Inner City Asthma Consortium (ICAC)

ICAC-07

Urban Environment and Childhood Asthma (URECA)

Protocol

Version 15.0
09/12/07

The Inner City Asthma Consortium is funded by
The National Institute of Allergy and Infectious Diseases

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1 Background

Asthma is more prevalent, and may be more severe, in the inner city, even after controlling for differences in ethnicity and socioeconomic status, thus strongly suggesting that environmental factors that are unique to the inner city may promote the development of more severe asthma in early childhood.[1] Although there are comprehensive studies of allergen exposure in the inner city environment, information relating early life exposures to viral infections and stress in the inner city to the development of asthma are lacking. Furthermore, despite studies which link asthma to abnormal development of the immune system and patterns of cytokine secretion, there has been no prospective evaluation of the effects of the unique environmental exposures unique to the inner city on immune development and the incidence of asthma. As a result, a number of important questions remain unanswered:

1) Does dysregulation of innate immune responses, which may already be present at birth, increase the risk of allergen sensitization (adaptive immune responses) and asthma, and if so, which cytokines are key in this process?
2) How do lower respiratory infections in infancy affect the subsequent risk of recurrent wheezing and asthma in inner city children?
3) Is the immune system of inner city children already abnormal at birth, due to a unique prenatal environment?
4) Of the many unique features associated with the inner city environment, which are the principal factors that adversely affect immune development, and thereby increase the risk of asthma?
5) Does the inner city environment (e.g. increased stress and pollution) increase the frequency or severity of lower respiratory infections (LRI), and if so, what are the relevant mechanisms (immune modulation versus lung-specific effects), and
6) Do environmental factors interact at a critical time point to establish a particular wheezing phenotype with future infections and/or exposures?

There is increasing evidence that allergy and asthma are associated with abnormal patterns of cellular immunity, as evidenced by a distinct and abnormal pattern of cytokine secretion. Since the pattern of cytokine secretion changes rapidly during the first few years of life, it is likely that the environment influences the development of the immune system in infancy, and this in turn modifies the risk for developing allergies and asthma. Differences in the inner city environment are numerous, and include prenatal factors (e.g. maternal stress, placental insufficiency), and numerous postnatal exposures. Some of the differences in postnatal exposures are well-documented, such as increased exposure to cockroach and tobacco smoke, while there is less specific information related to patterns of stress, exposure of young children to other pollutants and endotoxin, and the development of viral infections in the first years of life. If primary prevention of asthma is to be achieved, establishing the relationship of these factors to the onset of infantile wheezing, as well as their impact on the evolution to recurrent childhood wheezing (or asthma) will be critical.

1.1 Environmental exposures and asthma

1.1.1 Do respiratory viruses increase the risk of developing asthma?

There are experimental data to suggest that lower respiratory infections at a critical period during the development of the immune system or lungs could have long-lasting effects. For example, young mice infected with RSV have a propensity to develop Th2 rather than Th1 recall responses to virus,[2] which could enhance inflammation and impair antiviral responses upon re-exposure to the virus. In addition, CMV infections specifically inhibit expression of the epithelial growth factor receptor.[3] This finding implies that infection during early infancy, a time period of continued lung and epithelial differentiation could have detrimental and long-term effects on lung structure and function. Other infections (e.g. mycoplasma, chlamydia, adenovirus) have also been implicated in the promotion of chronic airway inflammation and/or recurrent wheezing, or conversely (e.g. hepatitis A), have been associated with reduced prevalence of allergies and asthma.[4] However, based on the experimental design, these observations could not determine if these changes had been present or "programmed" (i.e. cytokine dysregulation) prior to the onset of the infection.

There is a close association between RSV infections and wheezing syndromes in infants. Although nearly all infants are infected with RSV during the first three years of life, only a subset of infants develop wheezing illnesses, and consequently other risk factors including small lung size[5,6] and genetic factors, such as polymorphisms in the IL-8 gene,[7] are also involved. In animal models, enhanced lower airway responses to Sendai virus infection have been related to reduced IFN-γ production,[8] and preliminary data from a cohort of suburban infants (the Childhood Origins of Asthma [COAST] study) suggest that reduced cytokine responses (e.g. IL-13) by cord blood mononuclear cells may also increase the risk for wheezing with RSV infection.[9]

Children who develop wheezing with RSV infection are at an increased risk of recurrent wheezing and asthma in childhood.[10,11] Most of the risk factors for developing asthma after bronchiolitis in infancy relate to other signs or indicators of allergy, such as atopic dermatitis, food allergy, or allergic rhinitis.[12-14] In addition, immunologic responses at the time of infection which have been associated with recurrent wheezing after bronchiolitis include eosinophilic inflammation in airway secretions,[15-17] reduced IFN-γ production from peripheral blood mononuclear cells (PBMC) *ex vivo*,[18] and increased *ex vivo* PBMC IL-10 secretion during convalescence.[19] It has yet to be established whether this pattern of immune responses is a preexisting condition or whether abnormalities in immune responses develop in response to environmental influences. One of the keys to understanding the relationship between viral infections in infancy and asthma will be to determine the temporal sequence of these events, and the interplay with the unique pattern of environmental factors (stress, allergens, microbial products) in the inner city that could modify the clinical response to, and consequences of, viral respiratory infections. One final consideration is that the pattern of infectious diseases, and the age at which they are contracted, may be distinct in the inner city. This raises the possibility that a unique pattern of infections could modify lung and/or immunologic development in children, and that this could affect the risk of allergy and asthma.

1.1.2 How does the hygiene hypothesis relate to asthma in inner city children?

There are indications that some viral infections might actually protect against the subsequent development of allergies and asthma. This controversial theory, termed the "hygiene hypothesis," was first suggested by David Strachan, who noted that the risk of developing allergies and asthma is inversely related to the number of children in the family,[20] leading to speculation that infectious diseases, which are more likely to be transmitted in large families, modulate the development of the immune system to reduce the chances of developing allergies. This hypothesis implies that the immune system is skewed towards a Th2-like response pattern at birth.[21] This may in part be due to suppression of Th1-like responses by immunologically active factors in the intrauterine environment.[22] According to the theory, each viral infection would provide a stimulus for the development of Th1-like immune responses, and with this repetitive stimulation, the polarization would shift away from Th2 over-expression and thus reduce the risk for developing allergies.

Although early reports suggesting that contracting a single infectious disease, such as measles, can protect against allergies and asthma[23] have not been confirmed, the hygiene hypothesis is supported by studies that demonstrate an inverse relationship between attendance at day care centers, where exposure to viral infections is quite high, and a reduction in subsequent rates of allergy and asthma.[24] There is also evidence that it is not only the type of infection that is important, but also the route of exposure, as food-borne or enteric infections may have greater effects on allergy than respiratory infections.[25]

It is possible that distinct patterns of exposure to infectious diseases in inner city children could modify the incidence of allergy and asthma, and there are, in fact, several examples of differences in the epidemiology or clinical expression of infectious diseases that are related to ethnicity or culture. For instance, African-American and Native American populations acquire cytomegalovirus infections at an earlier age compared to middle-income Caucasian groups.[26,27] Furthermore, the seropositivity rates for other herpes viruses such as HHV-6 and EBV can vary between ethnic groups.[28,29] There may also be differences in the clinical severity of disease after infection: although RSV infections are nearly ubiquitous in the first three years of life, the rate of hospitalization is greater for African-American children, and those with low socioeconomic status.[30] Whether distinct patterns of infectious diseases in inner city infants affect the risk for developing allergy and asthma has not yet been determined.

1.1.3 Microbial exposure, atopy, and asthma

In addition to infectious diseases, exposure to microbes or microbial products in the environment has been related to wheezing, allergy, and asthma, although this relationship is complex. For example, elevated endotoxin levels in house dust has been associated with an increased risk of wheezing in the first year of life,[31] and increased severity of asthma in children.[32] On the other hand, growing up on a farm, and/or having exposure to higher levels of lipopolysaccharide (LPS), are associated with reduced rates of allergy and asthma.[33-35] Collectively, these findings suggest that exposure to microbial products in infancy may enhance respiratory illnesses to some degree in the short term, but may promote immune development and respiratory health in the long term. Unfortunately, there is very little information about the quantity and quality of innate stimuli in inner city homes.

The innate immune response is initiated by recognition of pathogen associated molecular patterns (PAMPs) through the toll-like receptors (TLRs).[36] Much attention has focused on endotoxin, which binds primarily to TLR4. TLR2 in combination with TLR6 or TLR1 responds to a wide range of PAMPs including peptidoglycans and microbial lipoproteins. The fungal wall polysaccharides [β (1-3) D-glucans] are probably also transduced by toll-like receptors. Because the signaling from TLR4 and from the other toll-like receptors is not identical – there is a MyD88 independent pathway for TLR4 not activated by other TLRs – it may be important to have a broad panel of markers to adequately characterize the environmental stimuli to innate immunity early in life.

One of the primary goals of the contract is to determine the apparent paradox between inner-city asthma and the current hygiene hypothesis of asthma. By all estimations, if the tenets of the hygiene hypothesis are correct, early exposure to infections and microbial products in the inner city should serve to markedly reduce the incidence of asthma compared to other locales in the United States. The converse, however, is the reality. Therefore, it is our proposal that the described clinical protocol associated with this application will begin to address this important question and determine the unique features of inner-city asthma and, from the proposal, provide important descriptive and selected mechanistic insights into this difference between events in the inner city and elsewhere as they relate to the pathogenesis of asthma.

1.1.4 How does allergen exposure affect asthma?

More than 80% of asthmatic children in the U.S. are allergic to one or more allergens. Despite this well-established relation between asthma and allergy, far less is known about whether allergens contribute to the development of asthma. Specific patterns of allergy are particular to asthmatic children in the inner city with higher rates of exposure to cockroach and rodents, as well as sensitization to these allergens compared to asthmatic children from other communities.[37,38]

Several recent prospective epidemiologic studies have demonstrated that increased home allergen exposure increases asthma morbidity in asthmatic children specifically sensitized to the allergen.[37,39] Exposure to indoor allergens may not only be a risk factor for aggravating existing disease, but for the development of childhood asthma as well. For example, birth studies have demonstrated that exposure to dust mite allergen in early life was associated with an increased risk of asthma at age 11 years,[40] and that exposure to cockroach allergen is associated with an increased incidence of wheeze in the first year of life and incident asthma among the sibling of the index children.[41] However, there is limited information regarding the role of allergens in the development of asthma in the inner city.

