GENETIC EPIDEMIOLOGY OF ATOPY AND THE HYGIENE HYPOTHESIS

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Abstract

Decreased exposure to microorganisms may be the reason the prevalence of allergic diseases has been increasing for the past several decades in westernized countries[1]. This postulate, the hygiene hypothesis, was formulated by Strachan when he observed after following a large cohort of children for 23 years that being born late in the birth order and being from a large family were protective for allergic diseases[2]. More recent studies have demonstrated that the prevalence of atopic sensitization is lower in the children of farmers [3-6]. Collectively, these studies appear to indicate that an appropriate microbial load is necessary to confer protection from allergic diseases. The Toll-Like Receptor 4 mutation (TLR4 D299G) is known to confer hypo-responsiveness to bacterial endotoxin in some individuals[7,8]. Individuals who carry this mutation may require additional bacterial challenge to switch to a non-allergic phenotype. Consequently, this thesis investigated the hypotheses that the TLR4 D299G was a significant predictor of atopic status in Humboldt, SK family-based data and that the effect of parental history on allergic diseases was modified by the environmental covariates: family size, farming exposures, pets and smoking in addition to age and sex. Overall, 734 children participated from Humboldt, SK in the study representing a response rate of 79.1%. The crude prevalence of atopy in this population of children was 30.4%. TLR4 D299G was not associated with atopy in this population of children even though the power to detect such an association if it existed was high. Of the 734 children, 309 children had both parents participate in the study representing 111 non-farming and 68 farming families. Based on the variables; total number of siblings, parental smoking, pets, humidity, parental history of allergic diseases and parental atopy, farming families did not differ from non-farming families in this study. Children whose parents farmed, whose parents worked with livestock or whose parents had atopy were not at a decreased or an increased risk of being atopic in this study. The reduced model consisting of the variables age, sex and parental history of allergic disease was the most parsimonious for the outcome variable, atopy.

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Dedication

I would like to dedicate this thesis to my parents: Angus Edward McDonald and Merrilyn Rose Ceming. Thanks for having one more!

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List of Abbreviations

FID	Father Identifier
FBAT	Family Based Association Test
h^2_N	Narrow-sense Heritability
HWE	Hardy-Weinberg Equilibrium
IID	Individual Identifier
LBP	LPS Binding Protein
LPS	Lipopolysaccharide
MID	Mother Identifier
NFκB	Nuclear Factor-κB
OR	Odds Ratio
PID	Pedigree Identifier
SNP	Single Nucleotide Polymorphism
TDT	Transmission Disequilibrium Test
Th0	T-helper cell subset 0
Th1	T-helper cell subset 1
Th2	T-helper cell subset 2
TLR	Toll-Like Receptor
TLR4	Toll-Like Receptor 4
TLR4 D299G	Toll-Like Receptor 4 Amino Acid Substitution Position 299 of Aspartic Acid to Glycine
TLR4 T399I	Toll-Like Receptor 4 Amino Acid Substitution Position 399 of Threonine to Isoleucine
TNF- α	Tumour Necrosis Factor- α

1. Introduction

1.1 Background

The prevalence of allergic diseases such as asthma, rhinitis, dermatitis and atopy has been increasing over the last 30 years in Western countries[1,9]. Epidemiologists have consistently struggled to find environmental risk factors that account for this rapid increase in the prevalence of allergic diseases. There is an abundance of information on potential biologically plausible environmental risk factors for allergic diseases. For example, pets provide a source of allergens but also provide a source of endotoxin [10,11] and both sources stimulate key immune system pathways. The immunopathology of allergic diseases is regulated by cytokines produced from inflammatory pathways. Studies of inflammatory pathways point to three key pathways as being instrumental in the immunopathology of allergic diseases: the Toll-receptor pathway, the T regulatory cell pathway and the T_{h0} cell differentiation pathway. Researchers studying the genetics of allergic diseases have repeatedly identified candidate genes from these three pathways but reproducing associations between populations has been difficult. These difficulties are likely a result of the challenges in assessing the environmental risk factors that may underscore or that may negate the importance of particular inflammatory pathways in different populations.

1.1.1 Atopy and the Rural Environment

Epidemiological studies of allergic diseases have consistently identified risk factors that are proxies for the introduction of bacterial or viral immune challenge e.g. total number of siblings and position in the birth order. In 1989, Strachan identified number of siblings and position in the birth order as risk factors for hay fever [2]. Children that grew up in large families and were the youngest child in such families had a significantly reduced risk of developing allergic diseases. It was postulated by Strachan in 1989 that older siblings introduced infectious organisms to younger siblings thereby providing

agents that would challenge the developing immune system of the younger siblings. This challenge in turn encouraged the development of a non-allergic immune system. This postulate became known as the hygiene hypothesis. More recent studies have identified daycare attendance and growing up on a farm as protective for allergic diseases. Both of these factors provide the opportunity for the immune system to be challenged at an early age and thus support the ideology behind the hygiene hypothesis. For example exposure to infections from older children and contact with farm animals are most likely proxies for the frequency of contact with agents such as bacteria that stimulate the immune system.

Humboldt, Saskatchewan is a rural community in which three previous studies (1977, 1983 and 1993) of the determinants of lung health were conducted. The data for this thesis is derived from that collected for the "Fourth Humboldt Survey 2003" conducted by Dr. James Dosman, Dr. Donna Rennie and Dr. Yue Chen and associates at the University of Saskatchewan [12-15]. Funding was sought from and, after review, granted from the Canadian Institutes for Health Research (CIHR) as part of a larger study of endotoxin and lung health granted to Dr. James Dosman. Ethical approval for the study was received in 2001 and the "Fourth Humboldt Survey 2003" was conducted from 2003-2004. Information was collected for the analysis of numerous allergic traits however, the analysis in this thesis will focus on the allergic trait atopy.

1.1.2 Hygiene Hypothesis and Toll-receptors

The toll-like receptor (TLR) pathway, among other regulatory-immune pathways, is essential for the defense against infectious organisms by recognizing key pathogen associated molecular patterns. Polymorphisms in genes coding for proteins in regulatory-immune pathways have been important for the survival of the species in the presence of high loads of bacteria and virus where the immune response has the potential to be systemic and deadly. An example is the CCR5Δ32 mutation that reduces the ability of certain types of viruses to infect T-cells and is believed to have allowed carriers of this mutation to survive the smallpox plague [16].

The rapid decrease in exposure to bacterial and viral challenge that has occurred over the last century has resulted in environments that may no longer reward genotypes with a reduced immune response to bacterial and viral challenge. It is interesting to consider that the prevalence of allergic diseases is lower in rural communities in comparison with urban communities[17]. Rural communities have been postulated to provide a sufficient load of bacterial challenge that may be necessary to deflect the immune response from an allergic immune response to a non-allergic immune response[18]. Thus, it is only in populations where the appropriate environmental challenges are reduced that the prevalence of allergic diseases is rising.

1.2 Objectives

In order to evaluate these hypotheses the following objectives must be met:

1. To link the Humboldt participants via a Pedigree Identifier (PID) into identifiable families.
2. To analyze the contribution of the TLR4 D299G mutation to the atopic phenotype.
3. To assess if the contribution of the parental history of allergic diseases is modified by the environmental covariates: family size, farming exposures, pets and smoking in addition to age and sex.

1.3 Hypotheses

The research hypotheses under investigation in this analysis include:

1. The Toll-Like Receptor 4 mutation (TLR4 D299G) is a significant predictor of atopic status in Humboldt family-based data.
2. The effect of parental history of allergic diseases is modified by the environmental covariates: family size, farming exposures, pets and smoking in addition to age and sex.

2. Literature Review

2.1 Atopic Disease

The term atopy specifically refers to those hypersensitivity mechanisms where IgE antibody involvement has been measured either through skin-prick testing or serum IgE levels to specific allergens [19-21]. The term allergy refers to any hypersensitivity reaction initiated by immunological mechanisms irrespective of the involvement of IgE [19,21]. The complexity of allergic diseases extends to the nomenclature of its phenotypes that include asthma, allergic rhinitis, atopy and eczema among others. Many patients may present with similar allergic symptoms however, it is a fallacy to assume that patients all suffer from the same disease pathology as this would be problematic for genetic association studies. This is because heterogeneity at the immunological level is indicative of heterogeneity at the genetic level. Selecting a trait for a genetic association study with a heterogeneous immunopathology limits the generalizability of the findings to other populations where the level of heterogeneity is likely to differ. Also, the internal validity of studying a trait with a heterogeneous immunopathology is a limiting factor as it would be difficult to interpret the relevance of any findings to individuals in the study. Afflictions of allergic diseases include asthma and dermatitis that may or may not be atopic [19]. This may be of minimal importance from the clinical perspective. However, the goal of studying the genetic epidemiology of atopic disease is to model subtle changes at the molecular level and their interaction with the environment that result in atopy. Fortunately, IgE levels are tightly regulated and the elevated IgE levels that are associated with the atopic phenotype are likely the result of minute changes in the delicate immune balance. For this reason, atopy was selected as the outcome to test for an association with the TLR4 D299G mutation and to investigate potential associations with the environmental covariables pets, total number of siblings, humidity and farming.

An individual's atopic status can be assessed through skin-prick tests. The variability in wheal reaction size from skin-prick tests is dependent on the reliability of the device, the

depth of the puncture needle and the force, duration of force applied and the angle of the application device [22]. As is recommended by the Joint Task force on Practice Parameters from the American Academy of Allergy, Asthma and Immunology (AAAAI), the American College of Allergy, Asthma, and Immunology (ACAAI) and the European Academy of Allergology and Clinical Immunology (EAACI), a positive skin-prick test is defined as a wheal reaction of no less than 3 mm greater than the negative control [22-24]. Thus, as per the recommendation of the Joint Task force any wheal reaction less than 3 mm of the control should not be considered a positive skin-prick test.

2.2 International Prevalence

There is an extreme interest in characterizing the prevalence of atopy and allergy among adults and children. Currently there are two large international multi-centered collaborative studies investigating the prevalence of these allergic diseases. The European Community Respiratory Health Survey is aimed at characterizing the international prevalence of asthma and atopy in adults aged 20 to 44 years [25]. The International Study of Asthma and Allergies in Childhood is focused on characterizing the prevalence of asthma and atopy among children aged 6 to 7 years and 13 to 14 years [26]. Both of these studies involve international multi-centered collaboration, detailed questionnaires and asthma and atopy testing.

In 1997, results were published from the European Community Respiratory Health Survey of adults that had been tested for specific IgE levels against house dust mite, timothy grass, cat and a local allergen in 13, 883 adults aged 20 to 44 years in 37 centres in 16 countries [27]. Researchers found a wide range for the prevalence of atopy in these 16 countries. The prevalence of atopic sensitization varied greatly from 16% in Spain to 45% in New Zealand. Later the European Community Respiratory Health Survey investigators published the results of a cross-sectional study during 1991 and 1992 on 13, 558 adults in 36 centres in 16 countries in order to determine the attributable fraction asthma symptoms caused by atopy [28] . More specifically, researchers were attempting to determine the proportion of asthmatics that had atopy. The overall attributable fraction

was 30% but varied widely between centres, ranging from 4 to 61%. The European Community Respiratory Health Survey investigators noted that the centres with the highest prevalences of atopy originated from primarily English-speaking countries. These findings underscore the importance of characterizing study center characteristics that may account for the wide variability in the attributable fraction of asthma symptoms caused by atopy but also the importance of elucidating the epidemiology of atopy for a large proportion of asthmatics in English-speaking countries such as Canada.

Furthermore, there have been numerous single country studies aimed at determining the prevalence of atopy. For example, a birth cohort of children on the Isle of Wight in the United Kingdom was followed until 4 years of age. In this study the children were tested for sensitization to 12 common allergens and assessed for asthma, rhinitis and eczema [20]. The overall prevalence of atopy in children in the study was 19.6% as defined by a positive reaction to one or more allergens [20]. The prevalence of atopy was higher in children diagnosed with asthma, 44%, rhinitis, 55%, and eczema, 43% [20]. These findings lend additional support to the importance of understanding the etiology of atopy in order to further the understanding of asthma, rhinitis and eczema.

On the whole, there is a strong body of evidence that the rising prevalence of allergic diseases such as asthma, rhinitis and eczema could be attributed to the rise in atopic sensitization. However, the international prevalence rates of asthma and atopy exhibit intense variability between countries and also within countries[27]. Environmental variables such as pollution, lifestyle, occupation, and socioeconomic status among others are likely key to being able to explain some of this international variability.

2.3 Environmental Factors Contributing to Allergic Diseases

Many environmental factors have been investigated in order to attempt to understand the relationship between environmental exposures and the prevalence of allergic diseases. Researchers have considered factors from pollution to family size. The following comprises a review of environmental factors and personal characteristics that have been

demonstrated to have some association with atopy or other allergic diseases in studies and their prospective relevance in rural communities.

2.3.1 Family Size

The increase in prevalence of allergic diseases that has occurred over that last few decades is the result of a reduced exposure to microbial burden during childhood [1]. This hypothesis was first postulated by Strachan in 1989 when he published the results of a birth cohort of 17, 414 British children born during one week in March, 1958 and became renowned as the hygiene hypothesis [2]. After 23 years of follow-up, he observed that birth order and the size of the family were significant risk factors for allergic diseases. That is, children that came from larger families and children with numerous older siblings had a lower risk of developing allergic diseases. However, it is important to note the allergic traits that were under consideration in Strachan's analysis were: "a) self reported hay fever during the past 12 months at age 23; b) parental report of hay fever or allergic rhinitis in the past 12 months at age 11; and c) parental recall of eczema in the first year of life at age 7"[2]. Thus, Strachan did not investigate atopy but self-reported symptoms by parents or the children once they became adults. Based on his findings at both 11 and 23 years of age there was an inverse relationship between hay fever and the household size at age 11. Strachan postulated that declining family size and improvements in the standard of personal cleanliness have reduced the chance of cross infection in families and this may have caused the increase in allergic diseases, which is what is now referred to as the hygiene hypothesis. In other words, measures that collectively reduce microbial burden increase the prevalence of allergic diseases.

Moreover, Strachan highlighted the importance of household size that subsequently other researchers investigated. A report from the European Community Respiratory Health Survey on 13, 932 participants aged 20-44 years from 36 areas found that hay fever was less common in participants with many siblings (OR 0.92 95% CI: 0.90-0.95 per sibling)[29]. This protective association of number of siblings and hay fever was only found in those participants with atopy and/or parental allergy. The report did not include analysis of atopy and number of siblings alone but was concerned mostly with hay fever.

Conceptually, the hygiene hypothesis infers any exposure that introduces a microbial load that stimulates the immune system can contribute a protective effect for allergic diseases, including daycare attendance and exposure to pets as a child. A study in The Netherlands investigated the contributions of day care attendance, older siblings and pet ownership to atopic disease in a cross-sectional survey of 1,555 children aged 8-13 years[30]. In this population, children who had attended day care or had a dog or a cat in the first two years of life had reduced odds of atopic sensitization but found no significant association with having an older sibling or older siblings. Nevertheless, a meta-analysis in 2002 reported that 14 of 16 studies of allergic sensitization or immunoglobulin E (IgE) reactivity demonstrated a protective association for number of siblings [31]. Hence, children from larger families and those that attend daycare may benefit from cross-infections from other children in that these infections challenge their immune systems and encourage the development of the non-allergic or more specifically non-atopic phenotype.

2.3.2 Farming

Farming as a parental occupation was significantly associated with reduced risk for atopic sensitization for outdoor allergens and for indoor allergens in 404 Swiss children aged 13-15 years [4]. In this 1999 study, Braun-Fahrlander and colleagues found that farming families were of lower socioeconomic status, had more children, often reported more humidity spots or visible molds in their home, were more likely to heat their homes with traditional heating systems using mainly coal and wood and were more likely to keep furred pets. Similarly, Riedler and colleagues in 2001 investigated atopic sensitization, to a panel of common aeroallergens and food allergens in 812 children, aged 6-13 years, from Austria, Germany and Switzerland and found that atopy was lowest in children exposed to stables and who consumed cow's milk in their first year [3]. Both of these studies indicated that exposures in the farm environment were associated with a lower risk for atopy.

In addition, a Canadian study by Ernst and Cormier found that high school students aged 12-19 years that had been raised on a farm were less likely to be atopic than their classmates that had never lived on a farm or worked on a farm [5]. A similar study of Danish farming students and rural controls aged 19-20 years found that the prevalence of atopy was lower in the farming students [6]. Collectively, studies from Switzerland, Austria, Germany, Canada and Denmark have demonstrated a protective effect from atopy in individuals that grew up on the farm[3-6].

The above studies and numerous others have led researchers to wonder what exactly is it about the farm life that is protective for allergic diseases? An interesting comparison group for this investigation are those individuals that abide by an anthroposophic lifestyle, whose children are often referred to as Steiner children, who have few vaccinations and consume a diet that is rich in lactobacilli [32,33]. In a case-control study of 295 children attending anthroposophic schools in Sweden and 380 children from neighboring schools all aged 5-13 years, the prevalence of atopy was significantly lower in children from anthroposophic families [32]. A publication from the Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyles group on 1,202 Steiner children and 634 reference children from non-Steiner schools in similar regions in Europe found that Steiner children had reduced risk of atopic sensitization [33]. This suggests that some of the lifestyle differences between Steiner children and other children in their communities are protective for atopy. Given that the anthroposophic lifestyle incorporates many characteristic that promote microbial challenge, e.g. minimal vaccinations, diet rich in lactobacilli, this lifestyle may be the embodiment of the hygiene hypothesis.

Likewise, other studies have attempted to further characterize protective elements of the farm environment. In a study of Bavarian children aged 5-7 years, the decreased prevalence of allergic disease was attributed to an increased exposure to livestock [34]. Also, the risk for a positive skin-prick test in Australian children aged 7-12 years was lower for children living on a farm provided that the farming community was composed mainly of livestock farms whereas no protection was seen for children from a farming

community comprised mainly of grain and cotton farms [35]. The authors postulated that a livestock farm provided exposure to pathogens such as *Camphylobacter enteritis* and *Escherichia coli* whereas crop farms may have actually exposed children to allergens. Gram-negative and gram-positive bacteria have been measured in high concentrations in farm buildings, stables, and confinement building [36,37]. Dust endotoxin levels in farm homes, rural homes, and farm barns are much higher than those in urban and non-farm homes [11,37]. Thus, contact with farm animals could be a proxy for increased exposure to endotoxin and other pathogen associated molecular patterns that stimulate the immune system and encourage the non-atopic phenotype.

In like manner, a recent publication from the Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyles study of 2,823 farm and 5,440 farm reference children aged 5-13 years found a protective maternal effect for atopic sensitization [38]. The protective effect for atopic sensitization was observed if the mother worked in the stables during pregnancy (OR 0.58; 95% CI 0.39-0.86 p=0.007). The study also looked at rhinoconjunctivitis, wheezing and asthma and did not find this association with the maternal effect and this highlighted the heterogeneity of the immunopathology between these allergic diseases and atopy. In conclusion, evidence from Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyles study contributed to the abundance of support that the farm environment may be protective for atopy in children.

2.3.3 Pets

Having a pet as a child has been associated as being protective for atopic diseases [39-45]. However, these associations have not been consistent [30,46-49]. Even in some of those studies where pets have shown to be protective only a cat or sometimes only a dog has been protective [42,45,49-51]. This indicates that pets have the potential to both be protective for atopy and a risk factor for atopy. Pets not only represent a source of allergen, e.g. *Fel d 1* antigen from cat, but also a potential source of bacterial challenge such as endotoxin [10]. These observations provide a rationale for the apparent paradox of exposure to pets and the relationship with atopy. In individuals that are atopic to pets

exposure would act as sources of allergens. However, in individuals that are not atopic to pets exposure may encourage the non-atopic phenotype in accordance with the hygiene hypothesis as they provide a source of bacterial challenge. Nonetheless, the effect of pet exposures on the development of atopy has been extensively investigated.

Accordingly, European Community Respiratory Health Survey researchers used childhood pet exposure to assess the association of pets with atopic and allergic diseases in adulthood[43]. The effect of pet exposure was sensitive to the type of pet, the allergens that an individual was currently sensitized and was dependent on the prevalence of the allergen in the environment. Researchers found that having a cat in childhood was associated with asthma only in atopic individuals and this association was strongest in centres where cat ownership was less common. From this observation, there appears to be a timing and dose requirement for the protective effect of cats for atopy. Cat exposure must be during the appropriate etiologic window and at a high enough dose to encourage the non-atopic phenotype. This effect is in keeping with hypothesis that consistent exposure to agents that introduce microbial challenge promotes deviation from allergic responses.

It is important to consider that several studies have noted that the protective effect of having a pet often becomes stronger when a family history of allergic diseases is considered [39,45,52]. In addition, pets such as dogs and cats are prevalent in most countries and children can be exposed to these pets without having a pet in their own home [44] and this may confound the association. Regardless, pet ownership is an important environmental exposure that may influence a child's atopic status.

2.3.4 Smoking

It is well established that exposure to environmental tobacco smoke has a harmful effect on the lung health of children[53]. However, the findings with regards to atopy in children have been less conclusive.

A prospective birth cohort of children in the United Kingdom followed to 3-years of age demonstrated that environmental tobacco smoke had no effect on the development of atopy [54]. Contrary to this finding, a German birth cohort found that at 3-years of age children that had environmental tobacco smoke exposure were at increased risk of sensitization to food allergens [55]. The German study tested two additional food allergens, soybean and wheat, and this may be the reason this group saw the association. Yet, a meta-analysis found that environmental tobacco smoke exposure, specifically via parental smoking, did not appear to increase the risk of allergic sensitization in children [56]. As a result of conflicting reports in the literature with regards to the relationship between environmental tobacco smoke exposure and atopy in children it is still an environmental factor that should be investigated.

2.4 Hygiene Hypothesis and the Immunopathology of Atopic Disease

The immunopathology of allergic diseases is the result of a tight network of immune cells. The primary line of defense of the body against invading pathogens is the innate immune system. The first immune cells to respond to a pathogen are tissue resident mast cells and dendritic cells and their activation begins a signaling cascade that activates the adaptive branch of the immune system, namely T and B cells. Initially T and B cells exist in an undifferentiated state and activation results when antigen is presented by innate immune cells or in the case of a viral infection by any of the body's cells along with stimulatory cytokines and other activation factors [57,58].

The immune system is comprised of the innate and adaptive branches. The innate branch comprises cells such as macrophages, dendritic cells and other cells that line the primary entry points for pathogens into the body and are responsible for initiating the host's response to infection. The adaptive branch of the immune system comprises cells that rely on the innate immune cells to prime them to respond to infectious agents e.g. B cells and T cells. CD4+ T cells, belonging to the adaptive branch of the immune system, are polarized into T_h1 or T_h2 cells depending on the nature of antigen presentation and co-stimulatory signals (Figure 2-1). T_h2 cells are differentiated from naïve T_h0 cells in the presence of IL4 and T_h2 cells in turn promote further differentiation through the

production of IL4, IL5 and IL9. The cytokines that stimulate the differentiation of T_h0 cells are secreted from the innate immune cells, e.g. macrophage, dendritic cells, or in the case of viral infection from any cell. T_h2 cells also produce IL13 that in conjunction with IL4 promotes B cells to switch to the IgE isotype, the hallmark immunoglobulin of allergic diseases. Whereas, T_h1 cells are differentiated from T_h0 cells in the presence of IL12 and IFN and leads to further differentiation through the production of IL12, TNFβ and IFNγ. Of most importance and interest is that IFNγ down regulates the differentiation of T_h2 cells and IL4 down regulates the differentiation of T_h1 cells. Thus, once CD4+ T_h0 cells have begun differentiation along one of the pathways it is difficult to switch pathways [57,58].

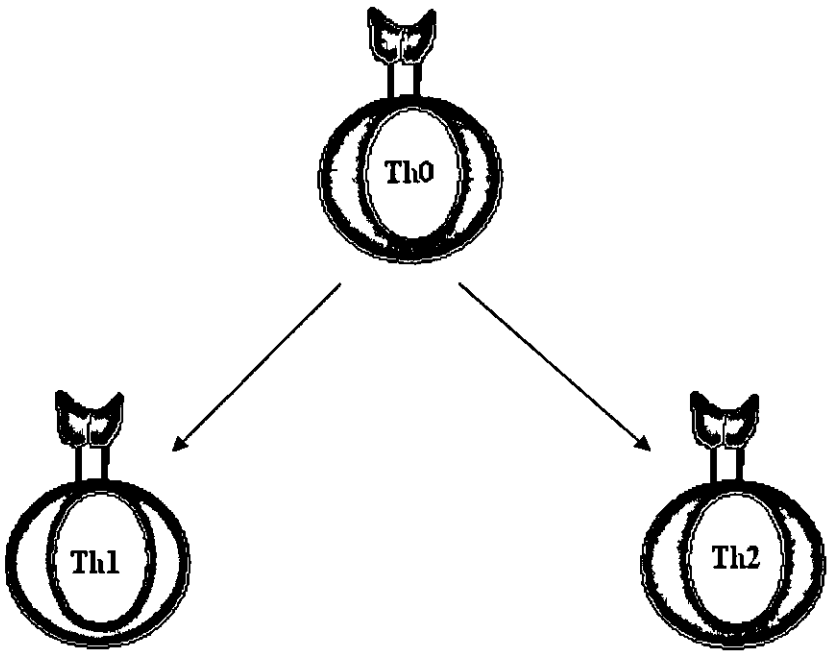

Figure 2-1 Th1-Th2 Paradigm

Innate cells, such as macrophages and dendritic cells, produce IFNs that are necessary for T_h1 polarization. Innate cells are activated to produce IFN via the detection of bacteria or viruses via toll-like receptors (TLRs) and these innate cells then act as antigen presenting cells to present antigen to T cells and B cells. However, the source of IL4 necessary for T_h2 polarization has not been fully elucidated [59]. Thus, it has been

postulated that in the absence of stimulation of the innate immune cells via their TLRs by bacteria and viruses that the immune system is predisposed to T_h2 polarization.

