435256=b2;
if a3=prob_best then il13_diplo435256=b3;
if a4=prob_best then il13_diplo435256=b4;
if a5=prob_best then il13_diplo435256=b5;
run;

**Making version 16;

proc sort data=cohort89.il13_435256snps;by id;run;
proc sort data=cohort89.il13_iow_v15;by id;run;
data cohort89.il13_iow_v16;merge cohort89.il13_iow_v15
cohort89.il13_435256snps;by id;
run;
```

SAS programs used to perform statistical analysis

```
libname cohort89 'l:\IOW\data';

data temp1; set cohort89.il13_iow_v16;
IF C8932056_10 IN ('TT','CT') THEN C8932056='TX';
IF C8932056_10 IN ('CC') THEN C8932056='CC';

IF C8932052_10 IN ('AA','AG') THEN C8932052='XA';
IF C8932052_10 IN ('GG') THEN C8932052='GG';

IF C8932053_10 IN ('TT','CT') THEN C8932053='TX';
IF C8932053_10 IN ('CC') THEN C8932053='CC';

IF C15862743_10 IN ('AA','AC') THEN C15862743='AX';
IF C15862743_10 IN ('CC') THEN C15862743='CC';

if ASCON_1 =1 and  ASCON_2 = 1 then asthma12= 1;
else if ASCON_1 ne .  or  ASCON_2 ne . then asthma12= 0;


age_mobi=(childdob-madob03)/365.25;
if  1< age_mobi < 24 then gr_age_mobi = 1;
if 24 <= age_mobi < 34 then gr_age_mobi = 2;
if 34 <= age_mobi      then gr_age_mobi = 3;

pcf2 = pcf3;
if pcf2 > 2 then pcf2 = 2;

if cb_ige=. then cige=.;
if 0<cb_ige<.5 then cige=0;
if .5=<cb_ige then cige=1;

if Delivery=1 then noninst_del=1;
else if Delivery ne . then noninst_del=0;

if neoncomp in (1,2,3,4,5) then ncomp=1;
if neoncomp in(9,99,100,.) then ncomp=0;

if ANTECOMP ne . then acomp=1;
if ANTECOMP=. then acomp=0;

if cat_0=1 or dog_0=1 then catdog0=1;
if cat_0=0 and dog_0=0 then catdog0=0;

if spt_48=1 or spt_10=1 then spt=1;
if spt_48=0 and spt_10=0 then spt=0;

if mb in(3,4,5) then season = 'spring';
if mb in(6,7,8) then season = 'summer';
if mb in(9,10,11) then season = 'fall';
if mb in(12,1,2) then season = 'winter';


if msmk_03=0 and (ets1=1 or ets2=1 or ets4=1 or anysmk=1)
then aft_p_effect='not in p but after';
```

```
if msmk_03=1 then aft_p_effect='in p, after y/n';
if msmk_03=0 and (ets1 ne 1 and ets2 ne 1 and ets4 ne 1 and anysmk ne
1) and
(ets1=0 or ets2=0 or ets4=0 or anysmk=0) then aft_p_effect='xneither';

if aft_p_effect='not in p but after' then smk_after=1;
else if aft_p_effect ne '' then smk_after=0;
if aft_p_effect='in p, after y/n' then smk_during=1;
else if aft_p_effect ne '' then smk_during=0;


if (C8932056_10 NOT IN ('','-')or C15862743_10 NOT IN ('','-')or
C8932053_10 NOT IN ('','-')or C8932052_10 NOT IN ('','-')) and cda10 ne
. then miss=0;


if il13_diplo435256 in (
'ACA_ATA',
'ACG_ACA',
'ACG_ACG',
'ACG_ATA',
'ATG_ATA',
'CCA_ACA',
'CCA_CCA',
'CCA_CTA',
'CTA_ACA',
'CTA_ATA',
'CTA_CTA',
'CTG_ATA',
'CTG_CTA',
'CTG_CTG') then minor_minor=1; else if il13_diplo435256 ne '' then
minor_minor=0;
if il13_diplo435256 in (
'CCG_ACA',
'CCG_ACG',
'CCG_ATA',
'CCG_ATG',
'CCG_CCA',
'CCG_CTA',
'CCG_CTG')then CCG_minor=1; else if il13_diplo435256 ne '' then
CCG_minor=0;
if il13_diplo435256 in ('CCG_CCG')then homo_z=1;
else if il13_diplo435256 ne '' then homo_z=0;
else if HETE_Z=. OR HOMO_Z=. THEN homo_m=.;


if (C15862743_10 NOT IN ('','-') and C8932056_10 NOT IN ('','-')  and
C8932052_10 NOT IN ('','-')) and aft_p_effect ne ''  then miss=0;

run;