1.1.5 What is the relationship between stressful life events and asthma?

Stress has been associated with a number of effects on the developing immune system and respiratory health, and could be an important cofactor in the development or disease activity of asthma. In fact, there are indications that the effects of stress on developmental immunology could begin in utero. Both the prenatal and postnatal environments exert long-term influences through pathways connecting the autonomic nervous system, endocrine regulation, and the immune system by triggering the release of immunoreactive hormones and neuropeptides. By modifying the production of cytokines,[42,43] psychological stress influences cell trafficking, T-cell

function, and lymphocyte production.[44] It has been speculated that stress-induced alterations in the maternal or infant hormonal response may affect T cell differentiation towards a Th2 cell predominance.[45-47]

Epidemiological data also links early life stress to childhood asthma phenotypes. Wright and colleagues have demonstrated that caregiver stress prospectively predicts early onset of wheezing in infancy[48] and persistent wheezing at age five years[49] among 505 children. In this same birth cohort, these authors have shown that children living in households reporting high-level chronic stress have increased IgE expression and a more pronounced allergen-induced lymphocyte proliferative response compared with their counterparts living in low stress households.[50]

This proposed study will consider pervasive sources of stress unique to inner-city populations. Differential experiencing of stress including unique stressors in high-risk inner city environments may in part explain socioeconomic disparities in the asthma burden.[51] It may be that stress experienced as early as the prenatal period 'primes' the maturing immune system towards a Th2 phenotype which is further potentiated by subsequent environmental exposures (e.g., allergens, viruses). A potential consequence of stress-induced changes in immune response is suppression of host resistance to infectious agents, particularly viral agents. Psychological stress has been associated with the incidence of viral respiratory infection,[52,53] with increasing stress related in a dose-response manner to increasing risk of infection. In this context, stress may be hypothesized to not only increase susceptibility to viral respiratory illnesses but also may potentiate the inflammatory response in a multiplicative manner.

1.1.6 How does pollution relate to asthma in the inner city?

Several different pollutants have been linked to acute exacerbations of asthma, and studies of selected pollutants, suggest an effect on initiation of asthma as well. For example, exposure to ETS in early childhood has been linked to an increased frequency of respiratory infection, increased respiratory symptoms, and slightly reduced rates of lung function growth in childhood.[54] In addition, a growing body of data suggests that tobacco smoke exposure in utero and in infancy may influence the risk of asthma.[55] Smoking remains an even larger public health problem in inner-city communities than in other segments of the US population, and ETS is therefore likely to be a particularly important exposure variable to consider in a study of asthma pathogenesis in this community.

Nitrogen dioxide (NO_2) is a combustion product of both gas stoves and automobiles. In very high concentrations, NO_2 and other reactive nitrogen species formed during combustion can damage respiratory epithelium[56] and induce airway inflammation. The NCICAS investigators have demonstrated relatively high levels of indoor NO_2 in the homes of inner-city asthmatic children and have shown a significant association between high indoor NO_2 levels and asthma morbidity in this population (Kattan, personal communication). Indoor NO_2 exposure has been linked to an increased risk of asthma exacerbation following upper respiratory infection in asthmatic children,[57] and among subjects with allergic asthma, exposure to NO_2 may enhance the response to inhaled allergen.[58] Collectively, these data indicate that there are significant adverse effects of NO_2 on persons with existing asthma, and suggest that NO_2 can interact with other environmental factors to exacerbate airway symptoms. NO_2 exposure in inner city homes is particularly high, due to the frequent use of gas stoves and heaters. Collectively, these data

suggest that NO_2 could be an important contributor to respiratory symptoms and asthma disease in children raised in an inner city environment.

Other pollutants of potential importance to asthma in the inner city include fine particulate matter suspended in the air, and diesel exhaust particles (DEP).[59,60] For example, recent studies have revealed an association between the proximity of the home to roadways and the occurrence of childhood asthma. Considerable attention has recently been focused on DEP as a potential risk factor for allergic sensitization and asthma. The concentration of DEP in outdoor air varies greatly in relation to proximity to motor vehicle sources, and inner-city communities often have particularly high exposures because of their proximities to highways, industrial sites, and public transportation stations and routes.

1.2 Asthma and cytokine dysregulation

Recent observations have stimulated research efforts to further define the relative importance and pathophysiologic contributions of cytokine dysregulation (so-called Th1/Th2 imbalance) to the development of various atopic phenotypes, including asthma. Although questions remain as to the full impact of a Th1/Th2 dysregulation in established asthma,[61] the contribution of cytokine polarization to the inception and evolution of various atopic diseases including asthma has received more uniform support. At birth, cytokine profiling of cord blood suggests that the newborn infant's mononuclear cell responses are not fully developed, and this includes both Th1 and Th2 cytokine responses.[62] Although these changes are apparent in all newborns, those who later go on to develop clinical or serological evidence of atopy appear to have a distinct pattern of immune responses at birth or in early infancy. The observed abnormalities have included diminished IFN-γ production,[63-66] and surprisingly, reduced Th2-like responses (e.g. IL-13) as well.[67-69] This has led to the concept that immune development, including both Th1 and Th1-like responses, in atopic children may be delayed.[70] Following the neonatal period, there appears to be a progressive skewing of immune responses to allergen (and perhaps other stimuli) towards a Th2 phenotype.[71] Although genetic factors, and especially genes that contribute to the regulation of the innate immune system, contribute to the differentiation of adapted immune responses to allergens, observations in clinical studies (e.g. the hygiene hypothesis) and animal models suggest that environmental factors during early infancy may shape the development of cytokine responses, and thereby modify the risk of developing allergy and asthma.[72] Although studies of immune development as it relates to childhood allergies and asthma have been conducted in several countries around the world,[21,69,73] and have stimulated insights into the nature of these disorders, there is a striking lack of data from American inner cities.

Specific cytokines may play key roles in the pathogenesis of asthma in early childhood. Innate immune responses may play important roles in establishing tolerance to proteins in the environment, and the roles of CD14, IL-12, and IL-10 have been studied in this regard.[74-76] Production of IL-10 by T regulatory cells may be particularly important in preventing hyperresponsiveness and promoting tolerance to allergens in the airway.[77,78] In addition, IL-4 and IL-13 play critical roles in IgE antibody isotype switching, and IL-13 is of particular interest as it has been implicated in murine models with enhanced inflammation, mucus secretion, subepithelial fibrosis, and airway hyperresponsiveness.[79,80] IL-12 and IFN-γ are antagonistic to many Th2 cytokine responses, and IFN-γ is an important component of the antiviral response. Taken together, these observations strongly support continued study of the ontogeny of innate

and allergen-specific cytokine responses, especially as they relate to the roles of viral infections and allergen sensitization in the inception of asthma.

1.3 Genetics of asthma

Asthma has long been known to be a familial condition. Genome screen linkage studies of asthma and related phenotypes have been performed in families from various populations, and a growing number of linkages to various chromosomal regions have been reported. Linkage of asthma, serum total IgE, and/or nonspecific bronchial responsiveness to the chromosomal region 5q23-31 has been observed in most[81,82] but not all[83,84] populations studied. This region includes genes for IL-4, -5, -9, and -13, cytokines that appear to play important roles in asthma pathophysiology. Other putative genes in this region include the adrenergic receptor and 5-lipoxygenase. Linkage of regulators of innate immune responses to atopy[76,85] and asthma[75] have recently been reported, and may have particular relevance to the onset of allergies and asthma in infancy and early childhood.

The frequency of many genetic polymorphisms varies among racial and ethnic groups, but there is no clear evidence at present that excess asthma prevalence or severity in inner-city populations is explained by genetic variation. In all populations it is undoubtedly the case that gene-by-environment and gene-by-gene interactions are crucial determinants of asthma occurrence and severity.[86] The specific combinations of genetic factors and environmental exposures that predispose to asthma and severe asthma are as yet unknown and are the focus of investigations now underway. Such investigations hold the promise of targeting specific immune-based therapies to the asthma patients most likely to benefit on the basis of their genetic profile.

One of the barriers to investigating the genetics of asthma is that asthma seems to be a collection of different disorders that have several features in common, including airway inflammation, hyperresponsiveness, and eventual remodeling. Perhaps the clearest demonstration that asthma is a syndrome instead of a uniform disease process is in early childhood. This has greatly influenced the design of the URECA study, as one of the major goals of this study is to provide a clear phenotypic and immunologic characterization of recurrent wheezing in infancy and early childhood. Once these, and perhaps new, asthma phenotypes have been defined in inner city children, this will provide the basis for investigating genetic linkages with specific asthma phenotypes. As part of the URECA protocol, genetic studies will be conducted to evaluate polymorphisms in genes that regulate both innate (i.e., IL-8, TLR-3, -4, and -9) and adaptive (i.e., IL-4, IL-13, IL-10, IFN-γ) immune responses that may be involved in the pathogenesis of virus-induced wheezing illnesses, asthma, and other allergic diseases.

2 Rationale

Environmental exposures are likely to alter immune development by affecting the mother and intrauterine environment, and also through continued effects on immune development during infancy. Epidemiologic studies suggest that infections and/or exposure to microbial products could have dual effects: exposure to lipopolysaccharide or some types of infections in infancy could protect against allergies and asthma, while in contrast, severe or chronic respiratory infections (e.g. RSV bronchiolitis, mycoplasma pneumonitis) could promote the inception of asthma. The inner city environment is associated with unique patterns of allergens, innate immune stimuli (e.g. LPS), infections, pollutants (e.g. tobacco smoke), and stress during

pregnancy or soon after birth. There is very little information, however, to determine effects of these exposures on immunologic development and effects on allergies or asthma. Based on these observations, as well as preliminary data from and ongoing study of immune development and asthma in a cohort of non-inner city children (COAST, Childhood Origins of Asthma), we propose the following two-stage hypothesis (Fig 1):

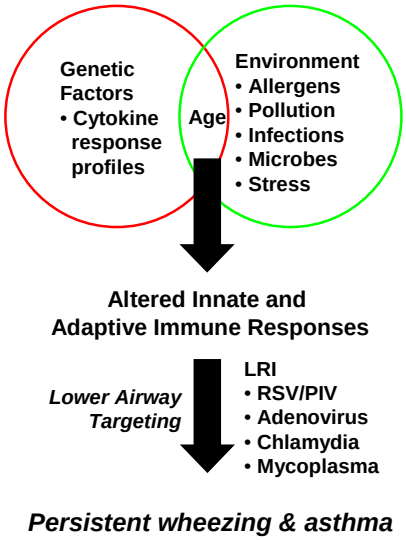

Figure 1. Recurrent wheezing and asthma: Hypothesis Stages 1 & 2

Stage 1
Environmental factors in the inner city adversely influence the development of the immune system to promote cytokine dysregulation (reduced production of IL-10 and IFN-γ by age one year, and increased IL-13 by age three years), allergy (increased cockroach-specific IgE), and recurrent wheezing by age three years. The relevant environmental factors include:distinct temporal patterns of microbial exposure and/or infectious diseases in infancy, an intensified and qualitatively distinct exposure to allergens (e.g., cockroach proteins) and pollution, and high levels of maternal and infant stress, both in the prenatal and postnatal periods.