Furthermore, the effector responses of T_h2 cells are responsible for the pathology of atopy. The elevated production of IgE from B cells specific to antigens leads to cross linking of IgE on mast cells and eosinophil surfaces via IgE (Fcε) receptors and the subsequent degranulation and release of mediators from mast cells and eosinophils that are responsible for tissue destruction and inflammation (Figure 2-2). Degranulation of mediators from mast cells and eosinophils results in the release of histamine, serotonin, heparin, thromboxanes, prostaglandins, leukotrienes, and cytokines that attract more inflammatory cells that in turn secrete cytokines that prolong the mast cell activation, promote B cells to secrete IgE and result in tissue damage [57]. Activated eosinophils are believed to mediate most of the disordered airway functions that are characteristic of asthma. Eosinophil recruitment and activation is a characteristic feature of asthma, whether allergic or non-allergic [60].

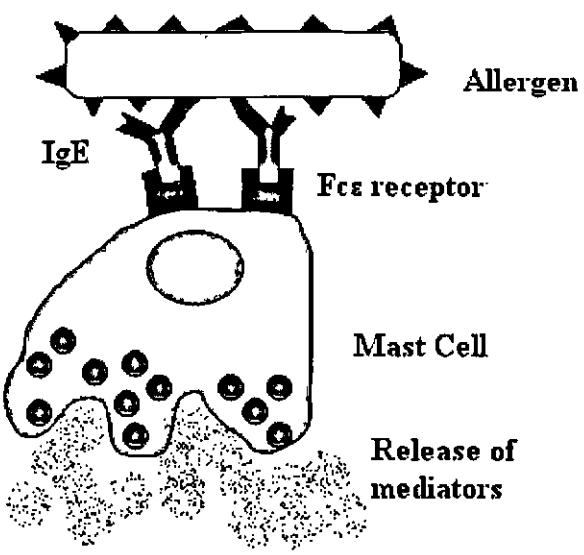

Figure 2-2 Degranulation of a mast cell in response to an allergen

Furthermore, a key element of the T_h1/T_h2 paradigm is that IFNγ produced by T_h1 cells down regulates the differentiation of T_h2 cells and IL4 produced by T_h2 cells down

regulates the differentiation of T_h1 cells. If the T_h1/T_h2 paradigm was complete and all encompassing then it would be impossible for a patient to present with both allergy, a T_h2 type disorder, and insulin dependent diabetes mellitus (IDDM), a T_h1 disorder. In an Italian study of children aged 3 to 15 years a reduction in the frequency of allergic symptoms in children with IDDM was seen but both atopy and IDDM were present in some of the same children in the study [61]. This may indicate that the relationship between T_h1 cells and T_h2 cells is oversimplified by the T_h1/T_h2 paradigm. However, it has been argued that IDDM's pathology is a result of β islet cell destruction early on and it is possible that the atopy phenotype arises after the T_h1 mediated islet cell destruction [1,59,61]. This would indicate that individuals with IDDM and allergic symptoms are not presenting with both a T_h1 disorder and a T_h2 disorder but rather presenting with a T_h2 disorder, atopy, and the resultant pathology of a previous manifestation of a T_h1 disorder, IDDM. Nonetheless, the prevalence of atopic disease is increasing in patients with IDDM and rheumatoid arthritis, an undisputed T_h1 disorder [62]. This suggests that there may be a common mechanism underlying infection-mediated protection from allergy and autoimmune diseases [62].

Regulation of T_h polarization is also carried out by other T-cell types, called T_{reg} cells [1]. T_{reg} cells are a highly heterogeneous family that includes Th3 cells, Tr1 (T regulatory 1) cells, and CD4+ CD25+ T cells [1]. T_H3 cells produce TGFβ, Tr1 cells produce IL-10 with or without TGFβ and CD4+ CD25+ T cells do not produce any cytokines and act via contact dependent mechanisms [1].

There has been much discussion in the literature that a general deficiency of T_{reg} cell activity might be responsible for the increased prevalence of allergic diseases and of autoimmune diseases [1,59,62,63]. A review of the role of T_{reg} cells on the prevalence of allergy emphasized that human T_h2 cells are less sensitive to the suppressive activity of T_{reg} cells than T_h1 cells[1]. This would mean that T_{reg} cells promote T_h2 cell development by suppressing T_h1 cells. However, the exact immunological mechanisms are still under debate but T_{reg} cells have the potential to modulate the T_h1/T_h2 paradigm in favour of the T_h2 spectrum of the paradigm thereby promoting the development of atopy.

In summary, the immunopathology of the hygiene hypothesis is still under debate among immunologists. However, the basic pathology of inflammatory disease consistently implicates certain cytokines and signaling proteins in the pathology of atopy regardless of whether the current immunological model is perfect. Genes coding for these cytokines and signaling proteins are likely to be key candidate genes for elucidating the genetic epidemiology of atopy. In order to begin searching for candidate genes for a disease there first must be some basis for genetic heritability.

2.5 Genetic Factors

Population based studies have provided inference on the heritability of atopy. Individuals with a family history of atopic disease have an increased risk of developing IgE-sensitization [64]. It has been found that if both parents are atopic there is a 50% risk for their child to be atopic; if one of the child's parents is atopic or a sibling is atopic the risk is 25% [65]. Narrow-sense heritability (h^2_N) represents the extent to which genes transmitted from an offspring's parents account for the observed phenotype [66]. Palmer and colleagues found in an Australian population-based study of 232 Caucasian nuclear families that log total serum IgE levels had h^2_N of 47.3% and that specific serum IgE levels against house dust mite and timothy grass had an h^2_N of 33.8% [67]. From these results, genetics account for 40-50% of the atopic phenotype. A study from the United Kingdom of 340 monozygotic and 533 dizygotic British twins, aged 18 to 72 years, reported higher concordance rates for monozygotic twins than dizygotic twins for hay fever, eczema and specific IgE positivity to *Der p 1*, mixed grass pollen and cat fur [68]. This suggests that there is not only a definitive genetic component to atopy but because monozygotic twins were often discordant for atopy further indicating that there is also a definitive role for environmental exposures. This supports the pursuit of genetic or epigenetic modifications that have the potential to interact with known environmental risk factors for atopy.

2.5.1 Genetic Epidemiology of Association Studies

Traditionally, genetic association studies have concentrated efforts on identifying major genes influencing disease etiology. For example, BRCA1 and BRCA2 mutations have been estimated to increase a woman's lifetime risk of breast cancer to between 60 and 85%[69]. No such major genes have been identified for allergic diseases and researchers are still attempting elucidate the genetics of atopy. A popular general technique for assessing the genetic components of a disease is the candidate gene approach. For this approach, genes are selected for analysis with a particular disease based on their functional relevance to the pathology of the disease [70,71]. For example, the Fcε receptor for IgE on mast cells, eosinophils and B cells has been investigated as a candidate gene for asthma, atopy and other allergic diseases[72,73]. The candidate gene approach investigates polymorphisms in genes postulated to have an effect on the disease pathology in order to compare the frequency of these polymorphisms in cases and in controls. The study design can vary from case-control to family-based association studies. Family based association studies are believed to be more powerful than other association studies because they are free from population stratification [74].

Population stratification occurs when a particular genotype in a population appears to associate with a disease but in reality is associated with some other unmeasured trait. For example, in a particular community a mutation, TLR19-588, associates with the prevalence of particular disease, D. The mutation has two alleles or versions: a major allele for which the majority of the population are carriers and a minor allele for which a minority of the population are carriers. In the affected individuals the frequency of the TLR19-588 minor allele is 23% in those that have the disease and in the unaffected individuals the frequency of the TLR19-588 minor allele is 7%. However, upon further analysis it is noted that the majority of the affected individuals (88.9%) in this community are Asian (Table 2-1).

Table 2-1 Population stratification example

Disease (D)	Asian (N)	Caucasian (N)
Affected	800	100
Unaffected	100	900
Total	900	1000

Further investigation reveals that the frequency of the TLR19-588 minor allele in Asians in this community is high, 25%, and is low in Caucasians, 5%. At this point researchers are concerned that any association in this population between the disease, D, and TLR19-588 is likely to be a result of population stratification and not because of an association with D. That is, it cannot be assumed that the disease is associated with TLR19-588 from this analysis. It can be inferred from this analysis that Asians are more likely to carry the minor allele for TL19-588. Subsequent analysis of the disease with this mutation must account for this by stratifying the analysis by ethnicity or only analyzing the Asians or the Caucasians separately.

Fortunately, family based association tests are robust to population stratification. These methods use information on the family structure in a sample population (e.g. mother-child relationship, siblings, etc.) to compare observed genotypes in relatives. The transmission disequilibrium test (TDT) is the prototype of all family based association tests [75,76]. TDT analysis is most appropriate when genotypes are available from two-parent offspring nuclear families wherein the offspring is affected. The TDT does not require information on parental phenotypes. The test is used to examine the number of times an offspring receives the risk allele from a heterozygous parent under the assumption that the observed transmissions do not deviate from chance alone. Humans are diploid organisms meaning that they have two copies of each autosomal chromosome, which are not sex chromosomes[77]. Females have two copies of the X chromosome and males have one copy of the X chromosome and one copy of the Y chromosome[77]. As such every human has two alleles for a gene from an autosomal chromosome, one allele that was transmitted from their mother and one allele that was transmitted from their father. If the alleles are the same the individual is called homozygous and if the alleles are different the individual is called heterozygous. The

term wild-type allele is synonymous with major allele[77]. Thus, for the family based association test (FBAT) only families with parents that are heterozygous at the locus under investigation are informative. The requirement of sufficient families with heterozygous parents and affected offspring limit the power of the analysis because a proportion of the population is not being used in the analysis. Extensions of the TDT that maximize this information followed. One of these tests was the FBAT.

2.5.2 Family-Based Association Test

FBAT was created as an extension of the TDT that allows for missing parental genotypes by inferring them from the genotypes of siblings where available [78]. FBAT excludes those families that are homozygous at the locus of interest, as does the TDT, because these families do not provide information on how alleles are transmitted. FBAT determines the expected score for each family under the null hypothesis of no association and no linkage or the null hypothesis of no association in the presence of linkage assuming Mendelian transmission, one allele transmitted from mother and one allele transmitted from father, by conditioning on offspring trait values and parental genotypes. The difference between the observed and expected score is summed for all informative families to calculate a test statistic with a chi-square distribution. The magnitude of the test statistic will indicate whether the observed transmissions from heterozygous parents to affected offspring deviate from what is expected from chance alone.

2.5.3 TLR4

The innate immune system is the primary line of defense against invading pathogens and innate immune cells recognize specific pathogen associated molecular patterns such as lipopolysaccharide (LPS) [79]. LPS is the signature pathogen associated molecular pattern of all gram-negative bacteria. Innate immune cells can recognize pathogen associated molecular patterns via their toll-like receptors (TLRs). Currently, 10 different TLRs have been identified in humans. When any member of the TLR family mounts an immune response to a pathogen a T_h1-type or a T_h2-type response can be initiated. For

example, it has been demonstrated in a mouse model of allergic sensitization that low level inhalation of LPS induces a T_h2 response and that high level inhalation of LPS induces a T_h1 response [80]. This may imply a crucial role for TLRs, specifically TLR4 an LPS receptor, in the T_h1/ T_h2 paradigm. When TLRs on innate immune cells such as dendritic cells recognize a pathogen they may secrete cytokines that favor the differentiation of T_h0 cells into T_h1 cells.

More specifically, TLR4 was identified as a crucial component for the recognition of LPS (endotoxin) because mice with a targeted deletion in the TLR4 gene have been shown to be unresponsive to LPS[81]. A simplified representation of the TLR4 pathway is diagrammed in Figure 2-3. In humans, LPS is sequestered by LPS binding protein (LBP) that is believed to transfer LPS to CD14. CD14, which may be membrane bound or soluble, facilitates recognition of LPS by TLR4 [79]. Mice deficient in CD14 are hypo-responsive to LPS [82,83]. Another protein, MD-2 is associated with the extracellular region of TLR4 and is essential for the cellular responsiveness to LPS [84]. Thus, the LPS recognition complex consists of an LPB/CD14/TLR4/MD-2 complex that in turn activates a signaling cascade. This signaling cascade activates NFκB induced phosphorylation of the IKK proteins that tag the IKK proteins for degradation[85,86]. Without the IKK proteins sequestering NFκB in the cytoplasm, this transcription factor activates the genes of inflammatory cytokines such tumour necrosis factor-α (TNF- α) that recruit other pro-inflammatory cells to the site of the pathogen's invasion in an attempt at clearing the infection.

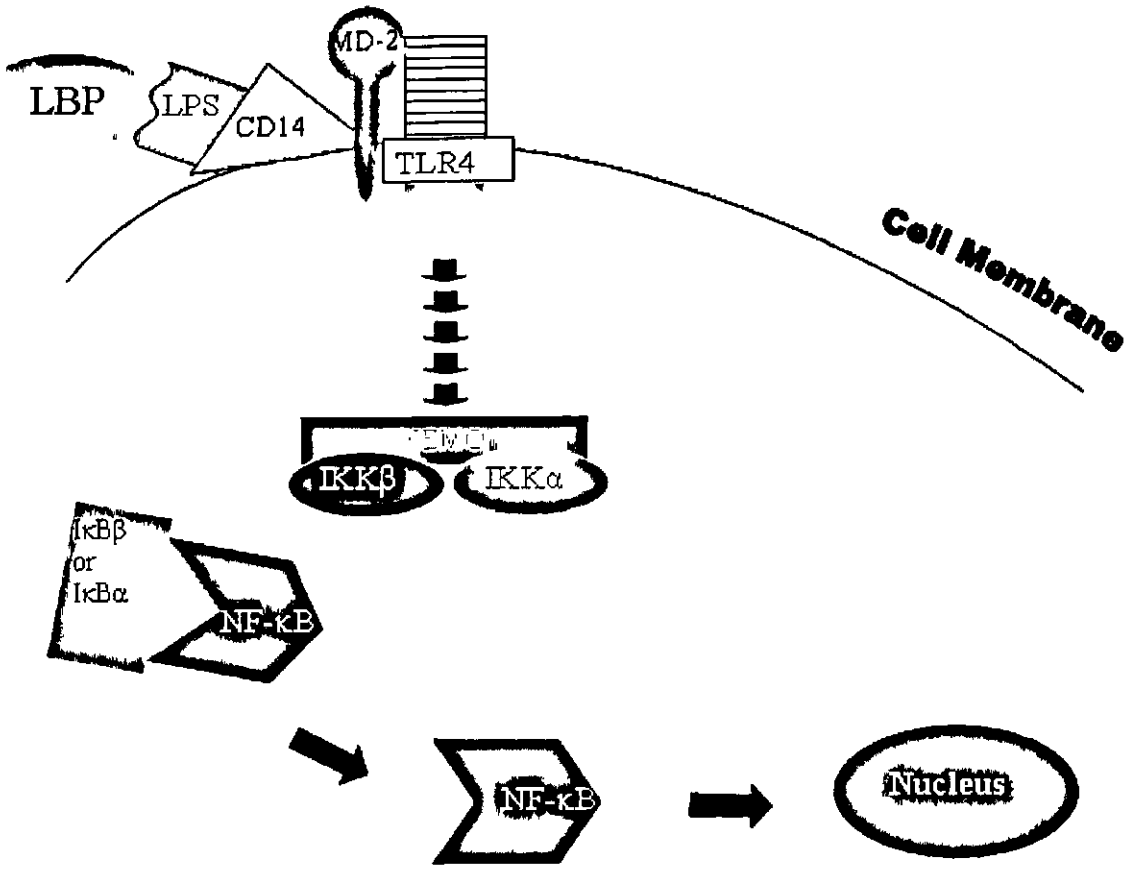

Figure 2-3 Schematic diagram of the TLR4 pathway

Due to the observation that activation of the TLR4 pathway by endotoxin at a high concentration produces a cytokine milieu that promotes the production of T_h1 cells[87], the influence of endotoxin exposure on the development of atopy has been investigated. This is because a cytokine milieu that favors T_h1 cells is one that in turn disfavors T_h2 cells and consequently disfavours atopy. For this reason, attempts have been made to determine whether endotoxin levels in the homes of children have significant associations with allergic diseases. One study of 61 infants found that children who were exposed to higher levels of endotoxin were significantly less likely to be atopic to a panel of allergens [88]. Thereby, supporting the postulate that exposure to higher levels of endotoxin would lead to a lower prevalence of atopy. Likewise, a study of children aged 5-10 years in Germany found a lower prevalence of atopy in children that were exposed to higher levels of house dust endotoxin[89]. This study provided further support for the protective effect of exposure to high levels of endotoxin for atopy in children. Thus,

there is a small body of evidence supporting the postulate that exposure to elevated levels of endotoxin has a protective effect on atopy.

Moreover, a single-nucleotide polymorphism (SNP) in TLR4 that results in an amino acid change from aspartic acid to glycine at position 299 (rs#4986790), TLR4 D299G, has been demonstrated to confer respiratory hypo-responsiveness to inhaled LPS in humans by Arbour and colleagues [7]. The SNP was identified from screening the coding region of TLR4 in 83 subjects of which 12% carried the minor allele. Only 1 of the 10 subjects was homozygous for the minor allele and thus, the frequency of the minor allele in this population was found to be 6.6%. The TLR4 D299G mutation was found to be in complete linkage disequilibrium with another mutation (rs#4986791) that resulted in an amino acid substitution of threonine to isoleucine. In other words, individuals that are carriers of the TLR4 D299G mutation are extremely likely to be carriers of the TLR4 T399I mutation. To demonstrate the functional relevance of the TLR4 D299G mutation, THP-1 cells, which are derived from a human leukemia cell line[90], were transfected with either wild-type or mutant TLR4 alleles. This resulted in those THP-1 cells that carried the TLR4 D299G mutation not responding normally to LPS as measured by NFκB activity following LPS stimulation. Interestingly, of the 10 subjects that carried the TLR4 D299G and TLR4 T399I co-segregating polymorphisms 3 subjects were LPS responsive and only 7 of the 31 subjects that were hypo-responsive to LPS carried the TLR4 SNPs. Thus, indicating that other factors may be acting in concert to confer hypo-responsiveness to LPS.

On the other hand, a more recent study by van der Graaf and colleagues used LPS to stimulate mononuclear cells isolated from individuals that were heterozygous for the TLR4 D299G mutation and those cells from wild-type individuals[8]. The researchers found no difference in the levels of the pro-inflammatory cytokine tumour necrosis factor (TNF) or the anti-inflammatory cytokine IL-10 produced between the wild-type cells or the TLR4 D299G mutant cells. The study differed greatly from that of Arbour and colleagues[7] because they used mononuclear cells, and not THP-1 cells, from healthy volunteers aged 30-50 years of age. In total there were 22 volunteers of whom 3 were

found to be heterozygous for TLR4 D299G mutation and one individual was homozygous for the minor allele. Arbour and colleagues found that only 10 of their 83 subjects were carriers of the TLR4 D299G mutation and of these 10 individuals only 7 were hypo-responsive to LPS. This may indicate that humans who carry the TLR4 D299G mutation are not all hypo-responsive to endotoxin. Due to the small sample size in the recent study and the findings of Arbour and colleagues, it is not surprising that all 3 individuals in the van der Graaf and colleagues study who carried that TLR4 D299G mutation were responsive to LPS. Rather this study underscores the possibility that the TLR4 D299G mutation may be acting in concert with other factors that may result in LPS hypo-responsiveness.

In a study of 336 British Caucasian families with at least two or more siblings that were asthmatics either by doctor diagnosis or current medication usage and 179 Caucasians without asthma or family history of asthma found no association of the TLR4 D299G mutation with developing asthma in parent-offspring trios or case-control analyses [91]. The frequency of the minor allele was 6% and it was not associated with asthma or atopy using family-based tests or case-control analyses. However, the researchers found that carriers of the TLR4 D299G mutation had a higher mean atopy severity score, 1.8, compared to the mean atopy severity score, 1.2, of carriers for the major allele ($p=0.003$). This analysis indicates that the researchers had difficulty in finding a direct association with the prevalence of atopy and the SNP. The trend the researchers did find was only for the first offspring and not the second. The atopy severity score ranged between 0 and 4.5 and was based on the number of skin-prick tests and specific IgE responses an individual had based on a panel of six allergens. This study suggests that the TLR4 D299G mutation may contribute to atopy severity in a population of asthmatics. It is important to note that this finding was not reproduced in the second affected sibling that may indicate that this finding was spurious or that the TLR4 D299G mutation's effect is part of an interaction with birth order.

TLR4 was resequenced in 90 ethnically diverse subjects, Black (n=24), White (n=23) and Hispanic (n=24) in addition to subjects with asthma (n=19) and a total of 29 SNPs

were identified [92]. The frequency of the TLR4 299 minor allele, G, ranged from 14.6% in the Black population to 4.2% in the Hispanic population. The researchers then selected 5 common mutations, including the TLR4 D299G and TLR4 T399I mutations, that would tag the majority of haplotypes for evidence of association with asthma in a heterogeneous North American cohort and also in a more homogeneous population from Quebec, Canada. Haplotypes are a set of alleles from loci that are closely linked and are usually inherited as a unit[77]. Using the transmission disequilibrium test (TDT), the researchers found no association with asthma or atopy-related phenotypes in either population. Consequently, TLR4 mutations were not associated with asthma or atopy-related phenotypes in the two birth cohorts.

In a study of 231 farmers' children and 385 non-farmers children in 6 rural areas in Austria and Germany there was no statistically significant association between the TLR4 D299G mutation and allergic diseases including atopy when the data were stratified by farming status [93]. When the investigators stratified the data according to endotoxin exposure they found that children who were heavily exposed (greater than 50^{th} percentile) and were carriers of the minor allele had a significantly lower prevalence of atopy than children that were wild-type for TLR4 D299G and heavily exposed. This finding lends to some speculation that a dose response to endotoxin may be an important consideration when assessing a potential gene by environment interaction or that the association was spurious given the known variability in response to endotoxin in carriers of the minor allele. Also, this study did not employ family-based tests and may have been prone to population stratification. Thus, further investigations of the association between the TLR4 D299G mutation will contribute to the understanding of the interaction of genes and the environment that influence the development of atopy.

3. Methodology

3.1 Ethics

Ethics approval was obtained from the University of Saskatchewan Biomedical Research Ethics Board to analyze data from the Humboldt Lung Study as a secondary data analysis (Bio REB#05-97). The larger study received ethics approval prior to this request (BMC#02-663a).

For this analysis, a pedigree identifier (PID) was created to link individuals into families. This process required the use of personal identifiers in a confidential manner. Once the PID was created for the respective families personal identifiers such as names and addresses were removed from the dataset.

3.2 Study Design

The design of the study was cross-sectional. Children, aged 6-17, were recruited from the elementary and high schools via a letter sent home to their parents asking for consent for their child's participation and a questionnaire that parents completed for their children. Children over the age of 13 years were given an additional questionnaire to complete themselves. The questionnaires were completed and consent was obtained prior to testing for lung function, testing for skin-prick reactions, measuring height and weight and blood or buccal swab collection. Subsequently, nurses visited the schools to conduct objective measurements of those children from whom parental consent had been obtained and to collect the completed questionnaires. Objective measurements included lung function testing, skin-prick testing and collection of samples for DNA isolation via buccal swabs.

Adults were recruited via canvassers that contacted all households within the town and surrounding areas asking all eligible adult subjects, 18-79 years of age, in each home to participate in the study and to complete a written consent of participation. The canvasser

left the questionnaire that was completed in the home by each subject and returned during a prearranged clinic visit when height, weight, waist circumference, lung function and allergen skin prick reactions were measured and blood for DNA isolation was collected.

3.3 DNA Isolation

The DNA isolation procedure involved taking two 8-mL samples of blood from adults. The samples were drawn into Qiagen *PAXgene* tubes. The *PAXgene* system was used to isolate DNA as per manufacturer's instructions. Typical DNA yields were 18-25μg per mL of whole blood. UV spectrophotometry was used to quantify DNA. Labeled stock samples were stored at -80°C and working dilutions of DNA of 10ng/μL were prepared for subsequent genotyping assays. Sample information was stored electronically referenced by a unique identifier.

To isolate DNA from the children's buccal swabs, of epithelial cells from the mouth inner cheeks, a swab was immersed in 50mM NaOH, vortexed, then heated at 95°C for 30 minutes. The swab was removed from the solution and the solution was neutralized with 1M Tris pH 8.0. Samples tubes were labeled and stored at 4°C.