*****Table3.1.1;
proc freq data=temp1;tables
gr_age_mobi
mum_03
dad_03
msmk_03
```

```
dog_0
cat_0
season
boy
pcf2
acomp
noninst_del
ncomp
cige
/list missing;
where cige ne .;
run;
proc freq data=temp1;tables
gr_age_mobi
mum_03
dad_03
msmk_03
dog_0
cat_0
season
boy
pcf2
acomp
noninst_del
ncomp
cige
/list missing;
where miss=0 and cige ne .;
run;

*****Table 3.1.2;
proc freq data=temp1;
tables cige*
(gr_age_mobi
mum_03
dad_03
msmk_03
dog_0
cat_0
season
boy
pcf2
acomp
noninst_del
ncomp);
run;
proc logistic data=temp1 descending;
class gr_age_mobi ;
model cige=gr_age_mobi /rl ;
run;
proc logistic data=temp1 descending;
model cige=mum_03 /rl ;
run;
proc logistic data=temp1 descending;
model cige=dad_03 /rl ;
run;
proc logistic data=temp1 descending;
```

```
model cige=msmk_03/rl ;
run;
proc logistic data=temp1 descending;
model cige=dog_0 /rl ;
run;
proc logistic data=temp1 descending;
model cige=cat_0/rl ;
run;
proc logistic data=temp1 descending;
class season ;
model cige=season /rl ;
run;
proc logistic data=temp1 descending;
model cige=boy /rl ;
run;
proc logistic data=temp1 descending;
model cige=pcf2 /rl ;
run;
proc logistic data=temp1 descending;
model cige=acomp /rl ;
run;
proc logistic data=temp1 descending;
model cige=noninst_del /rl ;
run;
proc logistic data=temp1 descending;
model cige=ncomp /rl ;
run;
**table 3.1.3*;
proc freq data=temp1;
tables C8932056_10 /list;
where C8932056_10 not in ('','-');run;
proc freq data=temp1;
tables  C15862743_10  /list;
where C15862743_10 not in ('','-');run;
proc freq data=temp1;
tables    C8932053_10 /list;
where C8932053_10 not in ('','-');run;
proc freq data=temp1;
tables r130q /list;
where r130q not in ('','-');run;
proc freq data=temp1;
tables C8932052_10/list;
where C8932052_10 not in ('','-');run;

**table 3.1.4*;
proc freq data=temp1;
tables ccg_minor*minor_minor*homo_z/list ;
run;

**Figure 3.1.1*;
proc freq data=temp1;
tables cige*homo_z/chisq;
tables msmk_03*cige*homo_z/chisq;
tables boy*msmk_03*cige*homo_z/chisq;
run;
**Figure 3.1.2*;
proc logistic data=temp1 descending;
```

```
model cige=homo_z /rl ;
run;
proc logistic data=temp1 descending;
model cige=msmk_03 /rl ;
run;
proc logistic data=temp1 descending;
model cige=homo_z /rl ;
where msmk_03=0;
run;
proc logistic data=temp1 descending;
model cige=homo_z /rl ;
where msmk_03=1;
run;

*****Table3.2.1;
proc freq data=temp1;tables
(boy
bwt_03
ncomp
catdog0
season
gr_age_mobi
msmk_03
mum_03
dad_03)*cige;
where cige ne .;
run;
*****Table3.2.2;
proc freq data=temp1;tables
cige;
where cige ne .;
run;
proc freq data=temp1;tables
cige;
where cige ne . and asthma12 ne .;
run;
proc freq data=temp1;tables
cige;
where cige ne . and ascon_4 ne .;
run;
proc freq data=temp1;tables
cige;
where cige ne . and spt_48 ne .;
run;
proc freq data=temp1;tables
cige;
where cige ne . and cda10 ne .;
run;
proc freq data=temp1;tables
cige;
where cige ne . and spt_10 ne .;
run;
**Table 3.2.4*;
proc freq data=temp1;tables
(spt_48 spthdm48 sptgrs48 sptcat48 sptdog48 sptalt48 sptcla48
spt_10 spthdm10 sptgra10 sptcat10 sptdog10 sptalt10 sptcla10)*cige;
run;
```

```
proc logistic data=temp1 descending;
model cige=spt_48 /rl ;
title 'unadjusted LR';
run;
proc logistic data=temp1 descending;
model cige=spt_10 /rl ;
title 'unadjusted LR';run;
proc logistic data=temp1 descending;
class season gr_age_mobi;
model cige=boy msmk_03 gr_age_mobi mum_03 dad_03 season bwt_03 ncomp
catdog0 spt_48 /rl ;
title 'Adjusted LR';run;
proc logistic data=temp1 descending;
class season gr_age_mobi;
model cige=boy msmk_03 gr_age_mobi mum_03 dad_03 season bwt_03 ncomp
catdog0 spt_10 /rl ;
title 'Adjusted LR';run;