Stage 2
Children who have had a viral lower respiratory infection and have developed cytokine dysregulation by age three years are at increased risk for the development of asthma by age six years.

To test these hypotheses, we will conduct a multicenter prospective study relating immunologic development and early environmental influences to the development of asthma in inner-city children. This study will combine elements and tools developed by the COAST study in Madison and the ACCESS study of inner city children in Boston. A major innovation in this protocol, compared to previous studies of asthma in the inner city, is to prospectively assess the importance of environmental factors in relation to the development of immune responses and asthma phenotype in this group of children.

3 Study Objectives

3.1 Primary Objective

The primary objective of the study is to establish in inner city children the immunologic causes for the development of recurrent wheezing by 36 months of age. To accomplish this objective, patterns of cytokine responses obtained throughout the entire 36-month evaluation period will be evaluated to determine their relationship(s) to recurrent wheezing.

Examples of specific hypotheses to be tested are as follows:

- IFN-γ responses at birth will be inversely related to the risk of recurrent wheeze at 3 years of age.
- Recurrent wheeze will be associated with an abnormal pattern in the development of IL-13 responses: IL-13 responses will be low at birth, and then elevated by three years of age.

3.2 Secondary Objectives

3.2.1 Indicators of Atopy

The secondary objective is to identify patterns of immunologic development associated with the development of atopy in inner city children. To accomplish this objective, patterns of cytokine responses obtained throughout the entire 36-month evaluation period will be evaluated to determine their relationship(s) to total IgE and allergen (cockroach)-specific IgE.

3.2.2 Effects of Environmental Exposures

Other secondary objectives are to identify environmental exposures associated with inner city life that modify immune development, and ultimately, the development of atopy and recurrent wheezing as defined above. Environmental factors to be analyzed will include: 1) the frequency and severity of respiratory infections in the first year of life, 2) exposure to microbial products (LPS, peptidoglycan, and ergosterol) in household dust, 3) exposure to selected allergens (cockroach, cat, dog, mouse, and dust mite proteins) in household dust, 4) prenatal and postnatal stress, and 5) household exposure to the pollutants NO_2 and tobacco smoke (nicotine). Outcomes at age three years to be considered will include recurrent wheeze, allergen-specific Th1 and Th2 cytokine responses, total IgE levels, and sensitization (allergen-specific IgE) to at least one allergen.

3.2.3 Immunologic and environmental risk factors for asthma.

This cohort was designed to have sufficient sample size and power to enable analysis of environmental and immunologic factors in early childhood that contribute to the risk of asthma by the age of 6 years. Upon completion of the current study, subjects and families will be invited to participate in a second protocol to continue observation and procedures which will begin at 4 years of age.

4 Protocol Overview

4.1 Study Design

The URECA study will be a longitudinal prospective evaluation beginning at birth up to the 4th birthday. Although the current study will involve a forty-eight month follow-up of study participants, the sample size will be large enough so that, if the initial phases of the study are successful, the children could be followed beyond the age of four years to assess the development of bona fide childhood asthma. Table 7.1a (visit schedule) illustrates the timetable for the protocol.

Families with a positive parental history of allergic diseases or asthma will be enrolled prior to birth. Maternal stress and other environmental exposures will be assessed prenatally. Beginning at birth and continuing for the first four years of life, the children will be evaluated longitudinally for blood cell cytokine responses, and for postnatal environmental influences (infections, allergen and microbial exposure, stress, indoor pollutants) that could affect the development of cytokine responses, and for clinical manifestations of allergy and asthma (recurrent wheeze). Finally, DNA will be obtained from study participants and their mothers to evaluate genetic correlates to the observed patterns of immune development as they relate to wheezing diseases and asthma.

As indicated in the timetable, periodic clinic visits will occur to perform physical examinations in order to track the development and persistence of significant lower respiratory tract symptoms. Scheduled visits will correspond with the home visit (age three months) for environmental assessment and dust collection, and yearly clinic visits beginning at age one year. Blood and nasopharyngeal mucus specimens will be obtained during the visits as indicated; these specimens will be used to evaluate changes in cytokine elaboration and for the presence of viral pathogens, respectively. Measurement of allergen-specific IgE and IgG will be performed at 12, 24 and 36 months of age in the enrolled children to determine the timing and the nature of the development of allergic sensitization and its relationship(s) to environmental conditions and/or patterns of in vitro cytokine responses. Frequent telephone contact will be maintained with the families to assess the development of respiratory infections, wheeze, the appearance of atopic diseases (food allergy, atopic dermatitis, chronic rhinitis, asthma), changes in environmental exposures (including stress), and to encourage participation in the scheduling and completion of study visits.

In addition to scheduled visits as indicated, the parents will be instructed to contact the study center at the earliest sign that their child is developing moderate cold symptoms or any lower respiratory tract symptoms (wheezing, coughing, retractions, etc.). Study coordinators and all physicians connected with the study will establish the necessary communication networks among families, primary care physicians, and study personnel to ensure that any significant symptoms are expeditiously reported so that a home or office visit can be arranged for the collection of a nasal mucus specimen for diagnostic virology, and if needed, an evaluation by a health care provider. A scorecard adapted from the COAST study will be used to assess the need for nasal lavage. A score of greater than or equal to 5, signifying a moderate to severe upper respiratory infection or a lower respiratory infection, will indicate the need to collect a nasal lavage sample for diagnostic virology.

Stress will be measured using questionnaires that will be administered repeatedly throughout the study to provide a dynamic and objective assessment of 1) pregnancy anxiety; 2) global stress; 3) difficult life circumstances; and 4) community-level stressors. Measures of potential buffers of the stress process (i.e., social supports, coping strategies) will also be assessed. Finally, exposure to relevant allergens (e.g. cockroach, dust mite, cat, and dog) and microbial products (e.g. LPS) will be determined through home visits with dust collection at the age of 3 months, 1-2 years, 2-3 years, and 3-4 years.

The URECA study will also include a genetics component. This will not require an additional venipuncture, as the blood sample will be obtained during venipunctures that are scheduled for the mothers and babies to provide samples for immunologic analyses. Tests will be conducted for genetic correlates to longitudinal measurements of: 1) cytokine secretion, 2) development of asthma and other atopic phenotypes, and 3) gene-by-environment interactions related to stress, and exposure to allergens, microbial products, pollutants (including prenatal exposure to tobacco smoke), and viral infections. Although the specific genes to be analyzed and methodology for gene analysis will be detailed in a separate protocol, it is anticipated that the analyses will focus on polymorphisms for genes that are involved in the regulation of inflammation, airway responsiveness, and/or have been identified as candidate asthma genes in other studies. Since it is anticipated that the large majority of participating mothers will be single, we will not attempt to perform genetic analyses on the fathers. Although the sample size for genetic analysis is relatively small, the detailed longitudinal information collected on these subjects should allow for meaningful analyses.

All URECA participants will also be invited to participate in the ancillary URECA Weight Study (Appendix 1). The objective of the URECA Weight Study is to examine the relationship between body weight/composition, childhood weight gain, asthma morbidity, and biomarkers of inflammation.

5 Study Population

5.1 Population Description

The URECA study will include a group of children who are at high risk for developing allergic diseases and asthma, on the basis of a parental history of asthma, allergic rhinitis or atopic dermatitis, and residence in the inner city. Babies who are born at 34 weeks gestation or later will be allowed into the study, provided that there is no significant respiratory distress as defined by the exclusion criteria, and they are otherwise healthy. Infants with chronic lung diseases such as respiratory distress syndrome, bronchopulmonary dysplasia and pneumonias will not be entered due to confounding effects on respiratory symptoms and lung function. Babies of HIV-infected mothers will be excluded due to immunomodulatory effects of HIV infection, and of anti-retroviral medications such as zidovudine (AZT).

5.2 Inclusion criteria

- Planning to deliver at the study hospital.

- A parental history of asthma, allergic rhinitis (hay fever), or eczema (atopic dermatitis). The presence of paternal allergy or asthma will be ascertained by maternal report.

- All study subjects will reside in census tracts with at least 20% of the residents with income below the poverty level.
- Gestational age at delivery of ≥ 34 weeks
- A suitable cord blood specimen must be obtained and processed to establish baseline cytokine secretion data.

5.3 Exclusion Criteria

- Respiratory distress requiring intubation and ventilation for four or more hours.
- Respiratory distress requiring either supplemental oxygen or CPAP for four or more days.
- Pneumonia requiring antibiotic treatment for one week or more.
- Significant congenital anomalies.
- Maternal HIV infection at time of delivery.
- Plans for the family to move out of the geographic area during the period of the study.
- Does not consent to all aspects of the study.
- Does not have access to a phone.
- Does not speak English (or Spanish at sites with Spanish-speaking staff).
- Administration of palivizumab (Synagis) for RSV prophylaxis.

Interested families who fail to qualify for the URECA study, because of not having a history of asthma, allergies, or eczema, but who meet all other inclusion and exclusion criteria, will be invited to join the Non-Allergic Families substudy of URECA, described further in Appendix 2.

6 Recruitment Methods

Our goal for this protocol is to recruit and retain the desired number of families (approximately 500) to not only permit analysis of the recurrent wheezing and other indicators of atopy at age three years, but also allow for sufficient statistical power to conduct a possible extension of this trial to age six years. This would allow for additional follow-up and also a more precise analysis of the effects of immune development and environmental factors on asthma, which can be more accurately diagnosed at this age. In Version 15.0 of the protocol, the study was extended from 36 months to age 48 months.

6.1 Recruitment Strategies

Recruitment of families for this study will be accomplished using methods and resources that have proven successful in the NCICAS, COAST, and ACCESS studies. The latter two studies

required prenatal enrollment, and the COAST study also required the acquisition of cord blood specimens. As a result of our experience with these studies, expectant families will be informed of their option to participate in our study using the following methodology.

Nurses in selected obstetrics clinics will inform patients about the study, and will refer interested women to the URECA staff. URECA staff will contact the potential participants over the phone or in the clinic and, after obtaining verbal consent, will administer a short screening questionnaire that includes a history of atopic diseases and asthma. If the pregnant woman meets eligibility requirements for inclusion into the study, the recruiter will explain the nature of the study and obtain written informed consent for participation. They will arrange with the patient to meet for the Prenatal Visit. If the Prenatal Visit is early in the pregnancy (before the 8th month), a follow-up phone call will be scheduled within a month of the projected delivery date to review study procedures. The chart of the patient will be flagged so that the patient can be easily identified as a study participant in order that study personnel will be notified of the delivery and cord blood obtained. The recruiters will also check the deliveries on a daily basis for study participants.