3.4 Genotyping

Genotyping for the TLR4 D299G mutation in children was performed by collaborators in Japan at Kumamoto University and genotyping for the adults was performed by collaborators in the United States at Duke University. For the adult samples, TaqMan assays were performed to genotype the TLR4 D299G DNA locus, using forward and reverse primers at a concentration of 200nM (GAAGAATTCCGATTAGCATACTTAGACTACT and TAATTCTAAATGTTGCCATCCGAA respectively). Fluorescent probes were ordered with the minor groove binding (MGB) protein and non-fluorescent quencher (NFQ). Thermal cycling was performed on the MJ Tetrad thermal cycler (MJ Research, Waltham, *Mass)*, with the following cycling parameters: initial cycles of 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, with a

final holding cycle of 4°C. Genotype was determined by post-amplification plate reading on the ABI Prism 7900 Sequence Detection System *(Applied Biosystems)*. The TLR4 D299G locus was genotyped using polymerase chain reaction (PCR) amplification (primers 5'-TTTAGAAGTCCATCGTTTGGTTCT-3' and 5'-CAAACAATTAAATAAGTCAATAGT-3', 2.5mM dNTPs, 5u/μL Taq polymerase) and subsequent restriction enzyme digestion. The products were then analyzed by on a 2.0% agarose gel, following ethidium bromide staining.

3.5 Questionnaires

For the children, questionnaires were distributed through the schools and questions were derived from that American Thoracic Society's Children's Respiratory Disease Questionnaire [94] and the Canadian Student Lung Health Study [95]. For the adults, the questions regarding respiratory symptoms and smoking were modified from the American Thoracic Society standardized questionnaire[94] and previous questionnaires used in the Humboldt Study (1977, 1983 and 1993) [12-15]. The questionnaires requested information on demographics, current and past respiratory conditions, other illness such as diabetes and heart disease, history of allergic diseases (both personal and familial) and lifestyle factors.

3.6 Operational Definitions

For the purpose of this thesis several operational variables were created for all of the children in the study and several operational variables were created for those children that had both parents in the study.

3.6.1 Variables that were computed for all 734 children

Several variables were used for analysis with all 734 children that were available in the study for this analysis. These include atopy, smoking, pets, total number of siblings, humidity, parental history of allergic diseases, farming exposures, farming and TLR4 D299G.

3.6.1.1 Atopy

Skin-prick tests were performed on children using five allergens (*D. pteronyssinus*, *D. farina*, grass, cat and *Alternaria*) by trained nurses. A positive skin-prick test was defined as wheal 3-mm in diameter or larger than the negative control, saline. Histamine was used a positive control.

3.6.1.2 Smoking

For the children in the study a composite variable for exposure to smoke was created from the following questions:

a) Does any family member smoke cigarettes regularly in your home at present?

b) Are this child's parents current smokers?

c) Does any family member smoke a pipe or cigars regularly in home at present?

In addition, children aged 13-19 years had a questionnaire that they completed which included questions on active smoking using the following question:

d) Have you ever smoked cigarettes? (Yes at least a whole cigarette, Yes just a few puffs, No not even a few puffs)

Any adolescent that answered, "Yes at least a whole cigarette" was coded as having active exposure to smoking and all other adolescents were coded as not having active exposure to smoking. Thus, for any positive response to questions a through d the child's smoking exposure was coded as yes (passive and/or active). All other children were coded as non passive and non active smoking exposure.

3.6.1.3 Pets

To assess exposure to pets the following questions were used:

a) Do you currently have any pets living inside your home: dog (yes or no), cat (yes or no), other (specify).

b) During this child's lifetime, have you had a dog, cat or bird living in your home?

The exposure to pets was coded as positive if at least one of the answers to either question was affirmative. In addition, the pets listed under other pets were coded as positive responses to the question if the pet was a furred pet e.g., hamster, rabbit or guinea pig. All remaining children were coded as no exposure to furred pets.

3.6.1.4 Total number of siblings

This variable was generated from the question, "What is the total number of brothers and sisters (excluding half-brothers and half-sisters) this child has?

3.6.1.5 Humidity

This variable was generated from a composite of two questions: "Does your house have any damage caused by dampness (e.g. wet spots on walls, floors)?" and "Are there signs of mold or mildew in any living areas of your home?" An affirmative answer to either of these two questions was coded as a positive response to humidity and all remaining children were coded as a negative response to humidity.

3.6.1.6 Parental history of allergic diseases

This variable is a composite of the responses to several questions:

a) Has the biological father of this child had: asthma (yes, no, don't know), allergy (yes, no, don't know), hay fever (yes, no, don't know) or eczema (yes, no, don't know).

b) Has the biological mother of this child had: asthma (yes, no, don't know), allergy (yes, no, don't know), hay fever (yes, no, don't know) or eczema (yes, no, don't know).

A positive response to any of these questions was coded as the presence of a parental history of allergic diseases and all remaining children were coded as having no such parental history.

3.6.1.7 Farming Exposures

Two different farming exposure variables were created. The first variable that will be referred to as "farming" from hereon applied to all 734 children in the study and the second that will be referred to as "farming parents" was only for those 309 children that had both parents in the study. Two different variables were created for these groups because for those children that had both parents in the study additional information was extracted from the adult questionnaire using the pedigree identifier (PID).

3.6.1.8 Farming

This variable is a composite of the following questions from the children's questionnaire:

a) During the past 12 months has this child ever taken care of cattle, hogs, poultry, horses or other livestock?

b) In the past 12 months has this child spent more than 1 hour on a regular basis near the following activities? Haying, Harvesting, Moving or playing with hay bales, Feeding livestock, Cleaning or playing in barns, Emptying of filling grain bins, Pouring or mixing farm chemicals?

c) In the first 12 months of this child's life did this child: Live on a farm, Visit a farm more than 3 times, Visit a farm regularly?

d) Where is home located? Farm, Acreage, In town?

An affirmative response to any part of questions a and b was coded as an affirmative for farming exposure. For question c, if a child had lived on a farm in the first 12 months of life the child was coded as having farm exposure. Lastly, if the child currently lived on a farm he/she was coded as having a farm exposure. All other children were coded as not having exposure to the farm.

3.6.1.9 TLR4 D299G

The major allele for the TLR4 D299G is denoted as the A allele for the nucleotide adenine and denoted as the G allele for the nucleotide change to guanine (rs#4986790)[7]. The A allele represents the major allele whereas the G allele represents the minor allele. The TLR4 D299G mutation represents a substitution of the amino acid aspartic acid to glycine at amino acid position 299 of the coding region of the TLR4 protein (Figure 3-1).

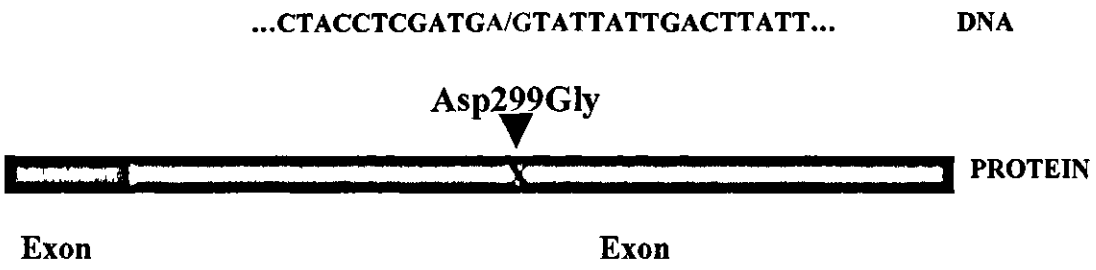

Figure 3-1 The A>G SNP in exon 2 of TLR4 encodes the non synonymous amino acid change Asp>Gly at amino acid position 299.

3.6.2 Variables that were generated for the 309 children that have both parents that participated in the study

Several variables were only available for analysis in 309 children that had both parents in the study. These variables include farming parents, parental education, mother's smoking status, father's smoking status, parental atopy, parental occupation, and parental work with livestock.

3.6.2.1 Farming Parents

This variable is a composite of the above questions for the farming variable in addition to occupational information extracted from the parents' responses to the adult questionnaire:

e) In the last 5 years, have you grown or handled wheat, durham, oats, barley, flax, canola, rye, mustard, alfalfa, or other grain, seeds, or legumes?

f) In the last 5 years, have you worked at looking after cattle, hogs, sheep, poultry, horses, or other livestock animals?

If either parent answered yes to question e or f the farming parents variable was coded as affirmative. This variable was also affirmative if there was an affirmative response to any part of questions a and b or if the child had lived on a farm in the first 12 months of life or if they currently lived on a farm.

3.6.2.2 Parental Education

The question: "What is the highest grade completed in school? (Grade school not completed, Grade school completed, High school completed, Trade school or only attended college, College graduate/ postgraduate)" was used to code this variable. A response of grade school not completed, grade school completed or high school completed was coded as low. A response of trade school or only attended college was coded as medium and a response of graduate/ postgraduate was coded as high. When the education level differed between parents the highest level of education was selected.

3.6.2.3 Mother's Smoking Status

The questions, "Do you now smoke cigarettes?" and "Do you smoke a pipe or cigars regularly at present?" were used to code for mother's smoking status. The initial syntax coded for all women in the study but only the information from those women that were determined to have children in the study was extracted from the adult dataset into the children's dataset via the aggregate function in SPSS based on the PID.

3.6.2.4 Father's Smoking Status

The questions, "Do you now smoke cigarettes?" and "Do you smoke a pipe or cigars regularly at present?" were used to code for father's smoking status. The initial syntax coded for all men in the study but only the information from those men that were determined to have children in the study was extracted from the adult dataset into the children's dataset via the aggregate function in SPSS based on the PID.

3.6.2.5 Parental Atopy

Skin-prick tests were conducted for adults using four allergens (*D. pteronyssinus,* cat, grass and *Alternaria*). A positive skin-prick test was defined as wheal 3-mm in diameter larger than the negative control, saline. Histamine was used a positive control.

3.6.2.6 Parental Occupation

The variable parental occupation was collected from the questions "In the last 5 years, have you grown or handled wheat, durham, oats, barley, flax, canola, rye, mustard, alfalfa, or other grain, seeds, or legumes?" and "In the last 5 years, have you worked at looking after cattle, hogs, sheep, poultry, horses, or other livestock animals?" If either parent answered yes to either question parental occupation was coded as farming.

3.6.2.7 Parental Work with Livestock

The variable parental occupation was collected from the question "In the last 5 years, have you worked at looking after cattle, hogs, sheep, poultry, horses, or other livestock animals?" If either parent answered yes to this question parental occupation was coded as having worked with livestock. All other parents were coded as not having working with livestock. In the cases where this variable was discordant between parents, the parents were coded as having worked with livestock.

3.7 Data Entry

Data for the adults were entered and double entered using SPSS-D/E for the adult questionnaires and using FileMaker Pro for the children's questionnaire at the Institute of Agricultural Rural and Environmental Health, University of Saskatchewan by a team of research assistants. After the data were double entered, it was dumped into SPSS v. 13.0 as two files: adults and children. Data cleaning was performed with frequency and contingency tables. Any outliers or seemingly erroneous entries were manually checked against the original participant's questionnaire. Data cleaning was completed in March, 2006.

3.8 Objective #1: Assigning Nuclear Family Identifiers

As an initial step in data cleaning the personal identifiers listed in the elementary, high school and adult databases were merged together in order to have a single file in order to link familial relationships. This task could not be automated because, though the data had been double entered, there were conflicts in the spelling of last names, first names and addresses. In addition, given that all future analyses with genetic data would rely on the family structures generated from this process a tedious manual process was the only option. As a result of the confidential nature of the information used to generate the PIDs all names, addresses and family structures are fictitious but representative of the system that was followed.

The software applications Family-Based Association Test (FBAT) and Pedigree-Based Association Test (PBAT) were used to perform the analysis of the TLR4 D299G

mutation with atopy[78,96]. In order to input the data into FBAT and PBAT, the data file had to be in a particular format that is summarized in Table 3-1.

Table 3-1 Example file for FBAT and PBAT

PID	FID	MID	IID	Sex	Affection Status	TLR4 D299G allele 1	TLR4 D299G allele 2
1	2356	1234	1	1	1	0	0
1	2356	1234	5	1	1	1	2
1	0	0	1234	2	0	1	2
1	0	0	2356	1	0	1	1

In Table 3-1, PID is the pedigree identifier that was used to identify nuclear families, FID is the father's identifier, MID is the mother's identifier, IID is the individual identifier. Sex is coded as "1" for males , "2" for females and "0" for missing. Affection status is coded as "1" for those that do not have atopy, "2" for those that do have atopy and "0" where the information is missing. For both TLR4 D299G alleles 1 and 2, a "1" represents the major allele (A), a "2" represents the minor allele (G) and a "0" represents individuals that were not genotyped. More specifically, looking at the PID "1", there are two children whose IIDs are "1" and "5". Their mother is IID "1234" and their father is IID "2356". Both children are unaffected and only IID "5" is genotyped for the TLR4 D299G mutation. For the parents, their respective FIDs and MIDs are missing (code "0") because this information was not collected for adults. This is the only format that is accepted by FBAT and PBAT. In order to generate a file like Table 3-1, personal identifiers were extracted from the SPSS v. 13.0 data files into two excel files, Tables 3-2 and 3-3.

Table 3-2 Initial file of personal identifiers for adults

										Spouse			Child1			Child 2		
IID	PID	FID	MID	Last Name	First Name	Sex	Age	Tele-pho	Ad-dress	First Name	Last Name	Ad-dress	Last Name	First Name	Age	Last Name	First Name	Age
1234				Deer	Janet	1	38	123-4567	1 Main	John	Deer	1 Main	Deer	Brown	9	Deer	Grey	12
2356				Ranger	Lone	0	66	222-2222	4 Main									
4321				Kid	Sarah	1	54	555-5555	45 St.	Sean	Kid	45 St.	Kid	Cabbage	17			
4444				Boo	Peeka	1	25	456-7890	5 St.	Scooby	Boo	5 St.						
5555				Boo	Scooby	0	29	456-7890	5 St.	Peeka	Boo	5 St.						
5678				Doll	Barbie	1	45	123-1234	2 Maple	Ken	Doll	2 Maple	Doll	Skipper	15			
7777				Deer	John	0	39	123-4567	1 Main	Janet	Deer	1 Main	Deer	Brown	9	Deer	Grey	12
8765				Doll	Ken	0	32	123-1234	2 Maple	Barbie	Doll	2 Maple						

In Table 3-2, the columns PID, FID and MID are empty because at this stage these identifiers have not been created. Information is available on the adult participants' names and addresses, that of their spouse's and children. Similarly, the PID, FID and MID columns are empty in Table 3-3 at this stage of the process. Information on the names of parents and siblings is available in the children's data file. The column denoted "P" is a variable that allows for types of families to be quickly counted and is only in the children's spreadsheet (Table 3-3). The categories for this variable are: p2s for both parents in the study and multiple siblings in the study, p2 for both parents in the study but only one offspring in the study, p1s for one parent in the study and multiple siblings, p1 for one parent in the study with one offspring in the study, sib for children that do not have either parent in the study but are siblings of the same family. For completion, those children without any apparent family in the study are labeled "single" under the P column.

Table 3-3 Initial file of personal identifiers for children

										Father		Mother		Sibling		
IID	PID	FID	MID	P	Last Name	First Name	Address	Tele-phone	Age	First Name	Last Name	First Name	Last Name	First Name	Last Name	Age
1					Deer	Brown	1 Main	123-4567	9	John	Deer	Janet	Deer	Grey	Deer	12
5					Deer	Grey	1 Main	123-4567	12	John	Deer	Janet	Deer	Brown	Deer	9
25					Kid	Cabbage	45 Street	555-5555	17	Sean	Kid	Sarah	Kid			
85					Doll	Skipper	2 Maple	123-1234	15	9999	9999	Barbie	Doll			
92					Boo	Tah	5 Street	456-7890	6	Scooby	Boo	Peeka	Boo			

The first step is to focus on the adults spreadsheet in excel and sort the data by the Child1 First Name column in order to identify those adults' that have listed children in the study (Table 3-4). Adults without children are highlighted in yellow.

Table 3-4 Initial file of personal identifiers for adults highlighting those without children

										Spouse			Child1			Child 2		
IID	PID	FID	MID	Last Name	First Name	Sex	Age	Tele-pho	Ad-dress	First Name	Last Name	Ad-dress	Last Name	First Name	Age	Last Name	First Name	Age
1234				Deer	Janet	1	38	123-4567	1 Main	John	Deer	1 Main	Deer	Brown	9	Deer	Grey	12
7777				Deer	John	0	39	123-4567	1 Main	Janet	Deer	1 Main	Deer	Brown	9	Deer	Grey	12
4321				Kid	Sarah	1	54	555-5555	45 St.	Sean	Kid	45 St	Kid	Cabbage	17			
5678				Doll	Barbie	1	45	123-1234	2 Maple	Ken	Doll	2 Maple	Doll	Skipper	15			
2356				Ranger	Lone	0	66	222-2222	4 Main									
4444				Boo	Peeka	1	25	456-7890	5 St.	Scooby	Boo	5 St.						
5555				Boo	Scooby	0	29	456-7890	5 St.	Peeka	Boo	5 St.						
8765				Doll	Ken	0	32	123-1234	2 Maple	Barbie	Doll	2 Maple						

The next step is to sort the adults that have children (not yellow) by last name (Table 3-5):

Table 3-5 Initial file of personal identifiers sorting adults that have children by last name

IID	PID	FID	MID	Last Name	First Name	Sex	Age	Tele-pho	Ad-dress	Spouse First Name	Spouse Last Name	Spouse Ad-dress	Child1 Last Name	Child1 First Name	Child1 Age	Child 2 Last Name	Child 2 First Name	Child 2 Age
1234				Deer	Janet	1	38	123-4567	1 Main	John	Deer	1 Main	Deer	Brown	9	Deer	Grey	12
7777				Deer	John	0	39	123-4567	1 Main	Janet	Deer	1 Main	Deer	Brown	9	Deer	Grey	12
5678				Doll	Barbie	1	45	123-1234	2 Maple	Ken	Doll	2 Maple	Doll	Skipper	15			
4321				Kid	Sarah	1	54	555-5555	45 St.	Sean	Kid	45 St.	Kid	Cabbage	17			
2356				Ranger	Lone	0	66	222-2222	4 Main									
4444				Boo	Peeka	1	25	456-7890	5 St.	Scooby	Boo	5 St.						
5555				Boo	Scooby	0	29	456-7890	5 St.	Peeka	Boo	5 St.						
8765				Doll	Ken	0	32	123-1234	2 Maple	Barbie	Doll	2 Maple						

and similarly sort the children by last name (Table 3-6).

Table 3-6 Initial file of personal identifiers for children sorting by last name of child

IID	PID	FID	MID	P	Last Name	First Name	Address	Tele-phone	Age	Father First Name	Father Last Name	Mother First Name	Mother Last Name	Sibling First Name	Sibling Last Name	Sibling Age
92					Boo	Tah	5 Street	456-7890	6	Scooby	Boo	Peeka	Boo			
1					Deer	Brown	1 Main	123-4567	9	John	Deer	Janet	Deer	Grey	Deer	12
5					Deer	Grey	1 Main	123-4567	12	John	Deer	Janet	Deer	Brown	Deer	9
85					Doll	Skipper	2 Maple	123-1234	15	9999	9999	Barbie	Doll			
25					Kid	Cabbage	45 Street	555-5555	17	Sean	Kid	Sarah	Kid			

From comparing Tables 3-5 and 3-6, it is possible to match most parents with their children. For example, adults, Table 3-5, with IIDs "1234" and "7777" are spouses and are the parents of children, Table 3-6, with IIDs "1" and "5". Thus in Table 3-6, for the children "1" and "5" their FID is "7777", corresponding to the IID of their father, and their MID is "5678", corresponding to the MID of their mother. This family consists of 2 parents and multiple children in the study and is thus labeled "p2s" in the column entitled P (Table 3-8). There is no way to tell if the parents of the adults "5678" and "7777" participated thus, their respective FIDs and MIDs are "0" and they are considered to be the founders of PID "1" (Table 3-7 and Table 3-8). In Table 3-5, IID "5678" has a spouse that does not appear to have participated in the study and is the mother of the child with IID "85". But by performing a search for the last name "Doll" amongst those adults that have not listed any children the spouse is found to have IID "8765". This man does not list any children and in this case, examining child "85" reveals that there is no father listed. Thus, IID "8765" is not the father of child "85" and the FID for IID "85" is still "0" for missing. The man with IID "8765" is still assigned the PID "2". This family has one parent participating in the study and one child thus is labeled "p1" under the column entitled P (Table 3-7 and Table 3-8). In similar manner, adult IID "4321" is the mother of child "25" but the father/ spouse does not appear to have participated in the study (Table 3-7 and Table 3-8).

Table 3-7 Completing PID, FID and MID in adults data file

IID	PID	FID	MID	Last Name	First Name	Sex	Age	Tele-pho	Ad-dress	Spouse First Name	Spouse Last Name	Spouse Ad-dress	Child1 Last Name	Child1 First Name	Child1 Age	Child 2 Last Name	Child 2 First Name	Child 2 Age
1234	1	0	0	Deer	Janet	1	38	123-4567	1 Main	John	Deer	1 Main	Deer	Brown	9	Deer	Grey	12
7777	1	0	0	Deer	John	0	39	123-4567	1 Main	Janet	Deer	1 Main	Deer	Brown	9	Deer	Grey	12
5678	2	0	0	Doll	Barbie	1	45	123-1234	2 Maple	Ken	Doll	2 Maple	Doll	Skipper	15			
4321	3	0	0	Kid	Sarah	1	54	555-5555	45 St.	Sean	Kid	45 St	Kid	Cabbage	17			
2356				Ranger	Lone	0	66	222-2222	4 Main									
4444				Boo	Peeka	1	25	456-7890	5 St.	Scooby	Boo	5 St.						
5555				Boo	Scooby	0	29	456-7890	5 St.	Peeka	Boo	5 St.						
8765	2	0	0	Doll	Ken	0	32	123-1234	2 Maple	Barbie	Doll	2 Maple						

Table 3-8 Completing PID, FID and MID in children's data file

IID	PID	FID	MID	P	Last Name	First Name	Address	Tele-phone	Age	Father First Name	Father Last Name	Mother First Name	Mother Last Name	Sibling First Name	Sibling Last Name	Sibling Age
92					Boo	Tah	5 Street	456-7890	6	Scooby	Boo	Peeka	Boo			
1	1	7777	1234	p2s	Deer	Brown	1 Main	123-4567	9	John	Deer	Janet	Deer	Grey	Deer	9
5	1	7777	1234	p2s	Deer	Grey	1 Main	123-4567	12	John	Deer	Janet	Deer	Brown	Deer	12
85	2	0	5678	p1	Doll	Skipper	2 Maple	123-1234	15	9999	9999	Barbie	Doll			
25	3	0	4321	p1	Kid	Cabbage	45 Street	555-5555	17	Sean	Kid	Sarah	Kid			

The next step is to examine the remaining children for whom parents were not identified. Comparing Table 3-7 and Table 3-8, it is apparent the child IID "92" does have parents in the study with IIDs "4444" and "5555" and this family is subsequently assigned PID 4 and labeled p2 under the P column (Table 3-9). There is one final case of family structure that still has not been described and that is of children that are siblings whose parents have not participated in the study. After all children with

parents have been identified and the corresponding PID, FID, MID and P values have been completed the children's data file is sorted by last name among those that do not have family identifiers.

Table 3-9 Completing PID, FID and MID in children's data file for children without parents

										Father		Mother		Sibling		
IID	PID	FID	MID	P	Last Name	First Name	Address	Tele-phone	Age	First Name	Last Name	First Name	Last Name	First Name	Last Name	Age
1	1	2356	1234	p2s	Deer	Brown	1 Main	123-4567	9	John	Deer	Janet	Deer	Grey	Deer	12
5	1	2356	1234	p2s	Deer	Grey	1 Main	123-4567	12	John	Deer	Janet	Deer	Brown	Deer	9
85	2	7000	5678	p1	Doll	Skipper	2 Maple	123-1234	15	9999	9999	Barbie	Doll			
25	3	7001	4321	p1	Kid	Cabbage	45 Street	555-5555	17	Sean	Kid	Sarah	Kid			
92	4	5555	4444	p2	Boo	Tah	5 Street	456-7890	6	Scooby	Boo	Peeka	Boo			
26	5	7002	7003	sib	Child	Oldest	55 8th	666-6666	17	Dad	Child	Mom	Child	Youn-gest	Child	9
70	5	7002	7003	sib	Child	Youngest	55 8th	666-6666	9	Dad	Child	Mom	Child	Oldest	Child	17

After assigning all PIDs, FIDs, MIDs and Ps the two files were merged and sorted by PID. The entire process was repeated three times. Upon completion of the three passes they were compared and any inconsistencies were remedied. The variables PID, FID, MID and P were then imported into the adult and children SPSS data files thereby removing any association with real names or addresses to ensure confidentiality during subsequent cleaning and analyses.

3.9 Objective #2: FBAT

After assigning relevant PIDs, FIDs and MIDs to participants in the study, the data was extracted from the SPSS data files (adult and children's) into an excel file that was saved as an .ped file. The file resembled that of Table 3-1.