**Table 3.2.5 and Figure 3.2.1*;
proc freq data=temp1;tables
asthma12*cige;
run;
proc logistic data=temp1 descending;
model asthma12=cige /rl ;
title 'Undjusted LR';run;
proc logistic data=temp1 descending;
model asthma12=boy msmk_03 gr_age_mobi mum_03 dad_03 season bwt_03
ncomp catdog0 cige /rl ;
title 'Adjusted LR';run;
run;

proc freq data=temp1;tables
ascon_4*cige;
run;
proc logistic data=temp1 descending;
model ascon_4=cige /rl ;
title 'Undjusted LR';run;
proc logistic data=temp1 descending;
model ascon_4=boy msmk_03 gr_age_mobi mum_03 dad_03 season bwt_03 ncomp
catdog0 cige /rl ;
title 'Adjusted LR';run;
run;
proc sort data temp=1;by spt;run;
proc logistic data=temp1 descending;
model ascon_4=cige /rl ;
title 'Undjusted LR';by spt;run;
proc logistic data=temp1 descending;
model ascon_4=boy msmk_03 gr_age_mobi mum_03 dad_03 season bwt_03 ncomp
catdog0 cige /rl ;
title 'Adjusted LR';by spt;run;
run;

proc freq data=temp1;tables
cda10*cige;
run;
proc logistic data=temp1 descending;
model cda10=cige /rl ;
```

```
title 'Undjusted LR';run;
proc logistic data=temp1 descending;
model cda10=boy msmk_03 gr_age_mobi mum_03 dad_03 season bwt_03 ncomp
catdog0 cige /rl ;
title 'Adjusted LR';run;
run;
proc sort data temp=1;by spt;run;
proc logistic data=temp1 descending;
model cda10=cige /rl ;
title 'Undjusted LR';by spt;run;
proc logistic data=temp1 descending;
model cda10=boy msmk_03 gr_age_mobi mum_03 dad_03 season bwt_03 ncomp
catdog0 cige /rl ;
title 'Adjusted LR';by spt;run;
run;

**Figure 3.2.2*;
proc freq data=temp1;
table cige ;
title 'this calculation is presented as an example';
where asthma12 ne . ascon_4 ne . and cda10 ne .;run;



**Table 3.3.1***;
proc freq data=temp1;tables
phenoty
bwt_03
aft_p_effect
boy
cat_0
dog_0
/list missing;;
*where whezwhi2 ne .;
where whezwhi2 ne . and phenoty ne . and aft_p_effect ne '' and
miss=0;run;

data bias;
input level $ miss $ n;
datalines;
a no 547
a yes 423
b no 239
b yes 156
;
run;
proc freq;
tables level*miss/chisq;
weight n;
run;
**Table 3.3.2***;
proc freq data=temp1;
tables C8932056_10 /list;
where C8932056_10 not in ('','-');run;
proc freq data=temp1;
tables  C15862743_10  /list;
where C15862743_10 not in ('','-');run;
```

```
proc freq data=temp1;
tables    C8932053_10 /list;
where C8932053_10 not in ('','-');run;
proc freq data=temp1;
tables r130q /list;
where r130q not in ('','-');run;
proc freq data=temp1;
tables C8932052_10/list;
where C8932052_10 not in ('','-');run;
**Table 3.3.2***;
proc logistic data=temp1 descending;
model phenoty=boy homo_z|smk_during homo_z|smk_after brst3mth bwt_03
cat_0 dog_0   /rl ;
title 'Eop';where phenoty in (2,0);
run;

proc logistic data=temp1 descending;
model phenoty=boy homo_z|smk_during homo_z|smk_after brst3mth bwt_03
cat_0 dog_0   /rl ;
title 'Lop';where phenoty in (3,0);
run;

proc logistic data=temp1 descending;
model phenoty=boy homo_z|smk_during homo_z|smk_after brst3mth bwt_03
cat_0 dog_0   /rl ;
title 'ET';where phenoty in (1,0);
run;

**Figure 3.3.2;
proc freq data=temp1;
tables (C8932056 C15862743 C8932052 homo_z)*aft_p_effect*phenoty;
title 'Eop';where phenoty in (2,0);
run;

proc freq data=temp1;
tables (C8932056 C15862743 C8932052 homo_z)*aft_p_effect*phenoty;
title 'Lop';where phenoty in (3,0);
run;

proc freq data=temp1;
tables (C8932056 C15862743 C8932052 homo_z)*aft_p_effect*phenoty;
title 'ET';where phenoty in (1,0);
run;
```

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