In addition, local obstetric practices will be provided with brochures to be included in the birthing information packets provided to parents prior to delivery as well as at the front reception desk. Local pediatric practices will be contacted and visited to explain the study and to clarify the role of the primary physician/clinic throughout the study. These visits will be incorporated into a breakfast or luncheon. These visits will establish rapport as well as allow the opportunity to highlight the benefits to referring physicians, such as obtaining a nasopharyngeal mucus sample for virus identification.

In addition, general recruitment methods, including flyers throughout the community and targeted newspaper advertising, etc., will be employed. Dedicated phone lines with daytime staff availability and voice mail will be used for all incoming study inquiries. Upon confirming interest in participation and verbal consent, the screening questionnaire will be administered over the phone or scheduled for a later time. If the mother is eligible and interested, the Prenatal Visit will be scheduled. The Prenatal Visit must be done prior to delivery and will involve informed consent, a parental history form and other questionnaires. To minimize demands on the mother's time, this visit will be scheduled in coordination with a prenatal check-up whenever possible. Parents will be asked if they have chosen a primary physician for their infant, and if so, the address and phone number of the physician will be recorded. Parents will be provided with "useful" items imprinted with the phone number of the research staff, such as adhesive stickers to apply to their phone, over-the-counter medication bottles, name tags/key chains for diaper bags or purses, refrigerator magnets, and other items to remind them to call the study center if their child is ill with a respiratory infection meeting criteria for nasopharyngeal mucus sampling.

Additional measures will be taken to enable and facilitate the recruitment of Hispanic study subjects. First, clinical coordinators who are fluent in both English and Spanish will be hired at the sites with significant numbers of Spanish speakers. In addition, recruitment materials and all questionnaires will be translated so that both English and Spanish versions are available.

Many families who live in inner cities consist of single mothers. To accommodate this family structure, we have designed the study so that the family history will be obtained from the

primary caretakers, and this could include grandmother, mother, or father. Whenever possible, the maternal medical records will be reviewed by obstetrics staff to gather specific information regarding maternal allergic diseases and asthma. Asking the father to undergo a medical evaluation may not always be feasible. We have therefore not planned to include genetic or immunologic analyses of the fathers.

We realize that this approach, and the study design in general, will result in a study population who will be heterogeneous in terms of their risk factors for asthma. This is advantageous in that not all of the children will develop allergies and asthma, and this will provide for a non-allergic non-asthmatic reference group within our study population. Differences in cytokine development between asthmatics, allergics, and normal babies may reveal specific linkages between immunologic development and atopic/asthmatic phenotypes.

6.2 Retention

Retention methods also involve a number of different approaches. First, we will actively utilize an appointment reminder system that consists of phone calls one week and one day prior to scheduled appointments for confirmation. Interval phone contacts will also be made to families between appointments to answer any questions and assess the status of the-participant. To facilitate telephone contact with subjects whose phone service may change during the study, or those families who do not have a telephone, at least three telephone contact numbers (relatives, neighbors, friends) will be collected for each subject. This has proven to be an effective strategy in the NCICAS, and the ongoing ACCESS protocol.

After delivery, a brief visit will be made to the mother in the hospital and an incentive package, consisting of a small diaper bag, useful items such as diapers or a bib, and a URECA refrigerator magnet, will be left. A participation thank you and congratulatory card will be mailed to the family at approximately one month of age. Pediatricians and family practice physicians for participating infants will be mailed a letter informing them of their patient's participation in the study. This will include a reminder to the physician that a nasopharyngeal mucus virus sample can be provided upon request. After the baby is discharged from the hospital, a phone call will be made to the family to schedule the home visit, and answer any questions related to the study. For all successive visits, an interval call will be made between visits as well as an appointment reminder call the day before the appointment. A study-specific family newsletter will be designed for publication every two to three months to update the family on the status of the study, remind them of study-specific criteria such as respiratory symptoms, as well as general asthma educational information. Regular postcards will be mailed in order to ascertain current mailing addresses and to thank the family for continued participation. Birthday cards will be mailed to the children at each birthday.

We will send a URECA holiday card and the participating families will receive small gifts as a token of appreciation. We will provide transportation to the research center or perform home visits when necessary. Our clinical coordinator staffing model will include convenient times for family appointments to include weekend and evening availability for study visits. Voicemail will be available after hours for notification of a viral illness. Parents will be offered modest monetary compensation for their time and participation in the study (Table 6.3a). Finally, our continued emphasis on establishing a positive long-term relationship with our subjects and their

families and letting them know we are interested in their well-being while they assist us with our research objectives no doubt provides the greatest motivation for continued participation.

6.3 Reimbursement

Table 6.3a. Reimbursement Schedule

Visit	Reimbursement	Actual Travel Cost Up To
Prenatal Visit	$50	$40
3 mo. Home visit	$50	
6 mo.	$20	
9 mo.	$20	
12 mo. Clinic visit	$60	$40
15 mo.	$20	
18 mo.	$20	
21 mo.	$20	
24 mo. Clinic visit	$60	$40
27 mo.	$20	
30 mo.	$20	
33 mo. Clinic visit	$60	$40
36 mo. Clinic visit	$75	$40
39 mo.	$20	
42 mo.	$20	
45 mo.	$20	
48 mo.	$20	$40
Year 2 dust collection	$15	
Year 3 dust collection	$15	
Year 4 dust collection	$15	
Nasal sample collection	$10 (may receive more than once)	
TOTAL	**$630+**	**$240**

Participants using cell phones to take study phone calls are also compensated for the use of their cell phone minutes based on the length of the call, as specified in the Manual of Operations.

7 Study Visits

7.1 Visit Schedule

Table 7.1a URECA Visit Schedule

	Pre-natal visit	Birth	3 mo home visit	Pick up PM	6 mo call	9 mo call	12 mo visit	15 mo call	18 mo call	21 mo call	24 mo visit	27 mo call	30 mo call	33 mo visit	36 mo visit
Reimbursement Amount	$50		$50		$20	$20	$60	$20	$20	$20	$60	$20	$20	$60	$75
Informed Consent	20														
Questionnaires	30		30		15	15	30	15	15	15	30	15	15	30	30
Form 1 - Screening/Eligibility - Mother	X														
Prenatal Visit															
Form 2 - Additional Contact Information	X														
Form 3 - Demographics	X						X				X				X
Form 4 - Smoking History and Alcohol Use	X														
Form 5 – Respiratory and Allergy History	X														
Form 6 - Pregnancy Anxiety	X														
Form 7 - Edinburgh Postnatal Depression	X		X				X				X				X
Form 8 - Perceived Stress Scale	X		X		X	X	X	X	X	X	X	X	X	X	X
Form 9 – Difficult Life Circumstances	X						X				X				X
Form 10 - Neighborhood/Block Conditions	X						X				X				X
Form 11 - Neighborhood Violence	X						X				X				X
Form 12 - Housing Stress	X						X				X				X
Form 13 - Brief COPE	X						X				X				X
Form 14 - Socioeconomic Status	X						X				X				X
Form 15 – Social Supports/Networks	X						X				X				X
Form 16 – Child Sleep Questionnaire							X				X				X
Form 20 - Birth Record		X													
Form 21 - Hospital Visit		X													
Form 22 - Eligibility Checklist		X													
Form 25 – Maternal Weight	X														
Quarterly Phone Calls															
Form 30 - Synagis Use			X		X										
Form 31 - Contact Information Update			X		X	X	X	X	X	X	X	X	X	X	X
Form 32 - Respiratory and Allergy Symptoms			X		X	X	X	X	X	X	X	X	X	X	X
Form 33 - Medication List			X		X	X	X	X	X	X	X	X	X	X	X
Form 34 - Infant Feeding/Diet			X		X	X	X								
Form 35 - Smoking Update			X		X	X	X	X	X	X	X	X	X	X	X
Form 36 – Child Allergy Symptoms and Triggers Update							X				X				X
Form 37 - Postnatal Respiratory History Review			X												

	Pre-natal visit	Birth	3 mo home visit	Pick up PM	6 mo call	9 mo call	12 mo visit	15 mo call	18 mo call	21 mo call	24 mo visit	27 mo call	30 mo call	33 mo visit	36 mo visit
Form 38 – Child Diet								X	X	X	X	X	X	X	X
Form 40 - Home Environment Questionnaire			X				X				X				X
Respiratory Illness															
Form 50 - Respiratory Illness Scorecard[1]															
Form 53 - Respiratory Illness Follow-up Phone call[1]															
Form 54 - Record of Medical Visit[1]															
Form 55 – Emergency Medical Recommendation Follow-up[1]															
Study Procedures															
Form 61 - Cord Blood Sample Collection[2]		10													
Form 60 – Maternal Blood Sample Collection[2]							10								
Form 64 – Child Blood Sample Collection[2]							10				10				10
Form 62 – EASI Scoring System			10				10				10				10
Form 39 – Child's 3-Month Weight and Length			5												
Form 63 - Physical Examination[3]							10				10				10
Form 68 – Bioelectrical Impedance Analysis (BIA)							10				10				10
Form 51 – Clinic Nasal Lavage Sample Collection[4]							5								5
Form 66 – IOS and Spirometry														30[5]	30
Form 65 – Allergen Skin Test Results														30	
Home Environment Procedures			45												
Form 41 - Home Environment Observation[6]			X						X				X		
Form 42 - Dust Sample Collection[6]			X						X				X		
Form 43 - Pollution Monitoring			X												
Form 44 - Pollution Monitoring Follow-up[7]				X											
Estimated Time Needed Per Timepoint (hrs)	1.0	0.3	1.5	0.3	0.3	0.3	1.5	0.3	0.3	0.3	0.8	0.3	0.3	1.5	1.6