Prior to beginning any hypothesis testing with the data the TLR4 D299G mutation was investigated to determine if it was in Hardy-Weinberg equilibrium (HWE) in this population[97]. HWE is represented by equation 3.1:

$$p^2 + 2pq + q^2 = 1 \qquad [3.1]$$

In equation 3.1, p and q represent the probabilities of two alleles for a particular locus where p is the probability of the more common allele, the major allele. In this study of the TLR4 D299G mutation, the A allele is the major allele and the G allele is the minor allele. The allele frequencies are used to determine the theoretical frequencies of the genotypes (AA, AG, GG) for the population and the theoretical numbers are compared with the actual distribution of genotypes in the population. Deviation from HWE is usually indicative of genotyping error but may also be a result of founder effects[98]. One of the major assumptions of HWE in a population is that mating is random [97] and with humans this is often not the situation as individuals often mate with others with similar lifestyle features e.g. live in the same neighborhood, attend the same church. Thus, deviations from HWE may be the result of genotyping error or not meeting the equilibrium underlying criteria.

The family based association test (FBAT) introduced in 2000 [78,99] is an extension of the transmission disequilibrium test (TDT) [76] put forth in 1993. The basis of the TDT involves looking at the transmission of an allele from parents to an affected offspring. That is, the basis of the TDT is an affected only design (Figure 3-2).

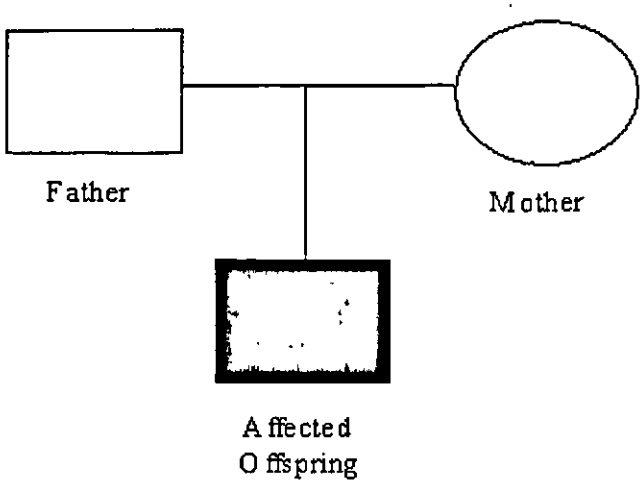

Figure 3-2: Family structure used for the TDT

The TDT compares the number of times an allele is transmitted to an affected offspring and compares this number with the frequency of such transmissions due to chance alone. Limitations of the TDT include: only affected offspring being considered, parents can not be missing and only families in which the parents are heterozygous for the allele of interest are informative for the test statistic. FBAT has the same premise as the TDT in that the transmission of an allele of interest is compared to the transmission that would have been observed due to chance. FBAT allows for missing parents if their genotypes can be inferred from multiple siblings. For this analysis, the null hypothesis for the test statistic is linkage and no association. FBAT is available as a software package and has a power calculator in the Pedigree Association Test (PBAT) software. For this analysis of the TLR4 D299G mutation, the genetic model was assumed to be dominant.

3.10 Objective #3: Logistic Regression for the Outcome Atopy

A descriptive analysis was performed for the variables atopy, parental history of allergic diseases, pets, farming exposure, sex, age, total number of siblings and smoking. This analysis was done as a final cleaning check and to ensure that the coding of the variables that were composites of many questions was valid. In children with both parents

participating in the study, a complete exploration of the data was performed stratifying the risk factors by farming. For all 734 children, univariable analyses between the outcome variate atopy and each risk factor were evaluated. Binary logistic regression was used to compute odds ratios (ORs) between atopics and non-atopic children.

Three models, the full model based on established risk factors, the full model including only those variables with a $p<0.25$ during the univariable analyses and the reduced model, were built using different inclusion criteria based on the results of the univariable analyses. Using binary logistic regression to model a dichotomous outcome, a variable can be selected for a model if it is clinically relevant and/or statistically significant based on a p-value<0.25[100]. The first model, the full model based on established risk factors, included all of the potential clinically relevant variables. The second full model included only those variables that had a $p<0.25$ during univariable analyses with atopy. The third and final model was the reduced model. Subsequently, interaction terms consisting of risk factors and parental history of allergic diseases were tested for significance at the $p<0.05$ level. The likelihood ratio test was used to select whether or not a single variable or a group of variables would be included in the final model. This test is based on the following formula 3.2, where the reduced model is nested within the full model.

$$G = -2(\log \text{likelihood of reduced model} - \log \text{likelihood of full model})^{100} \qquad \textbf{[3-2]}$$

G is a statistic with a chi-square distribution with k degrees of freedom and accounts for the number of additional variables in the full model in comparison with the reduced model. G is used in conjunction with the clinical interpretation of each multivariable model to assess the best model.

4. Results

4.1 Descriptives

A total of 734 children were ascertained from both the elementary schools and the high school. Overall the response rate for the children in the study was 79.1%. The crude prevalence of atopy in this population of children was 30.4% representing 223 atopic children of the total 734 children that had skin-prick tests for the five allergens: *D. pteronyssinus*, *D. farina,* grass, cat and/ or *Alternaria*. Table 4-1 provides a summary of the relevant categorical variables that will be assessed to build a logistic regression model of atopy in this population of children and also the frequency of the TLR4 D299G mutation. Of the 734 children that completed the questionnaire 712 (97.0%) consented to being genotyped for the TLR4 D299G mutation.

Table 4-1 Descriptives for categorical variables of overall study population of children

Categorical Variable	Frequency (n=734)	%
Sex:		
Female	388	52.9
Male	346	47.1
Smoking:		
No passive or active	525	71.5
Passive &/or active	209	28.5
Pets:		
Does not have a furred pet	415	56.5
Has a furred pet	319	43.5
Total Number of Siblings:		
0	41	5.6
1	283	38.6
2	287	39.1
3+	123	16.8
Humidity:		
No mold or dampness	666	90.7
Mold or dampness	68	9.3
Parental History of Allergic diseases:		
None	387	52.7
Yes	347	47.3
Farming:		
Non-farming	343	46.7
Farming	391	53.3
TLR4 D299G [A]		
AA	721	87.6
AG	90	12.3
GG	1	0.1

[A] N for the TLR4 D299G genotype is 712

Table 4-2 provides a summary of the continuous variable, age.

Table 4-2 Descriptives for continuous variables

Continuous Variable	Median (N=734)	Min	Max
Age	11.0	5	19

In order to fully explore the age variable, the frequency of each age and cumulative percent was examined in Table 4-3.

Table 4-3 Frequency of each age in the population of children

Age (years)	Frequency	Cumulative Percent
5	2	0.3
6	44	6.3
7	65	15.1
8	64	23.8
9	66	32.8
10	78	43.5
11	76	53.8
12	63	62.4
13	80	73.3
14	59	81.3
15	50	88.1
16	32	92.5
17	38	97.7
18	16	99.9
19	1	100.0
Total	734	

The cumulative percent was used to categorize the age variable according to the quartiles. The closest approximation to the quartiles was used to create four age categories: 5-8, 9-11, 12-13 and 14+ as is illustrated in table 4-4.

Table 4-4 Age variable categorized

Age Category (years)	Frequency	Percent	Cumulative Percent
9-11	175	23.8	23.8
12-13	220	30.0	53.8
14+	143	19.5	73.3
4	196	26.7	100.0
Total	734	100.0	

The prevalence of atopy in the 22 children that did not consent to being genotyped for the TLR4 D299G mutation was examined in Table 4-5.

Table 4-5 Prevalence of atopy in children that were not and were genotyped

	Genotyped			
	No N=22		Yes N=712	
	n	%	n	%
Non-atopic	19	86.4	492	69.1
Atopic	3	13.6	220	30.9

Of those children whose parents did not consent to genotyping 13.6% were atopic whereas in those children that did consent to genotyping the prevalence of atopy was 30.9%. This difference was not statistically different (p=0.10) but does appear to indicate a trend for atopic children to be more likely to have had their parents give consent for genotyping than non-atopic children in this population.

4.2 Family-Based Association Test with TLR4 D299G Mutation and Atopy

The TLR4 D299G mutation was found to be in Hardy-Weinberg equilibrium in the Humboldt population. Using the genotypes for both parents and children and the atopy affection status for the children, Family-Base Association Test (FBAT) was used to determine whether there was preferential transmission of the risk allele to the affected offspring. Only families where the parents were heterozygous for this genotype and had one affected offspring are considered informative and are included in the analysis assuming that the minor allele, the risk allele, was dominant (Table 4-6).

Table 4-6 FBAT analysis of the TLR4 D299G mutation with atopy

TLR4 D299G allele	Allele Frequency	Number of families	FBAT p value	Power
G	0.0671	62	0.1802	98.6%

The pedigree-based association test (PBAT) was used to calculate the power of the above FBAT analysis. The software was able to use or infer the genotypes in the study population to have 299 nuclear families in the analysis, of which 62 were informative. PBAT calculated the conditional power of the test to be 98.6%. Thus, these analyses appear to indicate that the TLR4 D299G mutation is not associated with atopy in this

population of children even though the power to detect such an association if it existed was high.

4.3 Assessment of Collinearity between Farming and Environmental Exposures

With respect to the hygiene hypothesis, the Humboldt population is particularly interesting as a result of potential exposures that are related to farming. One study in particular found that children from farming families differed based on parental education, number of siblings, mothers' smoking status, furred pets and family history of allergic diseases[4]. For exploratory purposes and also to assess potential collinearity between the variable farming and these exposure variables an analysis of the distribution of exposures between farming and non-farming families is essential. As a result of the methods of ascertainment, not every child in the study had both or even one parent that participated in the study. In total 309 of the 734 children had both parents participate in the study. These 309 children represent 179 families with both parents participating. The number of children per family varied as is illustrated in Table 4-7.

Table 4-7 Families both parents in the study

Number of Children/ Family	Number of families	%
1	77	43.0
2	76	42.5
3	24	13.4
4	2	1.1
Total	179	

A child was considered to be from a farming family for this analysis if he or she had one or both parents that had worked with grain or livestock in the last five years or had participated in farm activities in the last 12 months or currently lived on a farm or had lived on a farm in the first 12 months of life. The comparison between farming and non-farming families with both parents that participated in the study is summarized in Table 4-8. There were a total of 179 families as a result of not counting families multiple times because they had multiple children participate in the study. When the exposure was

assessed by family there were 111 non-farming families and 68 farming families that had both parents participate in the study.

From examination of Table 4-8, farming families, in which both parents participated in the study differed from their non-farming counterparts with regards to humidity but this difference did not approach statistical significance (p=0.38). Farming families were more likely to report visible mold or dampness in their homes (8.8%) compared with non-farming families (5.4%). Farming families were less likely to have both parents test positive for atopy, 5.9%, in comparison with non-farming families in which 11.7% had both parents test positive for atopy. However, this difference also did not approach statistical difference with a p-value of 0.32. There was also a trend for farming families to be more likely to report a parental history of allergic diseases (52.9%) compared with non-farming families (44.1%) but this was also not statistically significant (p=0.28). These families did not differ with respect to parental education, number of siblings, mother's smoking status, father's smoking status or pets.

Table 4-8 Comparison of farming and non-farming families in which both parents participated in the study

Variable		Total n=179		Non-farming N=111		Farming N=68		p-value
		n	%	*n*	%	*n*	%	
Parental Education	Low	31	17.3	22	19.8	9	13.2	0.48
	Middle	77	43.0	45	40.5	32	47.1	
	High	71	39.7	44	39.6	27	39.7	
Number of siblings	0	14	7.8	8	7.2	6	8.8	0.50
	1	76	42.5	49	44.1	27	39.7	
	2	68	38.0	44	39.6	24	35.3	
	3+	21	11.7	10	9.0	11	16.2	
Mother Smoking Status	Nonsmoker	157	87.7	98	88.3	59	86.8	0.82
	Smoker	22	12.3	13	11.7	9	13.2	
Father Smoking Status	Nonsmoker	153	85.5	94	84.7	59	86.8	0.83
	Smoker	26	14.5	17	15.3	9	13.2	
Pets	No furred pet	119	66.5	76	68.5	43	63.2	0.47
	Has furred pet	60	33.5	35	31.5	25	36.8	
Humidity	No mold or dampness	167	93.3	105	94.6	62	91.2	0.38
	Mold &/or dampness	12	6.7	6	5.4	6	8.8	
Parental History of Allergic diseases	No	94	52.5	62	55.9	32	47.1	0.28
	Yes	85	47.5	49	44.1	36	52.9	
Parental Atopy	Neither parent	77	43.0	45	40.5	32	47.1	0.32
	One parent	85	47.5	53	47.7	32	47.1	
	Both parents	17	9.5	13	11.7	4	5.9	

From Table 4-8, there appears to be no overall difference between farming and non-farming families. The variables parental atopy and livestock are available only for 309 children, belonging to the above 179 families, and not for the entire larger subset of children (N=734). Thus, Table 4-9 exhibits the results from univariable analyses with

the variables parental atopy, livestock exposure in addition to age, sex and parents' education level using all 309 children where both parents participated in the study.

From Table 4-9, parental atopy and the parents work with livestock conferred no additional risk for being atopic in this population of 309 children. The non-atopic and atopic children differed in that the median age for atopic children was 13 years whereas the median age for non-atopic children was 10 years. The odds of having atopy increased on average by a factor of 1.27 per year of age ($p<0.001$, 95% CI: 1.16-1.39). Children from farm families accounted for 48.4% of the non-atopic children and accounted for 47.7% of the atopic children. Consequently, the power to detect an OR of 1.5 and an OR of 1.1 for atopy between children from non-farming and farming families was 36% and 7%, respectively. After adjusting for age, sex and parental education, the crude OR for parental atopy, parental occupation and parental work with livestock did not change significantly or clinically (Table 4-10).

Table 4-9 Univariable analysis of outcome atopy in N=309 children with both parents in the study

Variable	Total N=309		Non-atopic N=221		Atopic N=88		OR	95% CI		p-value
								Lower	Upper	
	Median	*Range*	*Median*	*Range*	*Median*	*Range*				
Age (continuous)	11	5-18	10	5-18	13	6-18	1.27	1.16	1.39	<0.001
Sex:	*n*	%	*n*	%	*n*	%				
Male	163	52.8	111	50.2	52	59.1	reference			
Female	146	47.2	110	49.8	36	40.9	0.70	0.42	1.15	0.16
Parental Education:	*n*	%	*n*	%	*n*	%				
Low	53	17.2	38	17.2	15	17.0	reference			
Middle	134	43.4	97	43.9	37	42.0	0.97	0.48	1.96	0.92
High	122	39.5	86	38.9	36	40.9	1.06	0.52	2.16	0.87
Parental Atopy:	*n*	%	*n*	%	*n*	%				
Neither parent	126	40.8	95	43.0	31	35.2	reference			
One+ parent	183	59.2	126	57.0	57	64.8	1.39	0.83	2.31	0.21
Parental Occupation:	*n*	%	*n*	%	*n*	%				
Non-farming	185	59.9	134	60.6	51	58.0	reference			
Grain or livestock	124	40.1	87	39.4	37	42.0	1.12	0.68	1.85	0.66
Parental Work with Livestock:	*n*	%	*n*	%	*n*	%				
No	257	83.2	185	83.7	72	81.8	reference			
Yes	52	16.8	36	16.3	16	18.2	1.14	0.60	2.18	0.69

Table 4-10 Additional univariable analysis of outcome atopy in N=309 children with both parents in the study

Variable	OR Crude	95% CI		p-value	OR Adjusted for Age, Sex, Parental Education	95% CI		p-value
		Lower	Upper			Lower	Upper	
Parental Atopy:								
Neither parent	reference				reference			
One+ parent	1.39	0.83	2.31	0.21	1.46	0.85	2.52	0.17
Parental Occupation:								
Non-farming	reference				reference			
Grain or livestock	1.12	0.68	1.85	0.66	1.12	0.66	1.92	0.68
Parental Work with Livestock:								
No	reference				reference			
Yes	1.14	0.60	2.18	0.69	1.12	0.56	2.24	0.76

4.4 Logistic Regression for the Outcome Atopy

In order to examine the variables sex, age, smoking, pets, total number of siblings, humidity, parental history of allergic diseases and farming with the outcome atopy, univariable logistic regression was used. These results are summarized in table 4-11.

Table 4-11 Results of univariate logistic regression for children (N=734) with atopy

Variable		Total n=734		Non-atopic n=511		Atopic n=223		OR	95% CI		p
		n	%	n	%	n	%				
Sex	Male	346	47.1	231	45.2	115	51.6				
	Female	388	52.9	280	54.8	108	48.4	0.775	0.565	1.062	0.113
Age	5-8	175	23.8	146	28.6	29	13.0				
	9-11	220	30.0	173	33.9	47	21.1	1.368	0.819	2.284	0.231
	12-13	143	19.5	106	20.7	37	16.6	1.757	1.017	3.036	0.043
	14+	196	26.7	86	16.8	110	49.3	6.439	3.952	10.493	0.000
Total number of siblings	0	41	5.6	32	6.3	9	4.0				
	1	283	38.6	204	39.9	79	35.4	1.377	0.629	3.015	0.424
	2	287	39.1	196	38.4	91	40.8	1.651	0.757	3.602	0.208
	3+	123	16.8	79	15.5	44	19.7	1.980	0.867	4.525	0.105
Smoking	No passive or active	525	71.5	361	70.6	164	73.5				
	Passive &/or active	209	28.5	150	29.4	59	26.5	0.870	0.608	1.233	0.424
Pet	Does not have fur pet	416	56.7	292	57.1	123	55.2				
	Has a fur pet	319	43.5	219	42.9	100	44.8	1.080	0.790	1.488	0.618
Humidity	No mold or dampness	666	90.7	466	91.2	200	89.7				
	Mold &/or dampness	68	9.3	45	8.8	23	10.3	1.190	0.702	2.021	0.517
Parental History of Allergic diseases	None	387	52.7	278	54.4	109	48.9				
	Parental History	347	47.3	233	45.6	114	51.1	1.248	0.911	1.710	0.168
Farming	Non-farming	343	46.7	238	46.6	105	47.1				
	Farming	391	53.3	273	53.4	118	52.9	0.980	0.715	1.343	0.899

The only variable that differed significantly between atopic and non-atopic children in this population was that of the age. The older age groups had an increased odds of being atopic compared with the younger age groups. This association grew stronger with increasing age group. There was a mild association with sex, although not statistically significant at the 0.05 level, and atopy. The odds-ratio (OR) was 0.775 for females in comparison with males in this study. Similarly, there was a mild association with parental history of allergic diseases. Those children with a parental history of allergic diseases appeared to have an increased chances of being atopic (OR=1.248) however, this association was not statistically different. In this analysis, those children with 1 or more siblings were at an increased risk of developing atopy than children that were only children however, this association was not statistically significant. Both exposure to smoking, passive and/ or active, and exposure to pets appear to confer no additional risk for developing atopy. In like manner, no statistically significant association was seen with humidity or farming and atopy in this population.

From the univariable analysis, the variables sex, age, total number of siblings and parental history of allergic diseases had p-values less than 0.25. From a model building perspective, a p-value of less 0.25 is sufficient to include the variable in the initial steps of a logistic regression model [100]. The goal of this process is to achieve the most parsimonious model that still explains the data. A fine balance must be achieved between the number of variables in the model that are necessary to control for confounding from the epidemiological perspective and the number of variables in the model that minimize the standard errors and the model dependence on the observed data from the mathematical perspective must be achieved. The full model included all of the variables analyzed in the univariable analysis: age, sex, total number of siblings, smoking, pet, humidity, parental history of allergic diseases and farming (Table 4-12). These variables were selected for the full model on the basis of their clinical relevance and previous associations in the literature.

Table 4-12 Full model based on established risk factors using logistic regression for children with atopy compared with unaffected children

Variable	β	SE	OR/ Exp(B)	95% CI Lower	95% CI Upper	p-value
Constant	-3.665	0.534	0.026	-	-	0.00
Sex (ref: male)	-0.305	0.172	0.74	0.53	1.03	0.08
Age (continuous)	0.238	0.028	1.27	1.20	1.34	0.00
Parental History of Allergic diseases (ref: none)	0.322	0.173	1.38	0.98	1.94	0.06
Total Number of Siblings (ref: none)						
1	0.074	0.425	1.08	0.47	2.48	0.86
2	0.150	0.423	1.16	0.51	2.66	0.72
3+	0.235	0.451	1.27	0.52	3.06	0.60
Smoking (ref: not passive or active)						
Passive &/or active	-0.253	0.195	0.78	0.53	1.14	0.20
Pet (ref: does not have a furred pet)						
Has a furred pet	0.071	0.173	1.07	0.76	1.51	0.68
Humidity (ref: no mold or dampness)						
Mold or dampness	0.127	0.291	1.14	0.64	2.01	0.66
Farming (ref: non-farming)						
Farming	-0.124	0.175	0.88	0.63	1.25	0.48

Chi-square=89.97, df=10, p<0.001
-2 log likelihood = 811.478

A more stringent full model was fitted on the p-value <0.25 criteria from the univariable analysis and resulted in 4 of the 8 variables (sex, age, total number of siblings and parental history of allergic diseases) (Table 4-13).

Table 4-13 Full logistic regression model using p<0.25 for inclusion criteria in children with atopy compared with unaffected children

Variable	β	SE	OR/ Exp(B)	95% CI Lower	95% CI Upper	p-value
Constant	-3.718	0.519	0.024	-	-	0.00
Sex (ref: male)						
Female	-0.308	0.171	0.73	0.53	1.03	0.07
Age (continuous)	0.236	0.028	1.27	1.20	1.34	0.00
Parental History of Allergic diseases (ref: none)						
Yes	0.327	0.172	1.39	0.99	1.94	0.06
Total Number of Siblings (ref: none)						
1	0.048	0.422	1.05	0.46	2.40	0.91
2	0.164	0.420	1.18	0.52	2.68	0.70
3+	0.230	0.446	1.26	0.52	3.02	0.61

Chi-square=87.64, df=6, p<0.001
-2 log likelihood = 813.805

Finally, a reduced model was fitted using only the variables age, sex and parental history of allergic diseases (Table 4-14) as was generated using a reverse stepwise logistic regression approach.

Table 4-14 Reduced model using logistic regression in children with atopy compared with unaffected children

Variable	β	SE	OR/ Exp(B)	95% CI Lower	95% CI Upper	p-value
Constant	-3.625	0.372	0.027	-	-	0.00
Sex (ref: male)						
Female	-0.307	0.171	0.74	0.53	1.03	0.07
Age (continuous)	0.238	0.028	1.27	1.20	1.34	0.00
Parental History of Allergic diseases (ref: none)						
Yes	0.327	0.172	1.39	0.99	1.94	0.06

Chi-square=86.89, df=3, p<0.001
-2 log likelihood = 814.554

The chi-square from the likelihood ratio test is obtained by subtracting the -2 log likelihood from the full model in Table 4-12 from the -2 log likelihood from the reduced model in Table 4-14 (χ^2=814.554-811.478=3.076, df=7, p=0.878). This comparison indicated that these models were not statistically different. When the reduced model (Table 4-14) was compared to the full model (Table 4-13) using the likelihood ratio test, there was no statistically significant difference between these models (χ^2=0.749, df=3, p=0.862).

For the selection of the final model in this population, the reduced model is the most parsimonious. Part of the rationale for including the variables, total number of siblings, humidity, pets and smoking in a model of atopy in this population of children is that these variables have demonstrated associations in previous studies of rural populations with allergic diseases. However, an underlying assumption is that in this population there is some heterogeneity in exposure and this is likely a result of the farming environment.