	39 mo call	42 mo call	45 mo call	48 mo visit
Reimbursement Amount	$20	$20	$20	$20
Informed Consent				
Questionnaires	15	15	15	10
Form 1 - Screening/Eligibility - Mother				
Prenatal Visit				
Form 2 - Additional Contact Information				
Form 3 - Demographics				
Form 4 - Smoking History and Alcohol Use				
Form 5 – Respiratory and Allergy History				
Form 6 - Pregnancy Anxiety				
Form 7 - Edinburgh Postnatal Depression				
Form 8 - Perceived Stress Scale	X	X	X	
Form 9 – Difficult Life Circumstances				
Form 10 - Neighborhood/Block Conditions				
Form 11 - Neighborhood Violence				
Form 12 - Housing Stress				
Form 13 - Brief COPE				
Form 14 - Socioeconomic Status				
Form 15 – Social Supports/Networks				
Form 16 – Child Sleep Questionnaire				
Form 20 - Birth Record				
Form 21 - Hospital Visit				
Form 22 - Eligibility Checklist				
Form 25 – Maternal Weight				
Quarterly Phone Calls				
Form 30 - Synagis Use				
Form 31 - Contact Information Update	X	X	X	X
Form 32 - Respiratory and Allergy Symptoms	X	X	X	
Form 33 - Medication List	X	X	X	
Form 34 - Infant Feeding/Diet				
Form 35 - Smoking Update	X	X	X	
Form 36 – Child Allergy Symptoms and Triggers Update				
Form 37 - Postnatal Respiratory History Review				
Form 38 – Child Diet	X	X	X	
Form 40 - Home Environment Questionnaire				
Form 99 – Study Evaluation Form				X

	39 mo call	42 mo call	45 mo call	48 mo visit
Respiratory Illness				
Form 57 - Respiratory Illness Scorecard – 3-6 Year-olds[1]				
Form 53 - Respiratory Illness Follow-up Phone call[1]				
Form 54 - Record of Medical Visit[1]				
Form 55 – Emergency Medical Recommendation Follow-up[1]				
Study Procedures				
Form 61 - Cord Blood Sample Collection[2]				
Form 60 – Maternal Blood Sample Collection[2]				
Form 64 – Child Blood Sample Collection[2]				
Form 62 – EASI Scoring System				
Form 39 – Child's 3-Month Weight and Length				
Form 63 - Physical Examination[3]				
Form 68 – Bioelectrical Impedance Analysis (BIA)				
Form 51 – Clinic Nasal Lavage Sample Collection[4]				
Form 66 – IOS and Spirometry				
Form 65 – Allergen Skin Test Results				
Home Environment Procedures				
Form 41 - Home Environment Observation[6]		X		
Form 42 - Dust Sample Collection[6]		X		
Form 43 - Pollution Monitoring				
Form 44 - Pollution Monitoring Follow-up [6]				
Estimated Time Needed Per Timepoint (hrs)	0.3	1.3	0.3	0.3

Updated 08/03/2007

[1] Per protocol when child is ill.
[2] For RAST, absolute eosinophil counts (child only), cotinine, and immunologic studies (cytokine secretion assays and cellular studies)
[3] Exam of skin, nose, ears, and chest by URECA physician
[4] Specimens also collected during symptomatic LRIs
[5] Time allocated for teaching
[6] Additional dust samples will be obtained between Years 1 and 2, between Years 2 and 3, and between Years 3 and 4. The sampling will be performed to coincide, whenever possible, with a home visit to collect a nasal lavage specimen for an acute respiratory illness (see section 7.1.4).
[7] Follow-up visit scheduled for 2 weeks later; pick up pollution monitors

7.1.1 Prenatal Visit

Recruitment for this study will occur in obstetrics clinics at each of the clinical sites, and will be conducted by a collaborative effort between the obstetricians, obstetrics staff and the URECA coordinators, in accordance with IRB and HIPAA standards at each institution. The prenatal visits are of varying length, and so the procedures that are described as occurring during the "prenatal visit" may sometimes be spread over two visits. Optimally, the activities can be coordinated with the glucose tolerance test, which generally lasts several hours, thus affording the opportunity to complete all of the prenatal activities in a single session.

Informed consent will be obtained by the URECA staff. If the mother is a minor, informed consent will be obtained from her parent/guardian, following applicable state laws and local IRB procedures. Once a subject has given informed consent, a number of questionnaires will be administered to assess the family medical history, environmental exposures in the home, and anxiety and stress as perceived by the expectant mother. Mothers will be provided with written instructions for the first 2 months of the study at the Prenatal Visit including written information on how to contact the research center. Parents will also be informed and provided written information on the symptoms that need to be reported to the research center. These symptoms will be tabulated by the center staff to determine the "score" and the need for a nasal wash sample. It is estimated that these study procedures and questionnaires can be completed in approximately 1 hour.

7.1.2 Birth - Delivery Room

A sample of blood will be obtained from the umbilical cord at the time of birth, according to procedures established in the URECA Manual of Operations. The procurement of this blood sample will be critical to the longitudinal nature of this study and, as discussed previously, we will expand on the network of personnel and communication strategies that have previously proven to be successful in order to obtain the cord blood samples in the COAST and ACCESS studies. Briefly, this will involve clinical coordinator communications with the parents and obstetricians, the handling of the cord blood sample by on site obstetrics or nursery personnel, and the subsequent transportation and processing of the sample by clinical coordinators and research specialists to the laboratory personnel. Kits will be provided to the obstetric areas in the areas used by the staff at a time when delivery is imminent, and these kits will include protocols related to the collection of the cord blood specimen. All labor and delivery staff will receive a URECA inservice at a staff meeting prior to study initiation to assure comprehension of procedures. Staffing in the laboratory will be designed so that blood specimens will be processed 7 days per week, and within 16 hours of the time of collection. Study personnel will check the participating obstetric units daily for blood specimens to be transported to the immunology laboratories for processing.

7.1.3 Three-Month Home Visit

The three-month visit will be conducted in the home to enable dust collection and assessment of exposure to indoor pollutants. The visit will be scheduled by telephone with a reminder the day before the visit date. A detailed home environmental inspection will be performed, as well as an assessment of symptoms and health care visit utilization for respiratory illnesses. Dust will be collected according to established procedures. In addition, passive monitors for NO_2 and nicotine will be placed in the home, and a time will be scheduled with the family to enable the

monitors to be picked up from the home two weeks later. Two study coordinators will visit the home for this visit, and this will help to ensure the safety of the coordinators, as well as help to minimize the intrusion on the family's schedule, since one person can interview the parent(s), while the other proceeds with the environmental sampling procedures. One of the coordinators will be a nurse, so that the child can be examined and evaluated for atopic dermatitis. It is anticipated that this visit will take approximately one hour to complete.

7.1.4 Home Visits for Respiratory Illnesses

In addition to scheduled visits as indicated, the parents will be instructed to contact the study center at the earliest sign that their child is developing lower respiratory tract symptoms (wheezing, coughing, retractions, etc.). Study coordinators and all physicians connected with the study will establish the necessary communication networks among families, primary care physicians, and study personnel to ensure that any significant symptoms are expeditiously reported so that a home visit can be arranged for the collection of a nasal mucus specimen for diagnostic virology, and if needed, an evaluation by a health care provider. For example, parents will be given a laminated card to place in their child's diaper bag or purse outlining the need for nasopharyngeal mucus acquisition with respiratory infections, and a note to remind the child's primary physician of this study-required procedure at the sick visit. Parents will also be given refrigerator magnets with respiratory infection symptom criteria and the contact number for the study personnel.

The home visits will be conducted by a team of two to help maximize the safety of the study personnel and to facilitate completion of the study procedures. One of the team will be a nurse; in addition to collecting the nasal lavage fluid, a brief respiratory assessment will be performed. For infants with signs and symptoms of respiratory distress, the nurse will recommend that the family contact the health care provider for further evaluation. The research team will save part of the nasal lavage fluid, and will provide it to the health care provider for diagnostic testing upon request. No clinical care will be provided by the research personnel, other than general advice related to the home treatment of upper respiratory symptoms, and a review of signs and symptoms that might indicate a need to contact their health care provider. It is also anticipated, based on experience in the COAST study, that it will occasionally be necessary to conduct study visits for respiratory illnesses in a doctor's office, emergency department, or other health care facility. In this case, these efforts will be coordinated with the other health care workers so that we do not interfere with the evaluation or treatment of the illness.

It is also planned that dust sampling will be repeated for each home between the child's 1st and 2nd birthdays, between the 2nd and 3rd birthdays, and between the 3rd and 4th birthdays. The dust sampling will coincide, whenever possible, with a home visit for an acute respiratory illness. This will provide data, together with the scheduled three-month home visit, for a longitudinal assessment of environmental exposures to microbial products (e.g. endotoxin) and key allergens (e.g. cockroach, cat, dust mite) for the duration of the study. If an acute respiratory illness visit is not scheduled for an individual child by six months after the birthday, a visit will be scheduled just for dust collection.

7.1.5 Six- and Nine-Month Phone Calls

Phone calls will be placed to the family when the infant is approximately six and nine months of age to collect data related to study outcomes, remind the families to contact the study coordinators in case of respiratory infections, and also to continue to engage the family in the study and provide informational updates. Questionnaires to be administered will collect data on respiratory symptoms, environmental exposures and diet, health care utilization and medication use, contact information, and a brief Perceived Stress Scale.

7.1.6 12- and 24-Month Clinic Visits

These visits will be conducted in the study center to facilitate study procedures such as phlebotomy in these young infants. Blood and nasopharyngeal mucus specimens will be obtained from the child during the 12-month visit, and blood will be obtained again at age 24 months. The blood specimens will be used to evaluate changes in cytokine elaboration and immune development, allergic sensitization, and a plasma cotinine measure will indicate personal exposure to tobacco smoke. The nasopharyngeal specimen at age one year will help to establish the rate of viral detection in asymptomatic children. Questionnaires will be administered by study coordinators to survey for viral illnesses, and signs and symptoms of asthma, food allergies, atopic dermatitis and allergic rhinitis. The questionnaires will also ask about any changes in the home environmental survey since the last contact with study personnel. A brief physical examination will be conducted, to focus on ears, nose, throat, chest and skin.

At the 12-month visit, a blood specimen will be obtained from the mother. This will be used to isolate DNA, and also to subject the blood to a panel of immunological studies similar to the child's panels.

Procedures and samples that may be missed at a given visit, due to illness or another reason, may be rescheduled.

7.1.7 15-, 18-, 21-, 27-, 30-, 39-, 42-, and 45-Month Phone Calls

Phone calls will be placed to the family at these intervals to collect additional data, remind the families to contact the study coordinators in case of respiratory infections, and also to continue to engage the family in the study and provide informational updates. Questionnaires to be administered will collect data as indicated for the 6- and 9-month phone calls. These phone calls should be completed in 15-20 minutes.

7.1.8 33-Month Clinic Visit

At this visit, the coordinators will help the parent(s) to complete a health and environmental update, and teaching will occur related to pulmonary function measurements. Prick skin testing for aeroallergens will be performed. This visit will be completed in about 1.5 hours.

7.1.9 36-Month Clinic Visit

This visit will include all of the study procedures listed for the two-year visit (blood specimen, questionnaires), and additional effort will be spent on training the child to perform technically satisfactory pulmonary function measurements. Nasal lavage will be performed to ascertain the

prevalence of asymptomatic viral infections at three years of age. This visit will require approximately 1.6 hours to complete.

7.1.10 48-Month Clinic Visit

This end-of-study closeout visit will include a study evaluation form and other brief questionnaires. Study staff will give the parent/guardian a study completion certificate and reports of study results pertaining to their child, such as RAST results, skin test results, and results of pulmonary function testing, and they will answer any study-related questions the participants may have.

7.1.11 Emergency Department Visits and Hospitalizations

We will learn about emergency department visits and hospitalizations during scheduled interviews, and, whenever possible, an attempt will be made to abstract information from medical records of visits that involved serious respiratory illness.

8 Outcome Assessments

8.1 Primary and Secondary Outcomes

For this study, precisely defining a set of criteria for characterizing a child as having recurrent wheezing is critical for the analyses of the various relationships of this clinical phenotype with the immunologic and microbiological data that will be collected during the first three years of observation. As stated earlier, the diagnosis of asthma in infancy is problematic due to the lack of differentiating features between asthma and transient wheezing caused by recurrent viral LRI.[5] For this reason, we have selected recurrent wheezing, rather than asthma per se. For the purposes of the study, recurrent wheezing is defined as at least two episodes of wheezing in the first three years of life and one of these episodes of wheezing must have occurred during the third year.

Data regarding the frequency and severity of wheezing and wheezing illnesses will be collected using several different means. First, the parents will be specifically asked about infant wheezing during each of the quarterly telephone calls. These calls will also be reminders for the parents to notify study personnel in the event of a respiratory infection, and particularly those illnesses associated with wheezing or severe cough. Upon notification of a respiratory illness, the scorecard specifically asks about wheezing and respiratory distress. Examinations will also be conducted as part of the scheduled study visits at ages 1, 2, and 3 years, and questions about wheezing will be asked at these visits and during the quarterly telephone calls. All of this information will be recorded into a centralized database to facilitate data analysis. Finally, we will exclude children with any airway problems that could lead to confusion in making a proper diagnosis of recurrent wheezing (e.g. cystic fibrosis, immune deficiency, immotile cilia syndrome, anatomical abnormalities such as laryngotracheomalacia, foreign body aspiration, etc.).

A number of secondary outcome variables will also be assessed as follows:

1) **Stringent index for the prediction of childhood asthma** from the Tucson Children's Respiratory Study – early frequent wheeze plus one major criteria (asthma in a parent, or

doctor-diagnosed eczema) or two minor criteria (doctor-diagnosed allergic rhinitis, wheezing apart from colds, or peripheral blood eosinophilia [≥4%]).[12]

2) **Atopy**, defined as either a total IgE > 60 kU/L at age three years, atopic dermatitis (doctor-diagnosed or observed by our staff at any time during the study), or one or more positive skin tests to aeroallergens at age three years.

3) **Use of an anti-inflammatory medication for asthma** (corticosteroid, Cromolyn, Montelukast) for at least two weeks after age two years. This will be ascertained by questionnaire administered to the mothers.

4) **Pulmonary function**; and specific measurements to be analyzed will include peak expiratory flow and impulse oscillometry at age three years. It is anticipated that although not all of the children will be able to complete these measurements, the use of a training session at 33-months will help to maximize the number of successful procedures.

5) **Asthma** diagnosed by the child's health care provider.

8.2 Safety Assessments

8.2.1 Phlebotomy

The sample collection procedures to be performed in the proposed URECA project include the collection of peripheral blood samples of the children. A topical anesthetic such as EMLA cream will be offered before all blood draws to minimize discomfort in the children. All venipunctures will be performed either by a specially trained pediatric phlebotomist or research coordinators who have been trained in accordance with procedures outlined in the URECA Manual of Operations.

8.2.2 Nasal Lavage

The nasopharyngeal mucus samples will be obtained using techniques and materials used in the COAST study that are proven to be effective and well tolerated by infants and young children. All personnel performing these tests will be trained in accordance with standard procedures established by ICAC for this study and detailed in the URECA Manual of Operations.

8.2.3 Skin Testing

Skin testing to a panel of aeroallergens will be performed at 33-36 months of age. These will include a panel of well-characterized antigens that have been associated with respiratory allergies and asthma in this age group. Only personnel that are trained in the procedures established by the URECA Manual of Operations will perform this test. In addition, testing will be performed in accordance with generally accepted guidelines.[87]

8.2.4 Pulmonary Function Tests

Pulmonary function testing will only be performed by personnel who have been trained in accordance with the Manual of Operations, and certified in this regard. At the 33-month visit, a training session for pulmonary function tests will be conducted, and actual procedures will be attempted at age 36 months. Children in respiratory distress will not be tested, and rescue

medications will be available at the site in the unlikely event that performing the pulmonary function maneuvers triggers acute respiratory distress.

9 Special Study Assessments and Techniques

Several different immunologic assays will be utilized to help measure immunologic development in study subjects and their mothers, with a particular focus on immunologic features associated with atopy and asthma. It should be emphasized that this is an area in which knowledge is rapidly advancing, and whenever possible, methods will be updated or expanded to include new immunologic techniques or measurements related to these specific goals. This will be accomplished through yearly reviews of the assays and results by the URECA Scientific Advisory Panel, and through collaboration with the Immune Tolerance Network (ITN). In particular, URECA investigators will make an effort to take advantage of efforts by the ITN to standardize immunologic techniques and assays so that the results generated in this study will have maximum validity and comparability with studies conducted by other investigators. Specific details of procedures and analytes are listed in the following sections with the expectation that these are likely to change as a result of this reevaluation process. Care will be taken to ensure that once these procedures are established, they will be conducted in a consistent fashion throughout the study to ensure comparability of the data over the time course of this longitudinal study.

9.1 Blood Cell Responses and Serology

To establish patterns of cytokine secretion at birth and changes in these responses as well as the development of allergen-specific IgE in early childhood, blood samples will be obtained at birth (cord blood) and yearly until the age of 3 years to measure mononuclear cell *ex vivo* generation of cytokines (Table 9.1a) and allergic sensitization. Given the growing body of information generated from several different types of studies, including in vitro and studies, clinical protocols, and genetic analysis, implicating many different cytokines in the pathogenesis of allergic inflammation and asthma, a relatively large number of cytokines will be measured. These cytokines include those that regulate Th1 (IL-12, IFN-γ) or Th2 (IL-4, IL-5, IL-13) inflammation, those involved in airway dysfunction such as hyperresponsiveness and mucus cell hyperplasia (IL-9), cytokines with anti-inflammatory properties (IL-10, TGF-β), or those associated with innate immune responses (IL-8, TNF-α). To accomplish this, a microsphere-based multiplex immunoassay system will be used.

9.1.1 Obtaining cord blood samples

In order to ensure the procurement of the cord blood sample, this study has adapted and expanded upon the procurement procedures that were successful in the COAST and ACCESS protocols. Nurses working in the delivery suites will be alerted to the names of mothers who have previously consented to participate in the protocol and who are expected to deliver within the next two to three weeks by a specific, repeatable chart identification method. The physicians responsible for the prenatal care of the mothers will also be notified of the mother's desire to participate in this study. Cord blood samples will either be obtained by the nurses or physician staff on duty in the delivery suite. Specimens of peripheral or cord blood will be kept at room temperature according to established protocols (see Manual of Operations for more details) and

processed within 16 hours of delivery of the infant; cells will remain viable for this time period allowing flexibility with day and nighttime processing.

Mononuclear cells will be separated from the cord blood (or at subsequent visits, peripheral blood) using a standardized technique, and cytokine stimulation assays will be conducted. Supernatants from the stimulated cell cultures will be frozen (-80°C) and stored for subsequent analysis in batches at the laboratory in Madison.

Cord blood specimens can be contaminated with bacteria and maternal blood. We have chosen to use a "closed technique" for collection that involves using a syringe and needle to puncture an umbilical vessel in order to minimize the risk of contaminated specimens. Even when careful collection techniques are employed, small numbers of maternal blood cells (0.1-6%) can be detected in many cord blood specimens using sensitive molecular assays for maternal DNA.[88-90] Furthermore, there is evidence that the maternal cells in cord blood specimens cannot be explained solely on the basis of contamination during the collection procedure: there is solid evidence that gestation is characterized by a limited bidirectional exchange of nucleated cells and plasma DNA between the maternal and fetal circulation.[91] However, the small number of maternal cells has not precluded the use of these cells in genetic studies[92-96] or in studies involving measurements of mononuclear cell function.[97] For the URECA study, we will assume that the presence of small numbers of maternal cells in the cord blood specimens is expected, and there will be no prospective monitoring of this phenomenon. Since we will be saving frozen aliquots of cord blood cells when extra cells are available, it will be possible to retrospectively analyze the frequency of maternal cells in most of the cord blood specimens if this is desired in the future.

9.1.2 Mononuclear cell cytokine production

At the clinical sites cord or peripheral blood mononuclear cells will be incubated in the presence of AIM V serum-free medium and stimulants. Table 9.1a lists an approximation of the stimulants and concentrations to be used in these assays, and the final panel and experimental conditions will be established by preliminary experiments, and may be modified according to new advances and advice from study Investigators, Immunologists, and the Scientific Advisory Panel. Control specimens of cells will be incubated with medium but no additional stimulus. Cell supernatant fluids will be harvested, divided into aliquots, and frozen at -80°C pending analysis. The studies will require 1.4×10^7 cells to complete the entire panel of stimuli, and this should be possible, based on experience with the COAST study, in 90% of infants at ages 1, 2, and 3 years. If cell numbers are limited, the stimuli will be prioritized, such that innate immune stimuli have higher priority in early life, and allergen-specific immune stimuli will have greater priority by the three-year time point (Table 9.1a).

PBMC supernatants will be analyzed for the presence of cytokines utilizing a multiplex assay system (e.g. Luminex®). This technology can quantitate 15 or more cytokines from a single 50 µL aliquot.[98] The ability to perform multiple measurements on a small volume of culture supernate is advantageous given the limited number of PBMC that are anticipated, particularly at the 1-year time point. The sensitivity of this assay varies from 2-20 pg/ml depending on the specific cytokine, and this level of sensitivity is comparable to standard ELISA. All measurements will be performed in duplicate. Standard ELISA techniques will be used to analyze any cytokines (e.g. IL-9) or other immune factors (e.g. soluble CD14) for which Luminex reagents are not available.