From the previous examination of the 309 children that had both parents participate in the study, it appears that there is no statistically significant difference between farming and non-farming families in this study based on the variables parental education, parental smoking status, total number of siblings, pets, parental atopic status, humidity or parental history of allergic diseases. As a result of the lack of heterogeneity of exposures between farming and non-farming families, the reduced model is likely to be the most appropriate model of atopy in this population of children. In addition, interaction between parental history of allergic diseases was investigated with the variables pets, humidity, total number of siblings and farming. The investigation was stimulated by the assumption that those individuals with some genetic predisposition for atopy, using the parental history of allergic diseases as a proxy, would demonstrate divergent effects when exposed to these variables. For example, a dog may be protective for atopy by introducing microorganisms for children without a parental history of allergic diseases but may be a risk factor for children with the parental history by providing a source of allergen. However, none of the interaction terms were significant in the reduced or either full logistic regression models. Hence, the reduced model is the final model selected to represent atopy in this population of children in Humboldt. The overall equation for the model is:

$$\log (p/(1-p)) = -3.625 + (-0.307)\,\text{sex} + (0.238)\text{Age} + (0.327)\,\text{Family history of allergic diseases} \quad \textbf{[4.1]}$$

5. Discussion

5.1 Response Rate

When evaluating the results from any epidemiological study, an initial step is the evaluation of the validity of the study. Employing a strict definition of validity, a study that is valid is one that is free from systematic error [101,102]. Validity has been divided into two components: internal and external [101,102]. Internal validity is a measure of how well the inferences made from analyzing the study population apply to the source population that the study population was meant to represent. Whereas external validity or generalizability is a measure of how well the inferences made from analyzing the study population apply to populations outside of the source population. An important assessment of the validity of a study is the response rate of individuals to the request for participation in the study. If the study has high internal validity, the response rate will be high indicating that there is a low likelihood that those who did not respond to the request to participate in the study differ from those that did elect to respond. Overall the response rate for the children in the study was 79.1%. Questionnaires were available from 734 children that participated. However, consent from the children's parents to collect buccal swabs was obtained for only 712 of these children. Of the 22 children whose parents did not give consent for genetic analysis 19 were non-atopic. It appears that there is a trend for the parents' of atopic children to be more likely to give consent for genetic testing than the parent's of non-atopic children although this was not statistically significant ($p=0.100$) but may have introduced a selection bias. The parents of an atopic child may be aware that their child has allergies and as such are more likely to allow their child to give DNA for a lung health study. Although there was an indication of selection bias via the parents' of atopic children being more likely to allow their children to give DNA there also was some indication that teenagers were in general less likely to participate in the study. The participants ascertained from high school had a lower response rate, 64%, than children in the elementary schools where the response rate was 86%. This is of importance because when the relationship between age and

atopy was analyzed teenagers (14+) were found to have the highest prevalence of atopy in comparison with the other age groups (5-8, 9-11 and 12-13). More specifically, the teenagers had an increased likelihood of being atopic when compared with children between the ages of 5-8 years ($p<0.001$). It has been previously reported that the prevalence of atopy peaks near the 3rd decade of life [103,104] and a gradient in the prevalence of atopy in children aged 5-19 years of age should be expected. Similarly, Ernst and Cormier compared a group of teenagers aged 12-19 years of age (N=802) that currently lived on a farm with a group of their classmates that did not currently live or work on a farm (N=397) in Quebec[5]. They found that the prevalence of atopic sensitization was 40.8% in the group that lived on a farm and 53.4% in the other group. The prevalence of atopic sensitization in the Humboldt study for 12-19 year olds was 48.6% indicating that, although the response rate among teenagers was low the prevalence of atopy was similar to that in the Ernst and Cormier study. Thus, it was feasible to infer that the non-responders from high school were likely to have a similar pattern of prevalence to atopy as the responders. In summary, the response rate from high school students was low but a comparison with another Canadian study of rural high school students indicated that the selection bias was likely to be non-differential with respect to atopic status.

In short, there is some indication of selection bias in this study of 734 children. The parents of atopic children were more likely to allow their children to participate by giving samples for genetic testing and teenagers had a poor response rate in comparison with the younger children in the study. The first selection bias alone would result in over-representation of atopics in the study population in comparison with the source population for genetic analysis. However, FBAT used only those children with atopy to evaluate the transmission of the minor allele from a heterozygous parent thus, this would not have biased this analysis. Furthermore, there was a low response rate from the high school but the prevalence of atopy for this age group is similar to that of another study of rural teenagers in Canada indicating that any selection bias may be non-differential.

5.2 Prevalence of Atopy

A major goal of this population-based study was to determine the prevalence of atopy in the study population of children. Overall, the prevalence of atopy should be lower in this community, because it is a rural community, than the prevalence of atopy in urban communities as has been reported in the literature[17]. The following section comprises a summary of these results with relevant comparisons to prevalence reports of atopy from the literature. The issues surrounding the case definition of atopy will also be discussed.

The overall prevalence of atopy in this study of children aged 5 to 19 years of age was 30.4%. The odds ratio for being atopic was higher in the older age groups (14+, 12-13 and 9-11) in comparison with 5-8 year olds. This is consistent with the effect of age on atopy in the literature. The peak in prevalence of atopy to environmental allergens is somewhere around 30 years of age [103,104]. Thus, a gradient in the prevalence of atopy is to be expected with regards to age. Growing older per se is not a risk factor for atopy. However, as a person ages there are increasing opportunities for his/ her immune system to encounter allergens and multiple exposures to an allergen and this is required for a type I hypersensitivity reaction[57]. Type I hypersensitivity reactions refer to the IgE-mediated reactions that cause atopy. Before an individual becomes atopic to an allergen he/ she must first be first sensitized to the allergen or in other words have encountered it before. Thus, the increased likelihood of being atopic in the older age categories is likely the result of age confounding the association between the frequency of exposure an individual has to an allergen and whether an individual becomes atopic to that allergen.

Another Canadian study of children that were high-risk for allergic diseases, the Canadian Childhood Asthma Primary Prevention Study, found that at age 7 the prevalence of atopy in this population was 49.0% in their intervention group and 41.6% in their control group[105]. The intervention group had been advised to implement house-dust mite control measures, pet avoidance measures, avoidance of environmental tobacco smoke and encouraged mothers to breastfeed until between 4 months and 1 year of age where the control group had not been advised of these measures. In the Humboldt study the prevalence of atopy for children aged 7 was 16.9% (n=65). The children in

Canadian Childhood Asthma Primary Prevention Study were high-risk for allergic diseases and researchers tested a wider panel of allergens: house dust mite, cat, dog, cockroach, *Alternaria*, *Cladosporium*, tree, ragweed, cow's milk, egg white, wheat, soy, and peanut. The food allergens in particular are know to be prevalent in the first 3-5 years of life after which most children "outgrow" their allergies [106,107]. There was a limited panel of allergens used in the Humboldt study and as a result, there could be a misclassification bias of atopics in the study. More specifically, some individuals may have been atopic to allergens that were not tested. A study conducted in the United Kingdom involving atopic children aged 7 years found that 95% of atopics could be identified using grass, house-dust mite and cat compared with a panel of 29 allergens [108]. In like manner, another study found that grass, house-dust mite, cat and *Alternaria* could be used to screen 94% of atopic children at 4 years of age[20]. The current Humboldt study used *D. pteronyssinus*, *D. farina,* grass, cat and *Alternaria* to screen for atopic children. Furthermore, the results of another study, published by Kurukulaaratchy and colleagues in 2005, used a panel of 12 allergens including food allergens and found that the prevalence of atopy in children aged 10 was 26.9% [109]. Interestingly, the prevalence of atopy in the Humboldt Lung Study for 10-year olds was 28.2% (n=78). Thus, the prevalence of atopy in the 10-year olds in the Humboldt study approximated the prevalence of atopy in children from the study published by Kurukulaaratchy. All of the 1036 children that were skin-tested in the study published by Kurukulaaratchy who tested positive for food allergens were also aeroallergen positive at age 10 years[109]. This may indicate that not including food allergens in the panel of allergens tested in the Humboldt study did not significantly reduce the sensitivity of the test to detect atopics. But, there is the possibility that approximately 4-6% of atopics were missed due to the limited panel of allergens used in the study reflecting a sensitivity of 94-96% for the skin-prick test in this study. The analysis in the current study employed the FBAT to evaluate the possibility of an association with the TLR4 D299G mutation in atopic children in the study. Not being able to detect all of the atopic children in the study would have reduced the power of the test to some extent. It is difficult to predict by how much the power would have been reduced because the children that were misclassified as non-atopics would also have to have had TLR4 D299G heterozygous parents to be

included in the FBAT analysis. However, the power was high, 98.6%, and this is likely not a major concern. In addition, the power for the final logistic model may also have been reduced because this misclassification would bias any prospective associations towards the null.

More importantly, in this study the prevalence of atopy for 10 year olds (28.2%) and the prevalence of atopy 12-19 year olds (48.6%) was similar to the prevalence of atopy in 10 year olds in the study published by Kurukulaaratchy and colleagues[109] (26.9%) and was similar to the prevalence of 12-19 year olds reported by Ernst and Cormier (40.8% farming, 53.4% non-farming)[5]. The 7 year olds in Humboldt had a lower prevalence of atopy (16.5%) than the 7 year olds in the Canadian Childhood Asthma Primary Prevention Study (control 40.8%, intervention 53.4%)[105]. This is to be expected as the children in the Canadian Childhood Asthma Primary Prevention Study are from urban centers, Winnipeg and Vancouver, and these children are also high-risk for allergic diseases. The comparison with the teenagers in the Ernst and Cormier study indicated that this age group had a similar prevalence to that in other rural Canadian communities. However, the comparison with the study by Kurukulaaratchy and colleagues[109] indicated that the prevalence of atopy for 10 year olds was slightly higher (28.2%) than the 10 year olds in that study (26.9%) . Interestingly enough, the study by Kurukulaaratchy and colleagues[109] was of children from the Isle of Wight in the United Kingdom which had a lower population density (349 persons/ km^2) [110] than that of Humboldt (443 persons/ km^2) [111] and thus both areas can be defined as rural. Thus, the prevalence of atopy in 10 year olds in Humboldt was similar to the prevalence of atopy in 10 year olds from another rural area in the United Kingdom.

In summary, two major observations were made when the prevalence of atopy in children in the Humboldt study was compared to the prevalence of atopy for children in other studies. The first was that the panel of allergens selected had a sensitivity of at 94-96% and this may have reduced the power of the analyses. The second is that prevalence of atopy for 10 year olds and 12-19 year olds was similar to other rural communities indicating that these results are generalizable to other rural populations.

5.3 TLR4 Association with Atopy

Susceptibility genes for complex disease have been divided into three classes: major genes, oligogenes and polygenes[71]. In the past, the main research focus for most complex diseases has been on the search for major genes conferring susceptibility that are believed to be rare in number but, when influencing a particular complex disease, highly penetrant. The more penetrant a susceptibility gene is the more the observed phenotype can be accounted for by the differences among individuals with regards to this gene. Oligogenes are susceptibility genes that are thought to be in high frequency in the population, contribute a moderate risk to disease susceptibility and that may be accentuated by other risk factors. Polygenes are susceptibility genes that contribute only a small effect to the progression of the complex disease and as a result, are required in high number for a measurable effect. The following section comprises an evaluation of the results of the FBAT analysis of the TLR4 D299G mutation with atopy in children in this study and subsequent comparison to similar investigations with the mutation in the literature. It concludes with a discussion of some of the criteria that must be evaluated in order to assess the importance of a candidate gene association or lack thereof.

To begin, the frequency of the TLR4 D299G mutation was 7% in the Humboldt population under study. This frequency was comparable to that of another Canadian study, 10%, in French-Canadian families (n=167 families) from Saguenay-Lac-St-Jean, QB[92]. The French-Canadian families were ascertained via corticosteroid-dependent probands with asthma. In the Humboldt Study, using the FBAT to analyze the association of the TLR4 D299G mutation and atopy in 712 children who were genotyped and their parents the null hypothesis of no association in the presence of linkage could not be rejected. It is possible that the mutation is not associated with atopy or linked to a theoretical disease locus or loci that cause atopy.

Interestingly enough, this is not the first study to find no evidence to support an association for the TLR4 D299G mutation with asthma or atopy-related phenotypes. One group of researchers examined five common polymorphisms in TLR4, that included

the TLR4 D299G mutation, in two family-based cohorts and found no evidence of association using the TDT with any of the SNPs tested and asthma or atopy related phenotypes[92]. One of the cohorts, represented by 480 white family trios from the Childhood Asthma Management Program had a conditional power of at least 80% . The researchers ascertained nuclear families through asthma probands to analyze the influence of several TLR4 SNPs with allergic diseases including atopy. The second cohort in the study, composed of the 167 French Canadian families, had a conditional power of 50%. The current study also did not show an association for the TLR4 D299G mutation and atopy in the study population even though the power to detect such an association was high 98.6%.

In the current study, the TLR4 D299G mutation was investigated in order to test the hypothesis that the SNP was associated with atopy in children. The hypothesis was formulated based on the prediction that carriers of this SNP had a reduced response to endotoxin and this in turn would have resulted in a higher load of endotoxin being required to stimulate the non-allergic T_h1 environment. The FBAT analysis revealed that there was no association with the SNP and atopy in this population of children. However, when the TLR4 D299G mutation was initially characterized a gradient response to LPS was observed between individuals[7,8]. In other words, not all carriers of the SNP were hypo-responsive to LPS. Thus, it would be extremely unlikely that the TLR4 D299G mutation alone was causal for atopy or any allergic-disease. In those individuals who were carriers for the TLR4 D299G mutation and who were hypo-responsive to endotoxin it is possible that the SNP was in linkage disequilibrium with other loci that confer hypo-responsiveness to LPS that were not genotyped. In an extensive review of the literature of association studies for asthma and atopic diseases published in 2003, Hoffjan and colleagues did not include TLR4 in their list of candidate genes that had been associated with the diseases in at least one study[112]. In 2004, a study by Yang and colleagues found an association between the TLR4 D299G mutation and the severity score of atopy among asthmatics [91]. Yang and colleagues employed an atopy severity score in 336 British families with 2 or more affected siblings. But they only found this association in the first offspring and when they tested the association in

the second offspring it was not significant. They argued that this was likely due to the effect of birth order on the risk of atopy. In order to determine if this finding was spurious the analysis should repeated in a second group of families with 2 or more affected siblings with asthma.

In a broader sense, the study by Yang and colleagues of the TLR4 D299G mutation that found that the SNP may be a potential marker of atopy severity amongst asthmatics indicated that TLR4 may be one of many polygenes affecting an individual's atopic status. On the other hand, the complexity of the human genome adds another dimension. Loci that are close together on a chromosome are likely to be inherited together whereas loci that are farther apart on a chromosome have a greater chance of crossing-over occurring between them during meiosis. This means that in a given population, SNPs that are close together are more likely to be inherited together and this is referred to as linkage disequilibrium. It is for this reason that in order to completely study a gene in a given population several SNPs need to be studied [71]. Another issue that has not been considered in this study is the possibility of a gene-gene interaction. This would result in confounding because the studied phenotype would not be caused by one gene alone but the effect of interactions between two or more loci[98]. There was no way to investigate this in the current study as only a single SNP from a single gene was genotyped. Raby and colleagues characterized the coding region of the TLR4 gene in 3 ethnic populations: Black, White and Hispanic[92]. They found 5 rare variants of TLR4 among the three groups, but did not have enough power to analyze for association for these rare variants with asthma in their family cohorts (Childhood Asthma Management Program and Saguenay-Lac-St-Jean). Thus, the possibility still exists that one or more of these rare variants may be asthma-susceptibility variants but the power of a study to detect such an association if it exists is a limiting factor.

Alternatively, an underpowered study can result in a true positive association from another study not being replicated [74]. There are many other reasons that an association study is not replicated. It may be because the finding was a false-positive and it was subsequently not replicated. It also may be an association in one population but not in

another population because of heterogeneity in genetic or environmental background, population stratification [74]. In genetic association studies, genotyping error, missing data and misclassification of patients introduce noise thereby reducing the power of the study[74]. In fact, a non-differential misclassification bias of a dichotomous exposure, where the bias is equally likely to affect cases and controls, is known to bias the findings of a study towards the null [113]. The ultimate support of a genetic association study is the replication of the association with the same allele, phenotype and effect direction in an independent population sample [74].

Bias in genetic studies, particularly in family studies, may be introduced in two ways: genotyping errors and misclassification of individuals into pedigrees. Misclassifying individuals into pedigrees may be a result of sample management problems wherein samples are mixed-up in the laboratory performing genotyping or parents knowingly or unknowingly report false relationships. Genotyping errors in this study are likely to be non-differential between atopics and non-atopics and also among the different exposure groups. In studies measuring many genes and multiple SNPs genotyping errors and pedigree errors can be assessed to a greater degree. A limitation of the current study is that only one SNP was genotyped. With this in regard, parents transmit their alleles to their children according to Mendelian laws and the more SNPs that are genotyped in a family will lead to an increase in the likelihood that systematic genotyping errors and incorrect family assignment will be identified. On this note, every effort was made to correctly assign individuals to their families but it was done as a secondary analysis. The family members were linked using information regarding names and addresses of family members and ideally families should have been notified that they were being identified as such for genetic purposes. This would have given families the opportunity to correct any misclassifications and simplified the creation of the PID. Of the 299 families that FBAT analyzed there was one family with a Mendelian error. FBAT excluded the family from the analysis. The family in question consisted of two parents that were homozygous for the major allele whose offspring's genotype was heterozygous. There was no way to determine whether the Mendelian error was the result of a genotyping

error or the incorrect assignment of one of the three individuals to this family or a spontaneous mutation in a member of the family trio.

Collectively, the lack of association for the TLR4 D299G mutation and atopy in many studies and the range of responses to LPS in individuals that do carry the TLR4 D299G mutation seem to indicate that this SNP is not associated with atopy and TLR4 is not a major gene or even an oligogene for atopy. There is the possibility that TLR4 is a polygene in certain populations e.g. asthmatics. An important consideration is that there are many other candidate genes whose associations have been reproduced in various populations that are more likely to contribute to atopic status.

5.4 Collinearity between Farming and Environmental Exposures

In the Humboldt study, farming families should differ in comparison with non-farming families based on pets, humidity and total number of siblings. Farm exposures such as these have been repeatedly associated with a reduced risk of atopy, asthma and other allergic diseases [4-6,35,114-118]. Many of the exposures that are postulated to provide protection for atopy in the farm environment have been demonstrated to show protection in non-farming environments as well e.g. pets, large numbers of siblings. Thus, as an exploratory analysis of the variables parental education, number of siblings, mother's and father's smoking status, pets, humidity, parental history of allergic diseases and parental atopy in addition to age and sex distributions were compared among children with at least one parent that had farmed in the last five years compared with children whose parents had not.

A total of 309 of the 734 children in the study had had both parents participate. For this reason, it was for 309 children that parental occupation as farming or non-farming in the last five years could be determined. There were a total of 160 children from non-farming families and 149 children from farming families. Seventy-five percent of the children came from families in which multiple offspring had participated in the study and thus children could not be analyzed as individuals. This type of analysis would have weighted certain families more than others and biased the analysis. Farming families did

not differ significantly from non-farming families with respect to any of the variables tested parental education, number of siblings, mother's and father's smoking status, pets, humidity, parental history of allergic diseases and parental atopy in addition to age and sex distributions. This was surprising because a previous study demonstrated that farming children had parents with lower levels of education, had mothers that were less likely to smoke, had more siblings, had more indoor humidity and were more likely to have pet exposure than non-farming children [4]. In the present study, there were trends for farming families to be more likely to report humidity, more likely to report parental history of allergic diseases and to be less likely to have both parents test atopic. In order to obtain information on parental occupation to perform this analysis the number of eligible families was significantly reduced because not all families had both parents participate in the study and families that had multiple offspring were only counted once. Thus, the number of farm families was 68 and the number of non-farming families was 111. The power to detect a difference between the non-farming and farming families was low. Differences between the two types of families in the community may not have been observed because there were not many families in total. Other studies with larger samples sizes have found differences in exposures between farming and non-farming families. This may be due to these studies having had more power to detect such differences. For example, the study by Braun-Fahrlander and colleagues of children from three rural communities in Switzerland had 307 children from farming parents and 1313 children from non-farming parents[4]. In this study the researchers analyzed the characteristics associated with individual children and did not mention if some of the children came from the same families. There is a possibility that some of the children in their study were siblings especially for the farming children because they found that farming children had more siblings than non-farming children ($p<0.0001$). Thus, farming families may have been over-represented by the number of offspring in the study, which would have confounded the difference between the number of siblings farming and non-farming children had. On the one hand, analyzing families and not individuals in the Humboldt study reduced the sample size and the ability to detect a difference between farming and non-farming families if one existed. But on the other hand, families were not over-weighted if they had multiple offspring participate in the

study and this was an important consideration to maintain the internal validity of the analysis.

Another reason for the lack of differences found in this analysis is that a parental history of atopy, as opposed to other forms of allergic diseases, is more likely to correlate with a child's current atopic status after adjusting for age. Furthermore, in the study by Braun-Fahrlander and colleagues a protective effect of parental farming was found to be stronger when the farming involved livestock [4]. Several studies have associated a reduced risk of atopy with exposure to stable animals [3,35,40]. Table 4-9 represents univariate analyses on the 309 children with both parents in the study of the outcome atopy with the variables sex, age, parental atopy, parental occupation and parental work with livestock. There was a significant increase in the odds of being atopic with increasing age ($p<0.001$). This was expected given that prevalence of atopy is known to peak at around 30 years of age [103,104]. The prevalence of atopy did not differ significantly when females where compared to males. Children with parental history of atopy were not more likely to be atopic than those without any parental history of atopy ($p=0.21$). Children with at least one parent that farmed in the last five years were not more likely to have atopy in comparison with children whose parents did not farm ($p=0.66$). Children whose parents worked with livestock were not at a decreased risk of developing atopy in comparison with children whose parents did not work with livestock ($p=0.69$). In this part of the analysis, all 309 children were investigated without adjustment for those children that came from the same families. The variables age, sex and parental education were analyzed individually with the outcome to determine if they should be used to adjust the relationship between the outcome and parental atopy, parental occupation and parental work with livestock. In table 4-10, the crude OR did not differ to any significant extent from the OR adjusted for age, sex and parental education when the relationships between atopy and the variables parental atopy, parental occupation and parental work with livestock were examined. Although, age, sex and parental education are traditional confounders in epidemiological studies they did not significantly alter the relationship between atopy in children in Humboldt and parental atopy or parental occupation or parental work with livestock.

The results thus far have indicated that in Humboldt, SK farming families do not appear to differ from non-farming families with regards to parental education, number of children, parental smoking status, pets, and humidity. This analysis indicated that in Humboldt the farming and non-farming families are fairly homogeneous with regards to these exposures. But, other studies have been able to detect differences in these exposures between farming and non-farming individuals in rural environments [3,4,6,35,119,120]. Most of these studies have been conducted in European countries: Austria, Germany, Switzerland, Denmark and Finland. There must be some key characteristic or characteristics in these rural environments that differ from Humboldt. Some insight to this effect can be gained from an Australian study that reported a protective effect for atopy between children that had lived on a farm compared with those that had not in one rural community, Wagga Wagga, but not in another rural community, Moree [35]. The researchers attributed the protective effect in Wagga Wagga to be the result of this community having more livestock farms than Moree. Thus, it is possible that the missing element for a gradient of exposure in Humboldt was contact with livestock and this may be a proxy for high levels of exposure. However, the possibility that there was a protective effect associated with parental work with livestock was investigated in the present study of 309 children with both parents in the study (Tables 4-9 and 4-10). Parental work with livestock did not appear to affect atopic status for children in this study. One of the assumptions for investigating the farming environment in Humboldt was that farms in Humboldt are basically the same as farms in Europe. However, in Austria, Netherlands, Germany and Switzerland farm homes are more likely to have the barns attached to living quarters [38]. In Saskatchewan having the barn attached to the home is a rarity. The proximity of the barn is reflected in the patterns of exposure. High levels of endotoxin have been found in stables and confinement buildings [36,37]. Thus, it is possible that the previous studies of farming exposure being protective for atopy are confounded by the level of endotoxin to which individuals are exposed in their homes. This may be a reason why in the Humboldt Study no association was seen for parental occupation and atopy in the 304 children with both parents in the study. Furthermore, rural families in Humboldt did not differ significantly from farm families as expected on

many characteristics. However, farm families and rural families in Humboldt may still differ significantly from urban families on these characteristics. In order to assess this comparison a suitable subset of urban families would have had to participate in the study in order to assess the generalizability of these findings.

5.5 Model Building and Selection

The reduced model in Table 4-14 was selected to model atopy in this population of children. There were 734 children aged 5 to 19 who participated in the study. From Table 4-11, the univariable analysis indicated that older children in the study were more likely to be atopic. It has been previously reported that the prevalence of atopy peaks near the 3rd decade of life [103,104] and a gradient in the prevalence of atopy in children aged 5-19 years of age should be expected. From the crude analysis, there appeared to be no difference in age, the number of siblings, smoking exposures, pets, humidity, parental history of allergic diseases or farming between atopic and non-atopic children in the study (Table 4-11). Three models were considered in this analysis: the full model based on established risk factors (Table 4-12), the full model using $p<0.25$ for inclusion criteria (Table 4-13) and the reduced model (Table 4-14). The full model based on established risk factors was considered in order to analyze the combined contribution from covariables that had potential clinical significance. However, the previous analyses thus far in this population of children have indicated that factors that have been associated with atopy in other studies may not be suitable for this population of children. There appeared to be homogeneity between farming and non-farming families with regards to total number of siblings, smoking, pets, humidity and parental history of allergic diseases. For this reason, it was possible to consider farming as a variable independently in the model.

In this analysis the farming variable differed from that used to classify farming and non-farming families. In order to maximize the sample size for this analysis, all children were included in the analysis. As a result, information on parental occupation was only available for the 309 children with both parents in the study and for 113 additional children that had one parent participate in the study. The remaining 312 children did not

have either parent participate in the study and it was impossible to ascertain any parental occupation. For this reason, the farming variable was created that encompassed responses to whether a child currently lived on a farm, had lived on a farm during the first year of life and to farm activities. In addition for those 422 children who had at least one parent participate in the study the information on parental occupation as farming was also used to compute this variable. This varied from other studies that had seen a protective effect with farming. Much of the literature on farming as a protective lifestyle for atopy has been published independently by groups from Austria [118] and Switzerland [4] but also collaboratively with groups from Sweden and Germany [3,37,38,40,119]. Many of these studies do not specifically define how they determined that adults, in particular, were farmers. One Finnish study defined the children of farmers by if their fathers were farmers [117]. Realistically, an adult could be a farmer if they own a farm, live and work on a farm or work on a farm but do not live there. An adult that owns a farm may never or hardly visit whereas an adult that lives on a farm and works on a farm is likely to have considerable more exposure. In Saskatchewan, for many the family farm is often no longer able to generate sufficient funds to support the family and adults may spend a good portion of their time working elsewhere in a non-farming environment. It is likely that traditional questions regarding farming such as "Do you live on a farm?" or "Do you own a farm?" no longer capture farming exposures for adults and this may translate to their children's exposure as well. In Saskatchewan, individuals with a variety of exposures to the farm would answer yes to "Are you a farmer?" Thus, there are limitations to the farming variable used in this analysis. When it was included in the full model in Table 4-13 it did contribute significantly to the model. Holistically, given the discussed limitations with the variable, the apparent homogeneity in this study population with regards to exposures that are typically attributed to be part of the farm environment and the lack of statistical significance the variable farming was not included in the final model in Table 4-14.