Table 9.1a: Stimulants for Cytokine Secretion Assays

	Stimulus	Concentration*	Priority**			
48 hr			**Cord**	**1 Yr**	**2 Yr**	**3 Yr**
1	LPS	(Low)	1	1	4	4
2	Medium	---	2	2	5	5
3	PHA	5 µg/mL	3	3	6	6
4	Poly IC	1 µg/mL	4	4	10	10
5	Peptidoglycan	10 µg/mL	5	5	11	11
6	Bacterial DNA	10 µg/mL	6	6	12	12
7 day						
7	Cockroach	1:50	7	7	1	1
8	Bla g 2	50 µg/mL	8	8	2	2
9	Medium	---	9	9	3	3
10	RSV	10^5 SFU/mL	10	10	8	8
11	Derf1/Derp1 (dust mite)	1:50	11	11	8	8
12	Tetanus	50 µg/mL	12	12	9	9
Estimated # of subjects			500	400	375	350

* Estimated; dose-response titration will be performed
**In case there are not enough cells for all of the stimuli

It is essential to the design of this longitudinal study that antigens and reagents used in the cell stimulation assays are carefully controlled to ensure stability of antigenic activity. Large lots of the individual stimulants will be purchased and, if applicable, dialyzed to remove preservatives. Batches of RSV will be prepared yearly and standardized in terms of infectious units. Preliminary experiments will be conducted with each batch of stimulant to establish concentrations that produce vigorous cytokine secretion. These reagents will be aliquoted into single-use portions, and frozen (-80°C) until needed. Finally, the immunologic methods described above will be modeled in preliminary experiments to optimize experimental conditions for cytokine responses. If allergen-specific cytokine secretion responses in these preliminary experiments prove to be suboptimal, other techniques (e.g. cytokine mRNA levels by quantitative PCR) may be substituted.

9.1.3 Allergen-specific IgE and IgG

We will obtain specimens of plasma from (i) maternal blood and (ii) child blood specimens obtained at annual follow-up. These samples will be separated into aliquots, frozen at -80°C, and shipped in batches to the ICAC laboratories where the assays will be performed (Mt. Sinai School of Medicine and the University of Virginia-Charlottesville).

Table 9.1b. Serologic Measurements

Allergen	IgE	IgG**
Der f 1	C†, M	C†
Der p1	C, M	C
German cockroach	C†, M	C†
Bla g 2	C†, M	C†
Fel d 1	C†, M	C†
Dog	C, M	
Alternaria	C, M	
Mouse	C, M	
Egg white	C†	
Milk	C	
Peanut	C	
Ragweed	M	
Timothy grass	M	
Birch	M	

* Abbreviations: C = child, M = mother.
** If allergen-specific IgG is positive, then allergen-specific IgG_4 will also be measured. It is estimated that IgG_4 will be assayed on about 20% of the specimens.
† High priority, if the amount of plasma is limiting.

Analysis of these sera would include assays for total IgE and specific IgE to a range of common allergens (Table 9.1b). In addition, we plan to measure IgG antibodies to major allergens derived from the important allergens, (e.g. Der p 1 and Der f 1, Fel d 1, and Bla g 2). The IgE assays will be carried out using Pharmacia CAP, both for total serum IgE and for specific IgE on all sera except cord blood. Extensive experience with a previous birth cohort in Boston and in the COAST study (unpublished data) shows that allergen-specific IgE is not found in cord blood in appreciable quantities. The IgG antibody assays will be carried out using isotype-specific antigen binding immunoassays.[99,100] For samples that are positive for allergen-specific IgG (estimated 20% of samples), allergen-specific IgG_4 will be determined.

9.1.4 Development of T regulatory (T_R) cells

The objectives of these experiments are to: 1) determine if neonates that develop asthma and allergy are born with inappropriate numbers of T_R cells; and 2) establish if T_R cell function is dysregulated in children that develop asthma and allergy. These assays are labor intensive and exploratory, but have great scientific potential, and will be conducted at only one participating site (Boston).

Flow cytometry: The phenotype of mononuclear cells from cord blood, 1, 2, and 3 year peripheral blood samples, as well as maternal peripheral samples, will be determined by flow cytometric analysis. For these studies, the cells will be stained with a panel of monoclonal antibodies (e.g. CD4, CD25, FoxP3, and CD127); as well as appropriate isotype control antibodies. The numbers of T_R cells with specific phenotypes will be compared among children that have developed a wheezing phenotype and those who have not.

RT-PCR analysis: Separate samples of cells will be prepared for analysis of mRNA for semi-quantitative analysis of genes related to T_R function. The cells will be prepared for RNA isolation, and will be frozen at –80°C pending RNA isolation and reverse transcription. The sequences of the genes will be used to develop PCR primers and probes, and the expression of

selected mRNA (e.g. *gitr* and *foxp3*) will be determined by semi-quantitative RT-PCR. These studies will require approximately 1.5 X 10^6 cells to complete.

Functional Studies. T_R cell function will be determined on a limited number of blood specimens (based on availability) at each time point by an indirect assay that has been adapted from Taams et al.[101] This assay has several advantages for this type of large-scale clinical study, in that it can evaluate effects on antigen- and mitogen-induced T cell stimulation, requires relatively few cells, and can identify suppressive properties of specific cell phenotypes.

For these studies, it is estimated that we will require approximately 6 X 10^6 additional cells/subject. If cell numbers are limited in a particular sample, the studies will be prioritized as follows: flow cytometric analysis (1 X 10^6 cells), T_R functional studies (6 X 10^6 cells), and RNA analysis (1.5 X 10^6 cells).

Expected results and interpretation. It is expected that those children that have developed clinical signs of asthma will have dysregulated T_R function and/or phenotype compared with children who do not develop disease. The functional studies should demonstrate that depletion of $CD4^+CD25^+$ cells from children with asthma does not alter the antigen-specific proliferative response, while depletion of these cells from the blood of non-asthmatic children will enhance proliferation. These data should correlate with the phenotypic analyses in which we expect that cells isolated from asthmatic children will have: 1) fewer $CD4^+25^+FoxP3^+$ or $CD4^+CD25^+CD127^{low}$ cells and/or 2) a greater number of cells with an immature T_R phenotype ($CD4^+CD45RA^+$) compared with non-asthmatic children. Finally, we expect that genetic analyses will reveal a decrease in the expression of genes (*gitr* and *foxp3)* associated with T_R cells from children that develop asthma compared with those who do not.

Finally, it is anticipated that the results from this prospective study will determine if T_R cells have a role in the pathogenesis of atopy. Very few studies have been performed to date that have investigated the role of T_R cells in asthma and allergy. This will be the one of the first studies of its kind to address this question in a prospective manner in humans, and as such, the study procedures will be modified according to updates in this field and upon recommendations of the Scientific Advisory Panel.

9.1.5 Absolute eosinophil counts

Peripheral blood samples from the children's clinic visits will be sent to the clinical laboratory at each study site for a complete blood count and differential, to be used for the calculation of absolute eosinophil and lymphocyte counts.

9.1.6 Measurement of new factors or cytokines

It is anticipated that continuing research will reveal new insights related to the importance of specific cytokines and mediators in the pathogenesis of allergic diseases and asthma. Besides analyzing the mononuclear cell supernatants for the cytokines listed above, additional aliquots of supernate will be saved at -80°C so that measurement of other cytokines or mediators can be performed in the future. Advances in technology are in progress: the Luminex technology that is proposed for analysis of 15 cytokines simultaneously can be used to measure as many as 30

cytokines at once, and it is anticipated that expanded capabilities will be available in the near future through a collaboration with the Immune Tolerance Network.

9.2 Diagnostic Virology

To determine the contribution of the type and timing of viral infections in infancy to the subsequent development of cytokine dysregulation and recurrent wheezing, nasopharyngeal mucus specimens will be serially obtained and analyzed for respiratory viral pathogens including RSV, metapneumovirus, parainfluenza, influenza, coronaviruses, rhinoviruses, enteroviruses, and adenovirus. The collection of specimens of nasal lavage fluid during symptomatic time periods, as well as during routine clinic visits at 1 and 3 years of age, will provide us with important information regarding the epidemiology of the various viral pathogens within the community, the relevance of the detection of the pathogens to de novo and recurrent symptoms, and the influence these infections have on the development of cytokine responses relevant to allergic inflammation.

In addition to analyzing nasal secretions, plasma that is separated from the blood specimens at ages 1, 2, and 3 years will be analyzed for serologic responses to other pathogens (e.g. Epstein-Barr virus, cytomegalovirus, hepatitis A, mycoplasma pneumoniae, chlamydia pneumoniae, and RSV) that could affect immune and/or lung development. These data will allow us to estimate the total number of children that have been infected with each of these pathogens, as well as provide data as to the age at which these pathogens were encountered.

9.2.1 Nasopharyngeal lavage

Specimens will be obtained via nasal wash. Briefly, 2 mL of physiologic saline containing 0.5% gelatin is instilled into each nostril, and the nares are gently aspirated with a modified bulb syringe. Afterwards, the bulb syringe is forcefully expelled into a sterile container. Specimens are placed on ice, and transported to the site-specific laboratory where they will be vortexed to homogenize mucus, and aliquoted. The nasal lavage fluid (0.5-5 mL) will be divided into up to 8 aliquots (≥ 110 μL each) that will be frozen until they are shipped to the diagnostic virology laboratory in Madison. If there is sufficient nasal lavage fluid, an additional aliquot (0.5-1 mL) will be saved for clinical virology. This specimen will be sent to the local clinical diagnostic laboratory for virus isolation, if this is requested by the subject's health care provider. Otherwise, this extra aliquot will be discarded after the URECA diagnostic virology has been completed.

In batches, the nasal wash fluid specimens will be processed for immunologic (e.g. ECP) assays, and RT-PCR for respiratory viruses. After vortexing to achieve a homogenous suspension of wash fluid and secretions, a 100 μL sample is saved for RT-PCR for respiratory viruses as listed above. The remaining specimen aliquots will be stored at -80°C for subsequent immunologic assays.

9.2.2 Immunoassays

ECP in nasal wash fluid will be measured using monoclonal antibody-based fluorometric assays (CAP System, Pharmacia Diagnostics, Uppsala, Sweden). Cytokine levels (e.g. IL-8) will be determined by ELISA or multiplex ELISA (Luminex).

9.2.3 Microbial Detection

Specific RT-PCR assays will be used to detect influenza, parainfluenza, coronavirus, RSV, metapneumovirus, RV, enterovirus, adenovirus, chlamydia pneumoniae, and mycoplasma pneumoniae in nasal secretions. Specimens will be kept frozen at the collecting site, and then shipped in batches to the central virology laboratory in Madison. For extraction of total RNA, samples and appropriate negative controls will be placed in extraction reagent (e.g TRIzol®, Life Technologies, Rockville, MD).