In similar manner, there was no association seen with the variable pets. The pet variable was obtained from the responses to the questions, "Do you have a dog or cat or other pet living inside your home?" and "During this child's lifetime, have you had a dog, cat or

bird living in your home?" The lack of association with pets and atopy may be because the variable does not adequately assess a child's contact with pets. A study by Braun-Farhlander and colleagues found that farmer's children were more likely to have furred pets and pets in the bedroom in addition to a lower prevalence of atopy in comparison with the children of non-farmers [4]. Another European study did not find any association with atopy and having a cat or dog in the first year of life [40]. Many of the characteristics of this study mirrored the current Humboldt study because it included the children of farmers and the children of non-farmers in the same rural communities in Austria, Germany and Switzerland. The researchers found a significantly reduced association with IgE to grass in those children that had been exposed to a cat or dog in the first year of life. There was also a reduced association of IgE to cat in children that had had a dog in the first year of life but curiously not with cats. Another study found a lower prevalence of asthma and allergic rhinitis in children that had had a cat in the first year of life [42]. In this study a child was considered pet exposed only if a cat or dog had been kept inside the home. The researchers did not find any difference in the prevalence of atopy between children that had had pets in the first year of life and those that had not. They did see a lower prevalence of sensitization to cat in those children that had had a cat in the first year of life. In this study, researchers asked the parents of those children if they had not a pet in the first year of life why they had not. If the response to this question was due to the presence of allergy in the family these children were excluded from the analysis comparing exposure to pets with allergic diseases. This may have biased their results towards the null because there was a higher proportion of children with a familial history of allergic diseases in the exposed group. In the current study, the pet variable encompassed current and previous exposures to pets and the study by Hesselmar and colleagues indicated that exposure to pets early in life may be the key to reducing allergic diseases. It has been suggested that early life exposures may induce tolerance in individuals that would otherwise be susceptible to allergy. However, the timeframe that early life encompasses is difficult to pinpoint. Many studies refer to the first year of life but the window for inducing tolerance to allergens may be much larger. The underlying mechanism for tolerance is under debate. It has been postulated that some non-atopic individuals may produce IgG_4 to allergens instead of IgE as part of a

modified T_h2 response [121]. However, many non-atopic individuals do not have detectable antibodies to the allergens they are being tested indicating that there may be many mechanisms for inducing tolerance [121]. The presence of pets inside the home has been associated with higher endotoxin levels [10]. Thus, it is possible that associations in the literature that refer to the protective effect of a pet indoors in early life may in fact be confounded by higher levels of endotoxin in homes that have these pets that in turn may deviate the allergic immune response to a non-allergic response i.e. the hygiene hypothesis. In summary, there was no association with pets and atopy in this study of children however, this may be a result of the homogeneity in exposure to pets in this rural community or that pets in the first year of life rather than current and/ or previous pet ownership was the more appropriate question to assess the effect of this exposure on atopy. As a result, the pet variable was not included in the final model in Table 4-14.

Furthermore, there was no association with smoking and atopy in this study. However, the current smoking variable accounted for both passive smoking exposure to environmental tobacco smoke from family and friends in addition to active smoking exposure in children that were older than 13 years of age. It is possible that this variable did not truly capture the exposure to environmental tobacco smoke for each child. Smoking in general has not been associated with atopy in children [54,56]. Smoking is frequently adjusted for in epidemiological studies but analysis of this variable with outcome atopy in this study did not indicate that such adjustments were necessary nor does the literature indicate that smoking is a risk factor for atopy. As such, smoking was not included in the final model in Table 4-15.

In like manner, humidity was not associated with atopy in this study. In a previous association, indoor humidity was found to be higher in farming homes as opposed to non-farming homes [4]. Thus, the lack of association in this study may be a result of the homogeneity in exposure between the homes in this community. High levels of humidity are associated with increased levels of house-dust mite[122]. A more objective measure of humidity would be to measure the indoor humidity inside homes or to measure the level of house dust mite allergen as opposed to the question, "Are there any

visible signs of mold or dampness in your home?" Also, this question refers to the current home and some individuals may have only lived in their current home for a short period of time although, this likely did not represent a great proportion of the Humboldt community. In short, the variable humidity was not associated with atopy and was not included in the final model in Table 4-14 nor did it account for a significant proportion of the variance in the model when it was included (Table 4-13).

In order to discuss the variable total number of sibling it is important to recall that Strachan formulated the hygiene hypothesis after observing a protective effect of family size and position in the birth order on the prevalence of hay fever[2]. Thus, the variable total number of siblings was investigated in this study. Surprisingly, no association was found with this variable and atopy. This association has also been demonstrated with hay fever and adults, 20-44 years, in the European Community Respiratory Health Survey [29]. There are two potential explanations for the lack of association with family size in this study and atopy. The first being that atopy is a different allergic disease than hay fever and the protective effect of family size may only be important for hay fever. The second is that both Strachan's study and the European Community Respiratory Health Survey were significantly larger than the Humboldt study, 17,414 and 13, 932 respectively, compared with our sample size of 734 children. In Table 4-11, non-atopics are more likely to be only children (6.3%) in comparison with non-atopics (4.0%) but for this difference to have achieved statistical significance a much larger sample would be required. The European Community Respiratory Health Survey also investigated daycare attendance, which was not investigated in the Humboldt study. If the original observation made by Strachan is plausible then only children who attend daycare may have comparable exposures to children with numerous siblings further diluting an effect that may have been attributable to family size. The variable total number of siblings did have p-values less than 0.25 for children with three or more siblings and as a result was eligible for inclusion in the both full models (Table 4-12 and Table 4-13). However, the variable did not significantly contribute to either model and in an attempt to achieve the most parsimonious model it was not included in the final model in Table 4-14.

The variable parental history of allergic diseases was included in all models including the final model (Table 4-12, Table 4-13, Table 4-14). There is a large amount of evidence supporting a hereditary role for atopy and allergic diseases in general[64,65,67,68]. The variable used to test the association with atopy in the 734 children in the study was composed of self-reported responses from parents on asthma, eczema, rhinitis and allergy. The more objective variable atopic status of parents was only available in parents that had participated in the study and thus, not available for the final analysis. Regardless, there was mild tendency for atopic children to have parents that reported a history of allergic diseases at the univariable level ($p=0.168$; Table 4-11), in the full model ($p=0.06$; Table 4-12), in the model including all variables with a univariable association of $p<0.25$ ($p=0.06$; Table 4-13) and in the final model ($p=0.06$; Table 4-14). Interactions for this variable with pets, humidity, total number of siblings and farming were investigated but none of the interactions were significant in any of the three models. Atopy is a complex disease and as a result, is likely to be under the influence of many gene-environment interactions. However, both the variable parental history of allergic diseases and the homogeneity in environmental exposures among children in the study may be diluting the effect of any gene-environmental interactions. More specifically, if there had been an association with the TLR4 D299G mutation in this population of families this would have been the ideal opportunity to assess gene (TLR4 D299G) interactions with environmental covariables. In this situation, the ideal environmental covariable would have been a measure of endotoxin level. As such, parental history of allergic diseases was included in the final reduced model along with the confounders age and sex (Table 4-14).

In summary, the final model, which was the reduced model in Table 4-14, included the variables age, sex and parental history of allergic diseases. This model was the most parsimonious model that explained atopy in this population of children. The major hypothesis of this study was that there would be a significant interaction between parental history of allergic diseases and one or more of the environmental predictors. This was not evident from this analysis although the hypothesis that a gene-environment interaction exists still cannot be negated. The homogeneity of environmental exposures

in the study made studying a gene-environment interaction difficult because a larger sample size would have been necessary to detect a statistically significant association. In addition, an objective measure of atopy, such as skin-prick testing results from the parents, would have been more representative of the heritability of atopy.

5.6 Future Directions

One of the major limitations of this study was that only a single SNP from a single gene was investigated. The evidence from the literature on the TLR4 gene at best has indicated that it may have some influence on the severity of atopy among asthmatics. As a result, TLR4 is likely to be one of thousands of genes that may affect the severity of allergic diseases such as asthma.

Genome scans comparing linkage peaks among individuals with allergic diseases such as asthma and atopy with unaffected control groups have consistently linked the locus 5q31 with allergic diseases [123-125]. An extensive review of association studies for asthma and atopic diseases did not include TLR4 or its locus 9q32-33 [112]. It did include total of 7 genes from 5q31. If the ultimate support of a genetic association is reproducibility of the effect in the same direction in another population then consistently reporting no association for a gene for the same effect in multiple populations may be the ultimate discredit of such potential associations. It must be mentioned however, that there may be some plausibility to investigating TLR4 SNPs and their interaction with longitudinal exposure to endotoxin for their influence on atopy.

In addition to being severely limited by the number of SNPs genotyped for TLR4, the environmental variables were ascertained from questions formulated by other researchers that likely had different hypotheses in mind. This is one of the major limitations of using a dataset for a secondary analysis. However, from the graduate student's perspective the major advantage of using this dataset for a secondary analysis was that it allowed for the timely completion of the analysis. The study was cross-sectional in design and most variables were retrospective in ascertainment and this may have limited the accuracy of the exposure variables. Retrospective studies are often

limited by recall bias usually where cases of a disease are more likely to recall an exposure than controls[113]. However, in this study the "disease" under investigation was atopy and atopics may be asymptomatic or manifest other forms of allergic diseases such as asthma or rhinitis. The variables total number of siblings, smoking, pets, farming and humidity are probably not subject to differential bias between atopics and non-atopics because their influence on the phenotype status is not obvious to the general public such as smoking would be in a lung cancer study. Another issue with exposure ascertainment in a cross-sectional study is that many variables assess current exposure conditions that may not be etiologically relevant to the phenotype[101]. For the variables pets and humidity, it may be that with time the current exposure would not change. For example, pets, especially dogs and cats, live for considerable periods of time and families with these types of pets are likely to have had them for some time. In like manner for the variable humidity, the presence of humidity via visible molds and dampness was likely to have been present the entire time an individual lived in their current home and in Humboldt individuals on average stay in their current homes for some time. However, the total number of siblings may have fallen caveat to this issue related to the cross-sectional design of the study. If there were an etiologically relevant window where having numerous siblings was protective the current number of siblings would not be a valid indicator. The number of siblings during this window would have to be ascertained in order to assess the relevance of this exposure. There is also the issue of assigning exposure variables from the parents to that of the children. Paternal smoking exposure for example implies that the child spends a considerable amount of time with their biological father, which is not always the case as the father may elect to smoke outside. On the other hand, in order to adequately capture the potential effect of many of the exposures a longitudinal design would have been necessary. Also, the prevalence of atopy is confounded by age with the peak in prevalence around the age of 30. Although age was adjusted in the final model, an ideal study would have been a prospective longitudinal cohort, specifically a birth cohort, in attempt to capture the etiologically relevant window for atopy. However, this design has its limitations in that the cost is high and maintaining a high response-rate throughout the study is difficult. But, the gain

in power to detect associations is monumental as it was demonstrated by Strachan's original study that stimulated the hygiene hypothesis[2].

Finally, under the null hypothesis of linkage and no association the TLR4 D299G mutation was not associated with atopy in children using a family-based analysis. The outcome atopy in children in Humboldt who participated in the study was modeled with age, sex and parental history of allergic diseases. In order to fully explore the relationship with TLR4 and atopy in this population future effort should be directed towards a prospective longitudinal cohort design prospectively monitoring environmental covariables such as pets, family size, day care attendance and others not considered such as vaccination rates and the frequency of infections in early life. In addition, phenotyping the parents of children for atopy in the study and genotyping a larger panel of genes with more SNPs are important recommendations for evaluating the heritability of atopy in this population. Monitoring the actual load of endotoxin in the homes of children and other markers for bacterial challenge would provide a direct measure that may provide more information into the relationship between many of the covariables and atopy. The current analysis of atopy and children in Humboldt would be strengthened by a comparison with an urban population of children.

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Appendix A: Glossary of genetic terms

allele	One of the possible versions of a gene.
haplotype	Set of alleles that are inherited together.
heterozygous	An individual with two different alleles at a particular loci.
homozygous	An individual with two identical alleles at a particular loci.
locus/ loci	The location(s) on a chromosome.
major allele	The allele that has the greatest frequency in the population.
minor allele	The allelle that has the lowest frequency in the population.
SNP	Genetic mutation that is present in a population at a frequency of at least 5%.
wildtype allele	See major allele.

Appendix B: Copy of the Consent Forms

Humboldt Lung Study 2003 – 2004
Children and Youth (6 to 17 years) Survey

January 2004

Dear Parent or Guardian,

In 1993, we conducted a study of the respiratory health of children, youth and adults in Humboldt. As you may be aware, we are conducting a similar study now with Humboldt and area residents who are 6 to 79 years of age. Today, we are distributing the questionnaire about children's respiratory health. We also would like to measure lung function, obtain some information about how respiratory disease is inherited and examine allergies to certain allergens in the air that can affect children's breathing. This study is being done to learn more about the respiratory health of school age children and has the co-operation of your local school board.

All students 6 years and older will receive a questionnaire. A separate questionnaire should be completed for each child. The questionnaire takes about 15 minutes to answer and should be filled out by the parent most familiar with the child's health. Please read and follow the instructions on the first page. **When you have completed the questionnaire and the consent for measurements, please seal these in the envelope supplied and return them to the child's school.** We will collect all of the questionnaires from the schools.

As this study is meant to help us find our why some of us get certain respiratory conditions and why others do not, we will need to conduct some very special tests that are important for this study. We ask that you please consider our request. All testing will be done in the school setting by trained research assistants.

We will measure height, weight and blood pressure. As well, we will test your child's breathing with a spirometer that requires them to blow into a tube. Your child will have a few tries at this and will be coached by the nurse conducting the test. The breathing test will require some effort to blow air out of the lungs and may make your child cough during the test. To look at the inherited (genetic) characteristics of respiratory disease, we will need to swab the inside cheeks of your child's mouth for a sample of the mouth lining using Q-tips. The swabbing of the inside of the cheek is not uncomfortable. This sample will be kept by the University for 15 years and will be destroyed after that time.

We also would also like to find out if you child is allergic to four common allergens we breathe in. For this test we will place 6 droplets on the arm and lightly scratch each droplet. We will wait for 15 minutes to look at the droplets and measure any redness. Children may experience some itchiness or redness with the allergy testing.

We would appreciate participation in all testing, but if for some reason that is not possible, you can choose for your child to participate in certain tests only. At the time of the breathing test, students 12 years or older will also be asked to complete a short lifestyle questionnaire about television use, eating habits, physical fitness, smoking and alcohol use. *(Over)*

Institute of Agricultural Rural and Environmental Health (I.ARE.H)
Centre for Agricultural Medicine, University of Saskatchewan
103 Hospital Drive, Saskatoon SK S7N 0W8 Telephone: (306) 966-8286 Facsimile: (306) 966-8799

There are no direct benefits to you or your child for participating, although findings could benefit the future health of others. If you have any concerns or need more information, please call us at 1-306-966-7886 or you can leave a message at your child's school. We will return your call. If you have any questions about your rights as a research subject or concerns about your experiences while participating in this study you should contact the Chair of the Biomedical Research Ethics Board, C/o the Office of Research Services, and University of Saskatchewan at (306) 966-4053.

All personal information will be kept strictly confidential and used only for this research. The part of the questionnaire with your child's name or other information that could identify your child will be kept separate from your other answers and will be held in a secure place by the principal investigator. No information that could identify your child or family will be used when we report the results. Your answers will be combined with the answers from other parents.

Your participation in this study is free and voluntary and will be very helpful in understanding the respiratory health of other children living in Saskatchewan. If for some reason you decide not to be part of this study, it will not compromise you or your child's relationship with your school or health care. If you cannot participate, we ask that you kindly return the questionnaire to the school. Please keep this letter for future reference.

Thank you for your cooperation.

Sincerely,

Donna Rennie

Dr. Donna C. Rennie
Coordinator Humboldt Study
Associate Professor
College of Nursing and Institute for Agriculture, Rural and Environmental Health
University of Saskatchewan
Ph: 1-306-966-7886
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boldt Lung Study (Children 6 – 15yrs)
Consent for Health Measurements
Confidential When Completed

PERMISSION to PARTICIPATE:

This study will look at the respiratory health of children and those things around them that can affect it. The study is being done by Dr. Dosman and Associates at the University of Saskatchewan. I understand that as part of this study the researchers will need to conduct assessments on my child (*First and Last Name)*_____. I give permission for my child to participate in this health survey. I understand that this will involve measurements of my child's height, waist, weight, and blood pressure. It will involve a simple test of breathing that will measure how much air my child can blow out in a single breath. It will also involve swabbing of the inside cheeks of the mouth for the study of how respiratory diseases are inherited. This will involve carefully taking swabs from the inside cheeks of the mouth. This is not uncomfortable.

If agreed to, the health assessment will also include skin testing for allergies. The skin testing will take place in the school by a qualified registered nurse. In the skin testing procedure, 6 small drops of liquids containing allergen material will be placed on my child's arm. The surface of the skin underneath each drop will be scratched lightly. This procedure may cause some local itching at the scratched sites for some children. The itchiness will disappear within 1 hour following the test. We will keep all children at the test site until the itchiness is gone. A copy of the skin testing findings will be provided upon request once all study information is collected.

All information from this health assessment will be used for research purposes only and will be grouped with the information from other children. I understand that my child can refuse to participate at any time in any part of the study measurements and will have a chance to ask questions before the measurements are done. The researchers respect the decision by the child to participate or not. I have explained this permission slip to my child.

Please identify the assessments your child can participate in (Circle **ALL** that apply):

Blood Pressure	**Yes**	**No**
Lung Function (includes height, weight and hip measurements)	**Yes**	**No**
Cheek Swabs	**Yes**	**No**
Skin Testing	**Yes**	**No**

I have explained this consent to my child and he or she can participate in testing as circled.

_____
Signature of Parent or Guardian

_____
Signature of Child

_____
Signature of Research Assistant

Date _____

Parent's Name (*Please Print Below*) Address

_____

If you have any concerns about the health assessment, please contact:
Dr. J. Dosman, College of Medicine, University of Saskatchewan Phone: 1-306-966-8286. If you have any questions about your rights as a research subject or concerns about your experiences while participating in this study you should contact the Chair of the Biomedical Research Ethics Board, C/o the Office of Research Services, University of Saskatchewan at (306) 966-4053.

Humboldt Lung Study 2003 – 2004
Children and Youth (12 to 17 years) Survey

February 2004

Dear Parent or Guardian,

In 1993, we conducted a study of the respiratory health of children, youth and adults in Humboldt. As you may be aware, we are conducting a similar study now with Humboldt and area residents who are 6 to 79 years of age. Today, we are distributing the questionnaire about children's (6 to 17 years) respiratory health. We also would like to measure lung function, obtain some information about how respiratory disease is inherited and examine allergies to certain allergens in the air that can affect children's breathing. This study is being done to learn more about the respiratory health of school age children and has the co-operation of your local school board.

All students 6 years and older will receive a questionnaire. A separate questionnaire should be completed for each student. The questionnaire takes about 15 minutes to answer and should be filled out by the parent most familiar with the student's health. Please read and follow the instructions on the first page. **When you have completed the questionnaire and the consent for measurements, please seal these in the envelope supplied and return them to the student's school.** We will collect all of the questionnaires from the schools.

As this study is meant to help us find our why some of us get certain respiratory conditions and why others do not, we will need to conduct some very special tests that are important for this study. We ask that you please consider our request. All testing will be done in the school setting by trained research assistants.

We will measure height, weight and blood pressure. As well, we will test your child's breathing with a spirometer that requires them to blow into a tube. Your child will have a few tries at this and will be coached by the nurse conducting the test. The breathing test will require some effort to blow air out of the lungs and may make your child cough during the test. To look at the inherited (genetic) characteristics of respiratory disease, we will need to swab the inside cheeks of your child's mouth for a sample of the mouth lining using Q-tips. The swabbing of the inside of the cheek is not uncomfortable. This sample will be kept by the University for 15 years and will be destroyed after that time.

We also would also like to find out if you child is allergic to four common allergens we breathe in. For this test we will place 6 droplets on the arm and lightly scratch each droplet. We will wait for 15 minutes to look at the droplets and measure any redness. Students may experience some itchiness or redness with the allergy testing.

We would appreciate participation in all testing, but if for some reason that is not possible, you

(*over*)

Institute of Agricultural Rural and Environmental Health (I.ARE.H)
Centre for Agricultural Medicine, University of Saskatchewan
103 Hospital Drive, Saskatoon SK S7N 0W8 Telephone: (306) 966-8286 Facsimile: (306) 966-8799

can choose for your child to participate in certain tests only. At the time of the breathing test, students 12 years or older will also be asked to complete a short lifestyle questionnaire about television use, eating habits, physical fitness, smoking and alcohol use.

There are no direct benefits to you or your child for participating, although findings could benefit the future health of others. If you have any concerns or need more information, please call us at 1-306-966-7886 or you can leave a message at your child's school. We will return your call. If you have any questions about your rights as a research subject or concerns about your experiences while participating in this study you should contact the Chair of the Biomedical Research Ethics Board, C/o the Office of Research Services, and University of Saskatchewan at (306) 966-4053.

All personal information will be kept strictly confidential and used only for this research. The part of the questionnaire with your child's name or other information that could identify your child will be kept separate from your other answers and will be held in a secure place by the principal investigator. No information that could identify your child or family will be used when we report the results. All answers will be combined with the answers for other students.

Your participation in this study is free and voluntary and will be very helpful in understanding the respiratory health of other children living in Saskatchewan. If for some reason you decide not to be part of this study, it will not compromise you or your child's relationship with your school or health care. If you cannot participate, we ask that you kindly return the questionnaire to the school. Please keep this letter for future reference.

Thank you for your cooperation.

Sincerely,

Donna Rennie

Dr. Donna C. Rennie
Coordinator Humboldt Study
Associate Professor
College of Nursing and Institute for Agriculture, Rural and Environmental Health
University of Saskatchewan
Ph: 1-306-966-7886
Fax: 1-306-966-8799
Email: rennied@sask.usask.ca

Joint Lung Study (Students 6 to 17 yrs)

Consent for Health Measurements

Confidential When Completed

PERMISSION to PARTICIPATE:

This study will look at the respiratory health of children and those things around them that can affect it. The study is being done by Dr. Dosman and Associates at the University of Saskatchewan. I understand that as part of this study the researchers will need to conduct assessments on my child _____ *(First and Last Name of Child)*. I give permission for my child to participate in this health survey. I understand that this will involve measurements of my child's height, waist, weight, and blood pressure. It will involve a simple test of breathing that will measure how much air my child can blow out in a single breath. It will also involve swabbing of the inside cheeks of the mouth for the study of how respiratory diseases are inherited. This will involve carefully taking swabs from the inside cheeks of the mouth. This is not uncomfortable.

If agreed to, the health assessment will also include skin testing for allergies. The skin testing will take place in the school by a qualified registered nurse. In the skin testing procedure, 6 small drops of liquids containing allergen material will be placed on my child's arm. The surface of the skin underneath each drop will be scratched lightly. This procedure may cause some local itching at the scratched sites for some children. The itchiness will disappear within 1 hour following the test. We will keep all children at the test site until the itchiness is gone. A copy of the skin testing findings will be provided upon request once all study information is collected.

All information from this health assessment will be used for research purposes only and will be grouped with the information from other children. I understand that my child can refuse to participate at any time in any part of the study measurements and will have a chance to ask questions before the measurements are done. The researchers respect the decision by the child to participate or not. I have explained this permission slip to my child.

Please identify the assessments your child can participate in (Circle ALL that apply):

Blood Pressure	**Yes**	**No**
Lung Function (includes height, weight and hip measurements)	**Yes**	**No**
Cheek Swabs	**Yes**	**No**
Skin Testing	**Yes**	**No**

I have explained this consent to my child and he or she can participate in testing as circled.

_____

Signature of Parent or Guardian

_____

Signature of Student

_____

Signature of Research Assistant

Date _____

Parent's Name (*Please Print Below*) Address

_____

If you have any concerns about the health assessment, please contact:

Dr. J. Dosman, College of Medicine, University of Saskatchewan Phone: 1-306-966-8286. If you have any questions about your rights as a research subject or concerns about your experiences while participating in this study you should contact the Chair of the Biomedical Research Ethics Board, C/o the Office of Research Services, University of Saskatchewan at (306) 966-4053.

LETTER OF CONSENT FOR PARTICIPATION

Exposure to Endotoxin and the Lung – Common Measures

INSTITUTE OF AGRICULTURAL RURAL AND ENVIRONMENTAL HEALTH
UNIVERSITY OF SASKATCHEWAN

STUDY PURPOSE

The purpose of this study is to help determine whether lung function is related both to inherited (genetic) characteristics and to substances breathed in the environment, including endotoxins.

This project is being conducted by the Institute of Agricultural Rural and Environmental Health (I.ARE.H), University of Saskatchewan and is funded by the Canadian Institutes of Health Research. The nature of the study, risks, discomfort, and other information about the study are discussed below. Please feel free to ask any questions that you may have.

Endotoxin: A substance produced by bacteria and present in the dust.

CONFIDENTIALITY

A record of your participation in this research will be maintained, but this record will be kept confidential through the use of coded numbers. Your participation and the results of the research will not appear in any medical record and we will not communicate any individual results to any third party. Information identifying the personal source of these data will be kept separately and in a locked file by Dr. James A. Dosman, principal investigator, at the Institute for Agricultural Rural and Environmental Health of the University of Saskatchewan. The coding will be done so results of these tests cannot be linked to a specific individual. No information that can identify you or your family will be used when we report the results. Information from your tests will be combined with information from the tests of other participants.

The questionnaire and breathing test results will be kept on file at the Institute of Agricultural Rural and Environmental Health at the University of Saskatchewan. Your samples (blood/cheek) will be used only for the purpose of this research project and will be under the responsibility of Dr. James A. Dosman of The Institute of Agricultural Rural and Environmental Health, University of Saskatchewan. All the genetic samples will be destroyed on or by June 30th 2013.

YOUR RIGHTS

You have the right to withdraw from this study at any point without affecting the health care that would normally be provided to you. At anytime, you could request that we have no further contact with you, remove your personal identifiers (name, address) from the database or registry, stop testing your sample for its association with lung function, and destroy any genetic material we have obtained from you.

The intent of this project is to examine how genes may contribute to lung health or response to the environment. The results could lead to the development of commercial products from which you would receive no financial benefit. **The risks for participation in the study**

are as described with each test below. There are no direct benefits to you for participating, although findings could benefit the future health of others.

1. PROCEDURES FOR QUESTIONNAIRE, BREATHING TEST AND BLOOD PRESSURE

I agree:

- To complete an interview and a questionnaire about my health and exposure history. Questions will be asked about personal and family health history, smoking history, occupational and environmental exposures and other factors that influence lung dysfunction.
- To have my height, weight, waist and blood pressure measured.
- To have my lung function evaluated by blowing out air into a disposable mouthpiece connected by tubing to a device (spirometer) that measures how fast and how much air i can blow out. This test is a common screening test that may cause mild temporary discomfort such as dizziness, coughing, and mild shortness of breath for a few seconds following the test. In the event that I become ill as a result of participating in the course of this procedure, necessary medical treatment will be made available at no cost to me. By signing this document my legal rights are not waived.

2. PROCEDURES FOR COLLECTING GENETIC MATERIAL

I agree:

- To provide a blood sample *or have my cheeks swabbed* (circle appropriate test) to obtain genetic material. Blood will be drawn by a trained technician or registered nurse and there is a small risk of bruising and a remote risk of fainting and/or infection. *If cheek swab is taken, an applicator will be rotated on the inner surface of the cheek and there is a slight possibility that it may cause a small amount of bleeding.* In the event that I become ill as a result of participating in the course of this procedure, necessary medical treatment will be made available at no cost to me. By signing this document my legal rights are not waived.

Analysis of the genetic material will be limited to identifying those genes involved in aspects of lung function that are associated with substances breathed in the environment. The analyses will be performed at the University of Saskatchewan and at such other Universities as the University of Saskatchewan may utilise for laboratory testing.

3. PROCEDURES FOR ALLERGY TESTING

1 agree:

To have allergy skin testing to common allergens. Associated with lung function. In the skin testing procedure, 6 small drops of liquids containing allergen material will be placed on my arm. The surface of the skin underneath each drop will be scratched lightly. This procedure may cause some local itching at the scratched sites for some people. The itchiness will disappear within the hour following the test. To have my blood sample evaluated for blood allergy levels.

PARTICIPATION

"I, <name>, volunteer to participate in this study. The purpose of this study, procedures to be followed, risks and benefits have been explained to me. I have been allowed to ask any questions I have, and all of my questions have been answered to my satisfaction. I have been told whom to contact if I have additional questions. I understand that I may refuse to participate or withdraw from this study at any time, for any reason, and that my decision will have no consequences to me. I have been informed that all the information that I provide will be kept confidential, and that any research will not use my name or any other personal identifiers. I have read this consent form and consent to be a study participant. I have received a copy of this consent form."

1. CONSENT FOR QUESTIONNAIRE, BREATHING TEST AND BLOOD PRESSURE

(Circle correct response) **Yes** **No**

Signature of Subject Date

2. CONSENT FOR COLLECTING GENETIC MATERIAL

(Circle correct response) **Yes** **No**

Signature of Subject Date

3. CONSENT FOR ALLERGY TESTING

(Circle correct response) Yes No

Signature of Subject Date

Signature of Witness Date

Progress on the project will be posted on our website http://iareh.usask.ca once a year.

If you have questions later, you can contact the following persons at the I.ARE.H:
Dr. Jim Dosman, Principal investigator, (306) 966-8292, dosman@sask.usask.ca
Dr. Donna Rennie, Humbolt Study (306) 966-7886, rennied@sask.usask.ca
Ms. Liliane Chénard, Swine Study (306) 966-6645, chenard@sask.usask.ca
Fax: (306) 966-8799

Mailing address:
I.ARE.H
P.O. Box 120,
Royal University Hospital,
103 Hospital Drive,
Saskatoon, SK, S7N 0W8

Appendix C: Copy of the questionnaires

Confidential when complete

Questionnaire for the Fourth Humboldt Survey 2003-2004
Elementary School

This questionnaire can be answered by checking the best answer or by filling in a blank with a number or word(s).

Example 1:

Does this child usually have a cough?
No ___ Yes ✔

Example 2:

How long has this child lived in the current residence? Years 6

❖ - ❖ - ❖ - ❖ - ❖

FOR OFFICE USE:

Personal I.D.: _____

Family I.D.: _____

Child's name:

_____
Last First

Tel. No.: _____

Street address: _____

Father's name:

_____
Last First

Mother's name:

_____
Last First

Grade _____ Teacher _____

If the child has any brothers and sisters who are aged 6 to 17 years and presently living in Humboldt, list their names:

	Last	First	Age
1.			
2.			
3.			
4.			
5.			

Did the parents participate the 2003 survey?
Father No ___ Yes ___
Mother No ___ Yes ___

COUGH

A. Does this child usually have a cough?

No ___ Yes ___ Don't know ___

B. Does this child usually cough at all on getting up, or first thing in the morning?

No ___ Yes ___ Don't know ___

C. Does this child usually cough at all during the rest of the day?

No ___ Yes ___ Don't know ___

Or at night? No ___ Yes ___ Don't know ___

If YES to A, B, or C, answer D:

D. Does this child usually cough like this on most days as much as 3 months in a row out of the year?

No ___

Yes ___ past 12 months only

Yes ___ past 12 months and other years

PHLEGM

A. Does this child usually have congestion in the chest or bring up phlegm with cold?

No ___ Yes ___ Don't know ___

B. Does this child usually have congestion in the chest or bring up phlegm other than with cold?

No ___ Yes ___ Don't know _

If **YES**, has this congestion or phlegm been present for as much as 3 months in a row out of the year?

No ___

Yes ___, past 12 months only

Yes ___, past 12 months and other years

WHEEZING

A. Does this child chest ever sound wheezy or whistling:

1. When the child has a cold?

No ___ Yes ___ Don't know

2. Occasionally apart from colds?

No ___ Yes ___ Don't know ___

3. Most days?

No ___ Yes ___ Don't know ___

4. Or nights?

No ___ Yes ___ Don't know ___

If **YES** to 1, 2 or 3, for how many years has wheezing been present?

Number of years____

B. Has this child ever had an attack of wheezing that has made him/her feel short of breath?

No ___ Yes ___ Don't know ___

If **YES**, has he/she ever required medicine or treatment for the(se) attack(s)?

No ___ Yes ___

C. Does this child ever get attacks of wheezing after he/she has been playing hard or exercising?

No ___ Yes ___ Don't know ___

CHEST ILLNESSES

A. **During the past 12 months**, has a doctor ever said this child had any of the following chest illness:

1. Asthma	No ___ Yes ___
2. Bronchitis	No ___ Yes ___
3. Pneumonia	No ___ Yes ___
4. Hay fever	No ___ Yes ___
5. Sinus trouble	No ___ Yes ___
6. Pulmonary tuberculosis	No ___ Yes ___
7. Whooping cough	No ___ Yes ___
8. Croup	No ___ Yes ___

9. Other chest illness (including chest operations and injuries)

No ___ Yes ___

Please Specify: _____

If no to asthma, Skip to D

B. If **YES** to asthma, **during past 12 months** how many times has the child required services for asthma from the following places

Emergency room ____

Doctor's office ____

C. Which of the following statement best describes this child's asthma medication use **in the past 12 months?**

Never in the past 12 months_____
At least once in the past 12 months_____
At least once per month_____
At least once per week_____
Every day_____

D. **Before the past 12 months,** has a doctor ever said this child had any of the following chest illness:

1. Asthma	No ___ Yes ___
2. Bronchitis	No ___ Yes ___
3. Pneumonia	No ___ Yes ___
4. Hay fever	No ___ Yes ___
5. Sinus trouble	No ___ Yes ___
6. Pulmonary tuberculosis	No ___ Yes ___
7. Whooping cough	No ___ Yes ___
8. Croup	No ___ Yes ___
9. Other chest illness (including chest operations and injuries)	No ___ Yes ___

Please Specify: _____

E. If **YES** to asthma in either question A or D, at what age was the asthma first diagnosed?

Age___

PAST ILLNESSES - GENERAL

A. **During the past 12 months,** was this child seen by a doctor for:

1. Stomach acidity of reflux? No ___ Yes ___
2. An ear infection? No ___ Yes ___
3. An injury? No ___ Yes___

B. **During the past 12 months,** has this child missed more than 1 week of school because of a chest illness? No ___ Yes___

C. **During the past 12 months,** was this child kept over night in the hospital for any illness?

No ___ Yes ___

If **YES,** how many times? Times___

Please list hospitalizations

Diagnosis	Length of stay (day
1. _____	_____
2. _____	_____

E. **Before the past 12 months,** was this chi ever kept over night in the hospital for ar illness?

No ___ Yes ___ Don't know _

If **YES,** how many times? Times _

Please list hospitalizations

Diagnosis	Length of stay (day
1. _____	_____
2. _____	_____
3. _____	_____

F. Did this child have an operation to remov the tonsils or adenoids? No ___ Yes _

G. Has a doctor ever said this child had:

1. Diabetes	No ___ Yes _
2. Heart disease or defect	No ___ Yes _
3. High blood pressure	No ___ Yes _
4. Cystic fibrosis	No ___ Yes _

FAMILY HISTORY

A. Has the biological father of this child had

1. Chronic bronchitis, emphysema, or chronic obstructive lung disease
   No ___ Yes ___ Don't know _
2. Asthma No ___ Yes ___ Don't know _
3. Diabetes No ___ Yes ___ Don't know _
4. Heart disease or defect
   No ___ Yes ___ Don't know _
5. High blood pressure
   No ___ Yes ___ Don't know _
6. Allergy No ___ Yes ___ Don't know _
7. Hay fever No ___ Yes ___ Don't know _
8. Eczema No ___ Yes ___ Don't know _

Has the biological mother of this child had:

1. Chronic bronchitis, emphysema, or chronic obstructive lung disease
   No ___ Yes ___ Don't know ___
2. Asthma No ___ Yes ___ Don't know ___
3. Diabetes No ___ Yes ___ Don't know ___
4. Heart disease or defect
   No ___ Yes ___ Don't know ___
5. High blood pressure
   No ___ Yes ___ Don't know ___
6. Allergy No ___ Yes ___ Don't know ___
7. Hay fever No ___ Yes ___ Don't know ___
8. Eczema No ___ Yes ___ Don't know ___

What is the total number of brothers and sisters(excluding half-brothers and half-sisters) this child has? Number ___

Within this family what is the birth order of this child? (Circle) 1 2 3 4 5 6 7 8

How many older brothers and sisters of the child have had the following conditions?

1. Asthma Number ___
2. Diabetes Number ___
3. Heart disease or defect Number ___
4. High blood pressure Number ___

How many younger brothers and sisters of the child have had the following conditions?

1. Asthma Number ___
2. Diabetes Number ___
3. Heart disease or defect Number ___
4. High blood pressure Number ___

ASSIVE SMOKING

Does any family member smoke cigarettes regularly in your home at present?

No ___ Yes ___

If YES, how many persons smoke cigarettes?

Number ___

How many cigarettes do they smoke per day in total? Cigarette/day ___

B. Are this child's parents currently smoker(s)?

1. Father Yes ___
   No, but ex-smoker ___
   No, never smoker ___
2. Mother Yes ___
   No, but ex-smoker ___
   No, never smoker ___

C. Since this child's birth, how many years has the parents smoked?

1. Father ___ Years
2. Mother ___ Years

D How many cigarettes do they smoke per day at home? Cigarette/day ___

1. Father No ___ Yes ___
2. Mother No ___ Yes ___

E. Does any family member smoke a pipe or cigars regularly in your home at present?

No ___ Yes ___

If YES, how many persons smoke a pipe or cigar? Number ___

F. Did this child's mother smoke while pregnant with this child?

No ___ Yes ___ Don't know

DRINKING

A. How many cups of coffee does the child drink a day? Cups ___
   a week? Cups ___

B. How many glasses of soft drink does this child drink a day? Glasses ___
   a week? Glasses___

L. During the child's lifetime, have you had a dog, cat or bird living in your home?

No ___ Yes ___

M. **In the past 12 months** has this child had a farm-related injury?

No ___ Yes ___

If **YES**, please describe how and what happened.

_____

_____

_____

N. In the first 12 months of this child's life did this child:

Live on a farm No ___ Yes ___

Visit a farm more than 3 times No ___ Yes ___

Visit a farm 3 times or less No ___ Yes ___

If **YES** to living on or visiting a farm, what type of farm was it?

Grain_____

Mixed_____

Livestock_____

O. In the past 12 months has this child spent more than 1 hour on a regular basis near any of the following activities?

1. Haying No ___ Yes___
2. Harvesting No ___ Yes ___
3. Moving or playing with hay bales No ___ Yes ___
4. Feeding livestock No ___ Yes ___
5. Cleaning or playing in barns No ___ Yes ___
6. Cleaning pens No ___ Yes ___
7. Emptying or filling grain bins No ___ Yes ___
8. Pouring or mixing farm chemicals No ___ Yes ___
9. Moving or raking lawns No ___ Yes ___

SPORTS

A. Does your child participate in sports in school?

No ___ Yes ___

B Does your child play sports outside school?

No ___ Yes ___

C. Is your child now taking physical education or gym at school?

No ___ Yes ___

D. How good is your child's physical fitness?

Excellent ___
Good ___
Average ___
Below average ___
Poor ___

WEIGHT

A. Do you consider your child is:

Underweight? ___
Just about right weight? ___
Overweight? ___

B. Has your child ever tried to lose weight?

No ___ Yes ___

C. Is your child presently trying to lose weight, gain weight or neither?

Lose weight ___
Gain weight ___
Neither ___

D. If your child is presently trying to lose weight, which of the following ways of losing weight are being used?

Dieting No ___ Yes ___
Exercising No ___ Yes ___
Skipping meals No ___ Yes ___
Smoking No ___ Yes ___
Taking diet pills No ___ Yes ___
Attending programs No ___ Yes ___
Eating healthy No ___ Yes ___
Other, specify: _____

Confidential when complete

Questionnaire for the Fourth Humboldt Survey 2003-2004
(For children aged 12 to 17 years)

This questionnaire can be answered by checking the best answer or by filling in a blank with a number or word(s).

Example 1:

Does this child usually have a cough?
No ___ Yes ✔

Example 2:

How long has this child lived in the current residence? Years 6

◆ - ◆ - ◆ - ◆ - ◆

FOR OFFICE USE:

Personal I.D.: _____

Family I.D.: _____

Student's name:

_____
Last First

Tel. No.: _____

Street address: _____

Father's name:

_____
Last First

Mother's name:

_____
Last First

Grade _____ Teacher _____

If the child has any brothers and sisters who are aged 6 to 17 years and presently living in Humboldt, list their names:

	Last	First	Age
1.			
2.			
3.			
4.			
5.			

Did the parents participate the 2003 survey?
Father No ___ Yes ___
Mother No ___ Yes ___

COUGH

A. Does this child usually have a cough?

No ___ Yes ___ Don't know ___

B. Does this child usually cough at all on getting up, or first thing in the morning?

No ___ Yes ___ Don't know ___

C. Does this child usually cough at all during the rest of the day?

No ___ Yes ___ Don't know ___

Or at night? No ___ Yes ___ Don't know ___

If YES to A, B, or C, answer D:

D. Does this child usually cough like this on most days as much as 3 months in a row out of the year?

No ___

Yes ___, past twelve months only

Yes ___, past twelve months and other years

PHLEGM

A. Does this child usually have congestion in the chest or bring up phlegm with cold?

No ___ Yes ___ Don't know ___

B. Does this child usually have congestion in the chest or bring up phlegm other than with cold?

No ___ Yes ___ Don't know ___

If **YES**, has this congestion or phlegm been present for as much as 3 months in a row in the last year?

No ___

Yes ___, past 12 months only

Yes ___, past 12 months and other years

WHEEZING

A. Does this child chest ever sound wheezy or whistling:

1. When the child has a cold?

No ___ Yes ___ Don't know

2. Occasionally apart from colds?

No ___ Yes ___ Don't know ___

3. Most days

No ___ Yes ___ Don't know ___

4. Or nights?

No ___ Yes ___ Don't know ___

If **YES** to 1, 2 or 3, for how many years has wheezing been present?

Number of years ___

B. Has this child ever had an attack of wheezing that has made him/her feel short of breath?

No ___ Yes ___ Don't know ___

If **YES**, has he/she ever required medicine or treatment for the(se) attack(s)? No ___ Yes ___

C. Does this child ever get attacks of wheezing after he/she has been playing hard or exercising?

No ___ Yes ___ Don't know ___

CHEST ILLNESSES

A. **During the past 12 months,** has a doctor ever said this child had any of the following chest illness:

1. Asthma	No ___ Yes ___
2. Bronchitis	No ___ Yes ___
3. Pneumonia	No ___ Yes ___
4. Hay fever	No ___ Yes ___
5. Sinus trouble	No ___ Yes ___
6. Pulmonary tuberculosis	No ___ Yes ___
7. Whooping cough	No ___ Yes ___
8. Croup	No ___ Yes ___
9. Other chest illness (including chest operations and injuries)	No ___ Yes ___

Please Specify: _____

If no to asthma, Skip to D

B. If **YES** to asthma, how many times has the child required services for asthma from the following places **during past 12 months?**

Emergency room ____

Doctor's office ____

C. Which of the following statement best describes this child's asthma medication use **in the past 12 months?**

Never in the past 12 months_____
At least once in the past 12 months_____
At least once per month_____
At least once per week_____
Every day_____

D. **Before the past 12 months,** has a doctor ever said this child had any of the following chest illness:

1. Asthma	No ___ Yes ___
2. Bronchitis	No ___ Yes ___
3. Pneumonia	No ___ Yes ___
4. Hay fever	No ___ Yes ___
5. Sinus trouble	No ___ Yes ___
6. Pulmonary tuberculosis	No ___ Yes ___
7. Whooping cough	No ___ Yes ___
8. Croup	No ___ Yes ___
9. Other chest illness (including chest operations and injuries)	No ___ Yes ___

Please Specify: _____

E. If **YES** to asthma in either question A or D, at what age was the asthma first diagnosed?

Age___

PAST ILLNESSES - GENERAL

A. **During the past 12 months,** was this child seen by a doctor for:

1. Stomach acidity or reflux? No ___Yes ___
2. An ear infection? No ___ Yes ___
3. An injury? No ___ Yes___

B. **During the past 12 months,** has this child missed more than 1 week of school because of a chest illness? No ___ Yes___

C. **During the past 12 months,** was this child kept over night in the hospital for any illness?

No ___ Yes ___

If **YES,** how many times? Times_____

Please list hospitalizations

Diagnosis	Length of stay (days)
1._____	_____
2._____	_____

E. **Before the past 12 months,** was this child ever kept over night in the hospital for any illness?

No ___ Yes ___ Don't know ___

If **YES,** how many times? Times ___

Please list hospitalizations

Diagnosis	Length of stay (days)
1._____	_____
2._____	_____
3._____	_____

F. Did this child have an operation to remove the tonsils or adenoids?

No ___ Yes ___

G. Has a doctor ever said this child had:

1. Diabetes	No ___ Yes ___
2. Heart disease or defect	No ___ Yes ___
3. High blood pressure	No ___ Yes ___
4. Cystic fibrosis	No ___ Yes ___

FAMILY HISTORY

A. Has the *biological* father of this child had:

1. Chronic bronchitis, emphysema, or chronic obstructive lung disease
   No ___ Yes ___ Don't know ___
2. Asthma No ___ Yes ___ Don't know ___
3. Diabetes No ___ Yes ___ Don't know ___
4. Heart disease or defect
   No ___ Yes ___ Don't know ___
5. High blood pressure
   No ___ Yes ___ Don't know ___
6. Allergy No ___ Yes ___ Don't know ___
7. Hay fever No ___ Yes ___ Don't know ___
8. Eczema No ___ Yes ___ Don't know ___

B. Has the *biological* mother of this child had:

1. Chronic bronchitis, emphysema, or chronic obstructive lung disease
   No ___ Yes ___ Don't know ___
2. Asthma No ___ Yes ___ Don't know ___
3. Diabetes No ___ Yes ___ Don't know ___
4. Heart disease or defect
   No ___ Yes ___ Don't know ___
5. High blood pressure
   No ___ Yes ___ Don't know ___
6. Allergy No ___ Yes ___ Don't know ___
7. Hay fever No ___ Yes ___ Don't know ___
8. Eczema No ___ Yes ___ Don't know ___

C. What is the total number of brothers and sisters(excluding half-brothers and half-sisters) this child has? Number ___

D. Within this family what is the birth order of this child? *(Circle)*

1 2 3 4 5 6 7 8

E. How many older brothers and sisters of the child have had the following conditions?

1. Asthma Number ___
2. Diabetes Number ___
3. Heart disease or defect Number ___
4. High blood pressure Number ___

E. How many younger brothers and sisters of the child have had the following conditions?

1. Asthma Number ___
2. Diabetes Number ___
3. Heart disease or defect Number ___
4. High blood pressure Number ___

PASSIVE SMOKING

A. Does any family member smoke cigarettes regularly in your home at present?

No ___ Yes ___

If **YES**, how many persons smoke cigarettes?

Number ___

How many cigarettes do they smoke per day in total? Cigarettes/day ___

B. Are this child's parents currently smoker(s)?

1. Father Yes ___
   No, but ex-smoker ___
   No, never smoker ___
2. Mother Yes ___
   No, but ex-smoker ___
   No, never smoker ___

C. Since this child's birth, how many years has the parents smoked?

1. Father ___ Years
2. Mother ___ Years

D How many cigarettes do they smoke per day at home?

Cigarettes/day ____

1. Father No ___ Yes ___
2. Mother No ___ Yes ___

E. Does any family member smoke a pipe or cigars regularly in your home at present?

No ___ Yes ___

If **YES**, how many persons smoke a pipe or cigar? Number ___

F. Did this child's mother smoke while pregnant with this child?

No ___ Yes ___ Don't know

DRINKING

A. How many cups of coffee does the child drink a day? Cups ___
   a week? Cups ___

B. How many glasses of soft drink does this child drink a day? Glasses ___
   a week? Glasses___

AL

A. Has this child ever had an allergic reaction to any of the following?

House dust	No ___ Yes ___
Grain dust	No ___ Yes ___
Pollen	No ___ Yes ___
Trees	No ___ Yes ___
Grasses	No ___ Yes ___
Dog or Cat	No ___ Yes ___
Birds/feathers	No ___ Yes ___
Farm animals	No ___ Yes ___

Specify animaltype_____

B Has this child ever had an allergic reaction to things that:

1. Are eaten or ingested, (e.g. food or medicine)? No ___ Yes ___

2. Come in contact with the skin (e.g. wool, detergents or metals)? No ___ Yes ___

C. During the past 12 months has this child ever taken care of cattle, hogs, poultry, horses or other livestock? No ___ Yes ___

LIVING ENVIRONMENT

A. How long has this child lived in your current home? Years ___

B. Which best describes the building in which this child lives?

A mobile home or trailer ___
A one-family house not attached to any other house ___
A one family house attached to other house(s) ___
A building for 2 families ___
A building for 3 or more families ___

C. About which year was this building originally built?
Year_____ Don't know_____

D. Where is your home located?
Farm_____
Acreage_____
In town_____

E. How many rooms other than hallways or bathrooms are there in your home?
Rooms___

F. How many people live in your home?
Number ___

G. How is your home heated in winter?
Gas furnace ___
Electricity ___
Steam or hot water___
Other, specify:_____

H. Do you have any of the following in your home?

Central air conditioner	No ___ Yes ___
Room air conditioner	No ___ Yes ___
Air filter	No ___ Yes ___
Humidifier	No ___ Yes ___
Dehumidifier	No ___ Yes ___
Fireplace	No ___ Yes ___

I. Does your house have any damage caused by dampness (e.g., wet spots on walls, floors)? No ___ Yes ___

J. Are there signs of mold or mildew in any living areas of your home? No ___Yes ___

K. Do you currently have any pets living inside your home?

Dog(s)	No ___ Yes ___
Cat(s)	No ___ Yes ___
Bird(s)	No ___ Yes ___

Other, specify:_____

L. During the child's lifetime,, have you had a dog, cat or bird living in your home?

No ___ Yes ___

M. In the past 12 months has this child had a farm-related injury?

No ___ Yes ___

If **YES**,, please describe how and what happened:

_____

_____

_____

_____

_____

N. **In the first 12 months** of this child's life did this child:

Live on a farm? No ___ Yes ___

Visit a farm more than 3 times?

No ___ Yes ___

Visit a farm regularly? No ___ Yes ___

If **YES** to any of the above, what type of farm was it?

Grain_____

Mixed_____

Livestock_____

O. **In the past 12 months** has this child spent more than 1 hour on a regular basis near the following activities?

1. Haying No ___ Yes___
2. Harvesting No ___ Yes ___
3. Moving or playing with hay bales No ___ Yes ___
4. Feeding livestock No ___ Yes ___
5. Cleaning or playing in barns No ___ Yes ___
6. Cleaning pens No ___ Yes ___
7. Emptying or filling grain bins No ___ Yes ___
8. Pouring or mixing farm chemicals No ___ Yes ___
9. Moving or raking lawns No ___ Yes ___

PERSONAL INFORMATION

A. Child's sex: Male ___ Female ___

B. Child's date of birth: ____ ____ ____
Month Day Year

C. Child's age: ____

D. Country of birth:_____

E. What is this child's ethnic origin?

_____

F. How much did this child weigh when born (pounds and ounces)?

_____pounds _____ounces

G. Was this child breastfed? No ___ Yes ___

If **YES**, for how long?

Weeks_____ or Months___

(Please specify amount of time)

OTHER INFORMATION

A. Do you think your child's health is ..

Excellent ___

Good ___

Fair ___

Poor ___

C. What is your relationship to this child?

Biological father ___

Biological mother ___

Adoptive parent ___

Stepparent ___

Grandparent ___

Legal guardian ___

Other primary adult ___

D. Does this child live in a:

One parent home? ___

Two parent home? ___

E. What is today's date:

_____ _____ _____
Month Day Year

THE END *Thank you*

Humboldt Lung Study 2003-2004

Short Questionnaire 12 to 17 years (To be completed at time of Health Assessment)

The following questions deal with the things you do day to day and your daily health. Please try to answer these questions as honestly as you can. All answers will be kept confidential. *No one including your teacher and parents will know your answers.*

Name

[redacted]

Last First

Sex: Male [redacted] Female [redacted]

Date of Birth: [redacted] [redacted] [redacted]
Mo. Day Yr.

Grade: [redacted]

Homeroom Teacher:

SPORTS

1. Do you play sports in school?

No [redacted] Yes [redacted]

2. Do you play sports outside school?

No [redacted] Yes [redacted]

3. Are you now taking physical education or gym at school?

No [redacted] Yes [redacted]

How often is this?
Every day [redacted]
3 times a week
Less than 3 times a week

4. How good is your physical fitness?

Excellent [redacted]
Good
Average
Below average
Poor

[redacted]

WEIGHT AND HEIGHT

5. Present height: [redacted] cm

6. Present weight: [redacted] kg

7. Do you consider yourself to be:

Underweight? [redacted]
Just about right weight?
Overweight?

8. Have you ever tried to lose weight?

No [redacted] Yes [redacted]

9. Are you presently trying to lose weight, gain weight or neither?

Lose weight [redacted]
Gain weight
Neither

10. If you are presently trying to lose weight, which of the following are you doing to lose weight?

Dieting	No [redacted]	Yes [redacted]
Exercising	No	Yes
Skipping meals	No	Yes
Smoking	No	Yes
Taking diet pills	No	Yes
Attending programs	No	Yes
Eating healthy	No	Yes

Other, specify _____

TELEVISION AND VIDEO GAME PLAYING

11. Do you watch television or play video games every day or almost every day?

No [redacted] Yes [redacted]

12. How many hours do you spend on watching television or playing video games per day on an average?

[redacted]

Weekday: [redacted] hours,

Weekend: [redacted] hou[redacted]

13. How many hours did you spend on watching television or video game playing during last week in total?

[redacted] hours

CIGARETTESMOKING

14. Have you ever smoked cigarettes?

Yes, at least a whole cigarette
Yes, just a few puffs
No, not even a few puffs

If **YES**, answer Question 15,
If **NO**, go to Question 25.

15. Have you smoked cigarettes in the past 12 months?

Yes, at least a whole cigarette
Yes, just a few puffs
No, not even a few puffs

If **YES**, answer Question 16,
If **NO**, go to Question 22.

16. Have you smoked cigarettes in the past 4 weeks?

Yes, at least a whole cigarette
Yes, just a few puffs
No, not even a few puffs

If **YES**, answer Question 17,
If **NO**, go to Question 22.

17. Have you smoked cigarettes in the past week?

Yes, at least a whole cigarette
Yes, just a few puffs
No, not even a few puffs

If **YES**, answer Question 18,
If **NO**, go to Question 22.

18. Do you smoke cigarettes every day or almost every day?

No Yes

If **YES**, answer Questions 19, 20, and 21,
If **NO**, go to Question 22.

19. How old were you when you **first** started regular cigarette smoking?

Age

20. How many cigarettes do you smoke per day now?

Cigarettes/day

21. On the average of the entire time you smoked regularly, how many cigarettes did you smoke per day?

Cigarettes/day

22. How old were you when you **first** smoked a cigarette?

Age in years

23. If you do not smoke regularly at present, did you ever smoke every day or almost every day?

No Yes

If **YES**, how old were you when you stopped?

Age stopped

24. Have you smoked at least 20 packs of cigarettes in your lifetime?

No Yes

25. Does any of your friends (male or female) smoke cigarettes in your presence?

No Yes

If **YES**, how many hours **per day** on an average are you exposed to their smoking?

hours

If **YES**, how many hours **per week** on an average are you exposed to their smoking?

hours

DRINKING

26. Do you presently drink alcohol?

No Yes

If **YES**, is this as often as:

Occasionally	No	Yes
1 day per week?	No	Yes
2 days per week?	No	Yes
3 or more days per week?	No	Yes

OTHER INFORMATION

27. Do you think your health is ...

Excellent?
Good?
Fair?
Bad?

THE END

Humboldt Lung Study (*Children 6-17*)

Height: _____ **(cm)** Girth: _____ **(cm)**

Weight: _____ **(kg)**

	1	2
Systolic BP (mmhg):	_____	_____
Diastolic BP (mmhg):	_____	_____

Lung Function:

Done _____

Not done _____

Reason why:

1. Subject could not perform test _____
2. Refused _____
3. Other, specifiy _____ Tester's initials: _____

Room temperature: _____ (C.)

Skin Testing

Neg Control _____ Cat _____

Alternaria _____ Grass Mix _____

HDM _____ Farina _____

Pos Control _____

Buccal Swabs

Yes ___ No ___

Tester's initials: ____

Comments:

Today's Date: _____ _____ _____
Mo. Day Yr.

Confidential when Completed

Questionnaire for Completion (Adult Participant)

FOURTH HUMBOLDT LUNG STUDY*

Dear Participant:

The previous Humboldt studies in 1977, 1982 and 1993 were outstanding successes with a high number of people in the community participating. This 4th Humboldt Study is being conducted to study respiratory health and factors that affect that health. All persons between 6 and 79 years will participate in the Study. Children under 18 years will participate in the schools.

To find out about your respiratory health we would appreciate your cooperation in answering this questionnaire. All information will be kept strictly confidential and used only in research. Your information will be grouped with the information of other participants and will be presented only in a grouped manner. Names or anything that could identify you will not be used in reporting results of the Study.

As part of finding out more about your respiratory health, we would like to measure your lung function, height, weight, waist and blood pressure. The breathing test will consist of blowing a few times into a tube. To find out how lung function is inherited, we will need to collect a blood sample of 17 mls or approximately 1 tablespoon. The blood sample will be stored by the Institute of Agricultural, Rural and Environmental Health (I.ARE.H) at the University of Saskatchewan for 15 years. If possible, we would also like to find out if you have an allergy to any of four common allergens in the air we breathe by conducting a skin test for these allergens.

We would like you to consider our request and ask that you bring the completed questionnaire to our testing site at the Humboldt Mall. We will arrange an appointment by phone for you to attend the clinic. At that time you will have a chance to ask more questions about the tests and to complete a consent for testing. Tests will be conducted between September 15 and December 15, 2003.

If you have any questions, please call Dr. Dosman or Dr. Rennie (I.ARE.H) at the University of Saskatchewan at 1-306-966-8286. If you have any questions about your rights as a research subject or concerns about your experiences while participating in this Study, you should contact the Chair of the Biomedical Research Ethics Board, c/o the Office of Research Services, University of Saskatchewan at 306-966-4053.

Thank you for your cooperation.

Questionnaire for the Fourth Humboldt Survey 2003

(For Adults Aged 18 to 79 Years)

The questionnaire can be answered by checking the best answer or by filling in a blank with a number or word(s).

Example 1:

Do you usually have a cough? No ✓ Yes ___

Example 2:

How long have you lived in your current residence?
12 Years

* * *

FOR OFFICE USE:

Personal I.D.: _____

Family I.D.: _____

* * *

Name:

_____
Last First

Telephone No.: _____

Street address: _____

Spouse's name:

_____
Last First

Spouse address: _____
(if different)

If you have any **biological (natural)** children who are **aged 6 to 17 years** and presently living in Humboldt, list their names and ages:

1. _____ age____
2. _____ age____
3. _____ age____
4. _____ age____
5. _____ age____

Have you participated in a previous Humboldt Study?

In the 1977 survey? Yes ___ No ___

In the 1983 survey? Yes ___ No ___

In the 1993 survey? Yes ___ No ___

CONFIDENTIAL WHEN COMPLETED

Personal ID: _____

COUGH

A. Do you usually have a cough? No ___ Yes ___

B. Do you usually cough at all on getting up, or first thing in the morning? No ___ Yes ___

C. Do you usually cough at all during the rest of the day? No ___ Yes ___
Or at night? No ___ Yes ___

If YES to A, B, or C, answer D and E:

D. Do you usually cough like this on most days for 3 consecutive months or more during the year? No ___ Yes ___

E. For how many years have you had this cough? Number of years ___

PHLEGM

A. Do you usually bring up phlegm from your chest? No ___ Yes ___

B. Do you usually bring up phlegm at all on getting up, or first thing in the morning? No ___ Yes ___

C. Do you usually bring up phlegm at all during the rest of the day? No ___ Yes ___
or at night? No ___ Yes ___

If YES to A, B, OR C, answer D, E and F:

D. Do you bring up phlegm like this on most days for 3 consecutive months or more during the year? No ___ Yes ___

E. For how many years have you had trouble with phlegm? Number of years ___

F. Have you had periods or episodes of (increased) cough and phlegm lasting for 3 weeks or more each year?
No ___
Yes, last 3 years only ___
Yes, more than 3 years ___

WHEEZING

A. Does your chest ever sound wheezy or whistling:

1. When you have a cold? No ___ Yes ___

2. Occasionally apart from colds? No ___ Yes ___

3. Most days No ___ Yes ___

Or nights? No ___ Yes ___

If YES to 1, 2, OR 3, for how many years has this been present? Number of years ___

B. Have you ever had an attack of wheezing that has made you feel short of breath? No ___ Yes ___

If YES, have you ever required medicine or treatment for the(se) attack(s)? No ___ Yes ___

BREATHLESSNESS

If disabled from walking by any condition other than heart or lung disease, please describe and do not answer the following questions (A-E)

A. Are you troubled by shortness of breath when hurrying on the level or walking up a slight hill? No ___ Yes ___

If YES to A, answer B to E:

B. Do you have to walk slower than people of your age because of breathlessness? No ___ Yes ___

C. Do you ever have to stop for breath when walking at your own pace on the level? No ___ Yes ___

D. Do you ever have to stop for breath after walking about 100 yards(or after a few minutes) on the level? No ___ Yes ___

E. Are you too breathless to leave the house or breathless on dressing or undressing? No ___ Yes ___

CHEST ILLNESSES

A. **During the past twelve months**, has a doctor ever said you had any of the following chest illnesses:

1. Asthma	No ___ Yes ___
2. Attack of bronchitis	No ___ Yes ___
3. Pneumonia	No ___ Yes ___
4. Hay fever	No ___ Yes ___
5. Sinus trouble	No ___ Yes ___
6. Chronic bronchitis	No ___ Yes ___
7. Emphysema	No ___ Yes ___
8. Other chest illness (including chest operations and injuries)	No ___ Yes ___

Specify _____

If **YES** to asthma, at what age were you diagnosed?
Age_____

If **YES** to asthma, how many times have you required services for asthma from the following places during the past twelve months? Emergency room ___
Doctor's office ___

B. **Before the past twelve months**, has a doctor ever said you had any of the following chest illnesses:

1. Asthma If yes, at what age were you diagnosed? Age_____	No ___ Yes ___
2. Attack of bronchitis	No ___ Yes ___
3. Pneumonia	No ___ Yes ___
4. Hay fever	No ___ Yes ___
5. Sinus trouble	No ___ Yes ___
6. Pulmonary tuberculosis	No ___ Yes ___
7. Chronic bronchitis	No ___ Yes ___
8. Emphysema	No ___ Yes ___
9. Other chest illness (including chest operations and injuries)	No ___ Yes ___

Specify _____

C. Which of the following statements best describes your asthma medication use **in the past 12 months:**

Never in the past 12 months ___
At least once in the past 12 months ___
At least once per month ___
At least once per week ___
Every day ___

PAST ILLNESSES - GENERAL

A. During the past twelve months, were you seen by a doctor for stomach acidity or reflux?
No ___ Yes ___

B. **During the past twelve months**, were you seen by a doctor for an injury?
No ___ Yes ___

C. **During the past twelve months**, were you seen by a doctor for an ear infection?
No ___ Yes ___

D. Has a doctor ever said you had:

1. Diabetes	No ___ Yes ___
2. Heart disease	No ___ Yes ___
3. High blood pressure	No ___ Yes ___
4. Cystic fibrosis	No ___ Yes ___
5. Heart defect	No ___ Yes ___

If **YES**, do you currently have any treatment for it?

1. Diabetes	No ___ Yes ___
2. Heart disease	No ___ Yes ___
3. High blood pressure	No ___ Yes ___
4. Cystic fibrosis	No ___ Yes ___
5. Heart defect	No ___ Yes ___

E. **During the past twelve months**, were you kept overnight in the hospital for any illness?

No ___ Yes ___

If **YES**, how many times? Times ___

Please specify:

Diagnosis	length of stay (days)
1. _____	_____
2. _____	_____
3. _____	_____

FAMILY HISTORY

A. Has (did) your **biological** father had (have):

1. Chronic bronchitis, emphysema, or chronic obstructive lung disease No ___ Yes ___ Don't know ___
2. Asthma No ___ Yes ___ Don't know ___
3. Diabetes No ___ Yes ___ Don't know ___
4. Heart disease or defect No ___ Yes ___ Don't know ___
5. High blood pressure No ___ Yes ___ Don't know ___

B. Has (did) your **biological** mother had (have):

1. Chronic bronchitis, emphysema, or chronic obstructive lung disease No ___ Yes ___ Don't know ___
2. Asthma No ___ Yes ___ Don't know ___
3. Diabetes No ___ Yes ___ Don't know ___
4. Heart disease or defect No ___ Yes ___ Don't know ___
5. High blood pressure No ___ Yes ___ Don't know ___

C. What is the total number of brothers and sisters (exclude half-brothers and half-sisters) you have?

Number___

D. How many of your brothers and sisters have had the following disorders?

1. Chronic bronchitis, emphysema, or chronic obstructive lung disease Number___
2. Asthma Number___
3. Diabetes Number___
4. Heart disease or defect Number___
5. High blood pressure Number___

OCCUPATIONAL HISTORY

A. In the last 5 years, have you grown or handled wheat, durham, oats, barley, flax, canola, rye, mustard, alfalfa, or other grain, seeds, or legumes?

No ___ Yes ___

If **YES**, how many years? Years ___

B. In the last 5 years have you worked at looking after cattle, hogs, sheep, poultry, horses, or other livestock animals?

No ___ Yes ___

If **YES**, how many years? Years ___

C. Have you ever worked for more than six months in any of the following:

Mining No ___ Yes ___

Lumber No ___ Yes ___

Welding No ___ Yes ___

Grain elevator No ___ Yes ___

Feed mill No ___ Yes ___

Autobody No ___ Yes ___

Other No ___ Yes ___

If other, specify _____

D. Have you ever been exposed to grain dust in your work? No ___ Yes ___

Total years worked ___

Was dust exposure: Mild ___ Moderate ___ or Severe ___

E. Have you ever lived on a farm? No____ Yes___

If **YES**, at what ages? age _____to age_____

F. Have you had a farm-related injury **in the past 12 months?**

No ___ Yes ___

If **YES**, briefly describe below how and what happened.

_____

_____

_____

_____

AND

If **YES**, did you see a doctor or other health care worker?

No ___ Yes ___

CIGARETTE SMOKING

A. Have you ever smoked cigarettes? (If you have smoked less than 20 packs of cigarettes in your lifetime, answer no).

No ___ Yes ___

If YES to A, answer B to F:

B. Do you now smoke cigarettes? No ___ Yes ___

C. How old were you when you first started regular cigarette smoking? Age in years ___

D. How many cigarettes do you smoke per day now? Cigarettes/day ___

E. On the average of the entire time you smoked, how many cigarettes did you smoke per day? Cigarettes/day ___

F. If you have stopped smoking cigarettes completely, how old were you when you stopped? Age stopped ___

G. If there have been periods when you abstained from smoking, indicate total years of abstinence from smoking. Years ____

PIPES AND CIGARS

A. Have you ever smoked a pipe regularly? (Yes means more than 12 oz of tobacco in a life-time.)

No ___ Yes ___

B. Have you ever smoked cigars regularly? (Yes means more than 1 cigar a week for a year.)

No ___ Yes ___

C. Do you smoke a pipe or cigars regularly at present?

No ___ Yes ___

PASSIVE SMOKING

A. **Except for you**, does any family member smoke cigarettes regularly in your home at present?

No ___ Yes ___

If **YES**, how many persons smoke cigarettes?
Number ___

How many cigarettes do they smoke per day in total?

Cigarettes/day ___

How many cigarettes do they smoke per day at home?

Cigarettes/day ___

B. **Except for you**, does any family member smoke a pipe or cigars regularly in your home at present?

No ___ Yes ___

If **YES**, how many persons smoke a pipe or cigars?

Number ___

DRINKING

A. Do you presently use alcoholic beverages?

No ___ Yes ___

If **YES**, is this as often as:
1 day per week? No ___ Yes ___

2 days per week? No ___ Yes ___

3 or more days per week? No ___ Yes ___

B. How many cups of coffee do you drink a day?
Cups ___

C. How many glasses of soft drink do you drink a day?

Glasses ___

ALLERGIES

A. Have you ever had an allergic reaction to things that:

1. Are eaten or ingested (e.g. food or medicine)?
No ___ Yes ___

2. Are inhaled (e.g. pollen, dust, animal fur or smoke)?
No ___ Yes ___

3. Come in contact with the skin (e.g. detergents, wool or metals)?
No ___ Yes ___

4. Others

Specify _____

WEIGHT

A. Do you consider yourself to be:
Underweight? ___
Just about right weight? ___
Overweight? ___

B. Have you ever tried to lose weight?
No ___ Yes ___

C. Are you presently trying to lose weight, gain weight or neither?
Lose weight ___
Gain weight ___
Neither ___

D. If you are presently trying to lose weight, which of the following are you doing to lose weight?

Dieting No ___ Yes ___
Exercising No ___ Yes ___
Skipping meals No ___ Yes ___
Smoking No ___ Yes ___
Taking diet pills No ___ Yes ___
Attending programs No ___ Yes ___
Other, specify _____

LIVING ENVIRONMENT

A. How long have you lived in your current home?
Years ___

B. Which best describes the building in which you live?
A mobile home or trailer ___
A one-family house not attached to any other house ___
A one-family house attached to other house(s) ___
A building for 2 families ___
A building for 3 or more families ___
Other, specify _____

C. About which year was this building originally built?
Before 1980 ___ After 1980 ___ Don't know ___

D. How many bedrooms are there in your home?
Rooms ___

E. How many people live in your home? Number ___

F. How is your home heated in winter?
Gas furnace No ___ Yes ___
Electricity No ___ Yes ___
Steam or hot water No ___ Yes ___
Other, specify _____

G. What is usually used for cooking in your home?
Gas No ___ Yes ___
Electricity No ___ Yes ___
Other, specify _____

H. Do you have any of the following in your home?

Air conditioners No ___ Yes ___
Air filter No ___ Yes ___
Humidifier No ___ Yes ___
Dehumidifier No ___ Yes ___
Fireplace No ___ Yes ___

ving Environment, cont'd)

Does your house have any damage caused by dampness (e.g., wet spots on walls or floors)?
No ___ Yes ___

Do you have any pets living inside your home?
Dog(s) No ___ Yes ___
Cat(s) No ___ Yes ___
Bird(s) No ___ Yes ___

Other, specify _____

L. Have you ever had a pet living inside your home?
Dog(s) No ___ Yes ___
Cat(s) No ___ Yes ___
Bird(s) No ___ Yes ___

PERSONAL INFORMATION

A: Sex: Male ___ Female ___

B: Date of Birth: _____ _____ _____
Mo. Day Yr.

C: Age: _____

D: Place of Birth: _____

E: What is your race? Caucasian ___
Aboriginal ___

Other (specify) _____

F: What is your marital status? Single ___

Married/Common law ___

Widowed ___

Separated/Divorced ___

G: What is the highest grade completed in school?

Grade school not completed ___

Grade school completed ___

High school completed ___

Trade school or only attended college ___

College graduate/postgraduate ___

H: What is the range of your total, gross family income last year? Under $12,000 ___

$12,000 to $24,999 ___

$25,000 to $49,999 ___

$50,000 and over ___

I: What is the national origin of your grandparents?
Paternal grandfather _____

Paternal grandmother _____

Maternal grandfather _____

Maternal grandmother _____

REMARKS:

For Office Use Only

ID# _____

HUMBOLDT FOURTH LUNG STUDY

Height _____ (cm) Weight _____ (kg) Girth Measurement _____

NOTE: Has this person taken a bronchodilator in the past 6 hours? Yes _____ No _____

If yes, rebook.

	1	2
Systolic BP (mmhg)	_____	_____
Diastolic BP (mmhg)	_____	_____

Lung Function Testing: (check) Station A _____ Station B _____

Done _____ Not Done _____

Reason why:
1. Subject could not perform the test _____
2. Refused _____
3. Other, specify _____

Room Temperature _____

Today's Date: _____ Tester's Initials: _____

Blood Test for Genetic Testing: Yes _____ No _____

COMMENTS: _____

Skin Testing
Antihistamine or cold preparation in the last 72 hours: No _____ Yes _____
NOTE: If yes, rebook.

Allergy Tests

Neg Control	_____	Cat	_____
Alternaria	_____	Grass Mix	_____
HDM	_____	Histamine	_____

Not Done _____

Reason why:
1. Subject could not perform the test _____
2. Refused _____
3. Other, specify _____

Appendix D: Copy of ethics approval

University of Saskatchewan
Biomedical Research Ethics Board (Bio-REB)

06-Oct-2005

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
James A. Dosman	Institute of Ag. Rural and Environmental Health	05-97

INSTITUTION (S) WHERE RESEARCH WILL BE CARRIED OUT

University of Saskatchewan

Saskatoon SK

SUB-INVESTIGATOR(S)

Lalita Bharadwaj

SPONSORING AGENCIES

CANADIAN INSTITUTES FOR HEALTH RESEARCH (CIHR)

TITLE:

Genetic Epidemiology of Atopy and the Hygiene Hypothesis

ORIGINAL APPROVAL DATE	CURRENT EXPIRY DATE	APPROVAL OF
06-Oct-2005	01-Oct-2006	Protocol as submitted

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board has reviewed the above-named research project at a full-board meeting (any research classified as minimal risk is reviewed through the expedited review process). The proposal was found to be acceptable on ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research project, and for ensuring that the authorized research is carried out according to governing law. This Approval is valid for the above time period provided there is no change in experimental protocol or in the consent process.

ONGOING REVIEW REQUIREMENTS/REB ATTESTATION

In order to receive annual renewal, a status report must be submitted to the Chair for Committee consideration within one month of the current expiry date each year the study remains open, and upon study completion. Please refer to the following website for further instructions: http://www.usask.ca/research/ethics.shtml. In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. This approval and the views of this REB have been documented in writing.

APPROVED.

Michel Desautels, Ph.D., Chair
University of Saskatchewan
Biomedical Research Ethics Board (Bio-REB)

Please send all correspondence to:

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