RT-PCR will be performed as previously described.[102] After extraction and reverse transcription, the cDNA will be amplified with primers and protocols specific for specific respiratory pathogens. All specimens will be analyzed in duplicate, and results will be reported as positive or negative if both specimens have the same result. Discrepancies will be resolved by repeating the process starting with a new specimen. To minimize the risk of false positive results, reagents will be prepared and aliquoted in a separate room, RT will be conducted in a tissue culture hood, and the PCR mixtures will be prepared in a dedicated PCR hood that is treated daily with UV light. During the first year of the protocol, the PCR protocols will be adapted to a multiplex assay (or assays) using a 96-well microtiter format. Advantages of this approach include the ability to process the large number of specimens that will be generated by this protocol, with savings on time, labor, and reagents. Furthermore, stored cDNA can be further analyzed with quantitative PCR techniques should it be advantageous to develop quantitative estimates of viral shedding in clinical specimens.

In addition to the use of PCR, serologic tests will be performed annually to detect IgG antibody responses to pathogens (e.g. EBV, CMV, chlamydia, mycoplasma, hepatitis A) that could potentially affect immune development or the risk of asthma. In addition, since we hypothesize that RSV LRI may be especially important in the pathogenesis of the recurrent wheezing phenotype, and our surveillance techniques are likely to miss infants who develop mild or asymptomatic infections, RSV serology will also be performed annually by ELISA, as previously described.[102]

We will be using methods for detecting respiratory viral infections that are well established in our laboratory. Although standard virologic methods are sufficient to detect many of the viruses that produce lower airway infections (i.e., RSV, influenza, PIV), PCR will be the principal method used as it is a more sensitive technique, particularly for detecting rhinoviruses due to the low sensitivity of viral culture and the unavailability of serologic diagnosis. Potential pitfalls associated with PCR detection of viruses are: 1) false-positive results, and 2) detection of picornaviruses in asymptomatic individuals. To minimize the first pitfall, we will use specific primers and probes to eliminate false positive results due to nonspecific amplification of genomic DNA. In addition, to minimize the possibility of cross-contamination of specimens, PCR and RT reaction mixtures will be prepared in a separate hood from the one used in RNA extraction, and no more than one sample will be opened in the PCR hood at the same time. Gloves will be worn during the preparation of PCR reaction mixtures, and will be changed frequently. Controls included in each PCR run included samples containing reagents with no cDNA and positive control samples containing cDNA prepared from stock virus.

The data will need to be interpreted considering that asymptomatic infection with respiratory viruses can occur. For example, the incidence of asymptomatic infection for picornaviruses

(rhinoviruses and enteroviruses combined) has been recorded at 14-31%.[103,104] To estimate the rate of asymptomatic infection in our study population, we will also obtain and analyze nasal lavage specimens from children during routine health care visits in the absence of respiratory symptoms. Finally, as new viruses that are important contributors to respiratory illnesses are discovered (e.g. metapneumovirus), they will be evaluated for inclusion into the PCR-based and or serology testing panels.

9.3 Dust Analysis for Allergen and Microbial Products

9.3.1 Sampling of house dust

Dust specimens will be collected when the baby is three months of age, and each year thereafter, following procedures described in the URECA Manual of Operations. Three dust samples will be collected from each home in these locations: 1) the child's bed (defined as the location the child sleeps the most) and bedroom floor, 2) another sample of dust from the floor of the child's room, in case extra dust is required, and 3) the living or TV room (chair and floor). Dust collection thimbles will be placed in airtight plastic bags immediately after collection. Dust samples will be shipped in batches to the central processing laboratory at Johns Hopkins University.

9.3.2 Allergen assays

The recovered dust from all locations will be sifted at the laboratory. The fine dust will be appropriately weighed and aliquoted for antigen analysis. A portion of the fine dust will be extracted and analyzed for allergenic proteins such as: dust mites (Der p 1 and Der f 1), cockroach (Bla g 1), cat (Fel d 1), mouse (Mus m 1) and dog (Can f 1). The assays are all two-site monoclonal antibody ELISAs, and results will be reported as units or μg per gram of dust. These assays are well established. Samples of frozen house dust will be retained, so that other allergens can be quantified if these are deemed at a later date to be biologically relevant to the development of asthma.

9.3.3 Microbial exposure

We propose to assess microbial exposure in settled house dust using a combination of several different assays. Endotoxin measurement in the past has been performed by a very sensitive but not entirely specific and rather variable assay using a natural extract (LAL) made from horseshoe crab blood. LAL is about to be supplanted by an assay made using a cloned protein from the horseshoe crab. The major difference between the assays is that the old natural extract contains a large number of proteins. The natural extract's composition and sensitivity is variable from lot-to-lot; it reacts with β (1-3) D-glucans and sometimes with cellulose; and it is inhibited or enhanced in its reactivity with endotoxin by many compounds present in environmental samples. The recombinant factor C (rfC) assay avoids many of these problems. It is not at all responsive to glucans, can be produced in very large batches, and has little lot-to-lot variability. In addition, the initial dust samples (3-month visits) we will measure a chemical marker of peptidoglycan (muramic acid), and a chemical marker of fungal biomass (ergosterol). The muramic acid and ergosterol can be measured using GC-mass spectroscopy. These assays will be performed at a central laboratory, as specified in the Manual of Operations. Each dust analysis will require 5 mg of dust, so that all five assays can be performed on 25 mg.

9.4 Psychosocial Stress

Repeated measures of the stress experience will be administered beginning in the prenatal period (Table 7.1a). To determine the role of psychosocial stress on development of cytokine dysregulation and asthma, we will longitudinally administer questionnaires designed to measure specific stressors relevant to the demographics of the study sample, stress buffers, and the psychological response to stress. All chosen stress measures have been validated and are relevant to populations of lower socioeconomic status. Those measures that are frequently repeated (depression scale and perceived stress measures) are very brief and have not been found to overburden study participants. Experience with these scales in project ACCESS (RJ Wright PI) and the Home Allergens and Asthma Study (DR Gold PI) have demonstrated a low refusal rate. Stress measures to be included in the prenatal baseline questionnaires include the Difficult Life Circumstances (DLC) scale, the Perceived Stress Scale (PSS), the Pregnancy Anxiety Scale, The Edinburgh Depression Scale, the Social Networks Scale, Brief COPE scale, Neighborhood Violence, Housing Stress, and the Community Survey/Neighborhood/Block Conditions. Indicators of socioeconomic status will also be ascertained as part of this baseline survey. Repeated measurement of perceived stress will be obtained using the brief 4-item Perceived Stress Scale at the time of the planned quarterly follow-ups. The annual assessment will include all the stress measures from the Prenatal Visit with the exception of the Pregnancy Anxiety Scale. Each of these forms will provide quantitative measures of family stress, and/or individual responses to stressful conditions, and selected forms are highlighted in the following sections.

9.4.1 Pregnancy Anxiety Scale

This scale has been developed and validated as a measure of situation-specific stress related to pregnancy.[105] Pregnancy anxiety has been linked to dysregulation of the hormonal stress response in pregnant women as well as preterm labor and low birthweight.[106,107] This will be administered to participating families during the prenatal visit.

9.4.2 Perceived Stress Scale

Beginning when the mothers are enrolled prenatally, the Perceived Stress Scale (PSS) will be used to measure the degree to which the respondents felt their lives were unpredictable, uncontrollable and overwhelming in the preceding one month (reliability, = 0.85).[108] The maximum possible PSS4 score is 16. Longitudinal (repeated) measurement of caregiver perceived stress will be obtained utilizing the shorter four-item scale (PSS4). This brief measure will be administered with the pregnancy anxiety scale prenatally and will be part of the phone follow-up calls scheduled quarterly to ascertain respiratory illness experience as detailed above.

9.4.3 Difficult Life Circumstances

The burden of adverse life experiences over the preceding year will be ascertained annually using the Difficult Life Circumstances scale, which was developed as a contemporary measure of life stressors relevant to populations with similar demographics to the proposed inner-city cohort. The psychometric properties of the measure show acceptable validity and good test-retest reliability.

9.4.4 Neighborhood/Block Conditions

This consists of an abbreviated version of the Community Survey Questionnaire developed in the Project on Human Development in Chicago Neighborhoods.

9.4.5 COPE

Coping strategies will be assessed using the COPE instrument. The COPE is widely used, easy to administer, and has standardized scoring procedures. A reliable and validated brief form of the COPE exists.[109]

9.5 Pollution

9.5.1 Measurement of airborne nicotine concentration

Although nicotine is not the only component of ETS that leads to adverse effects on the airways, tobacco smoking is the sole source of nicotine in indoor air, so nicotine provides an excellent metric for estimating relative exposure to ETS among homes. Nicotine in indoor air can be measured using a passive diffusion filter approach that requires no air pump and provides an integrated measure of nicotine concentration over the period that the diffusion filter is exposed.

At the time of the first home visit in the first few months of life, we will obtain an integrated 2-week measure of indoor air nicotine concentration in the TV/playroom using the same method used in the Inner City Asthma Study. The monitor is a passive, diffusive sampler. The monitor will be placed in a secure location approximately three feet off the floor. After two weeks of sampling in the home, the nicotine filter will be retrieved, unloaded from the cassette, and shipped to a central laboratory where nicotine concentration will be determined.

9.5.2 Measurement of NO_2

An integrated two-week measurement of NO_2 in the TV/playroom will also be obtained at the time of the first home visit in the first few months of life. The measurement of NO_2 concentration will be made using a modified diffusion filter sampler (Ogawa monitor) that will be located in a secure location approximately three feet off the floor. After two weeks of sampling in the home, the samplers will be retrieved, sealed, and shipped to a central laboratory for extraction and ion chromatography analysis.

10 Laboratory Samples

10.1 Sample Collection, Storage At Site And Preparation For Shipping

Once collected, all samples collected from study subjects or their homes will be put into containers that will be free of any personal identifiers except for a subject identification number. Samples of blood will be collected in the delivery room by obstetrics staff, and placed into a special medium to preserve cell viability until the samples are picked up by study personnel (see URECA Manual of Operations). Peripheral blood specimens drawn by research coordinators or phlebotomists in clinical laboratories will be carried to the laboratory by study personnel. The blood cells and plasma will then be processed according to the laboratory procedures that are described in the Manual of Operations. The cytokine secretion assays will be cryopreserved and then shipped for processing at a single laboratory. After the cells have been stimulated, the

supernatant fluids will be placed into 60 μL aliquots, and stored at –80ºC pending batched shipments to the storage facility.

Selected samples of cells will be processed for later analysis of gene expression (quantitative PCR), genetic studies (DNA) and cell phenotypic analysis (flow cytometry). Each of these samples will be collected at the study sites, processed, and then stored at the central storage facility pending analysis. DNA studies will be performed only on samples from subjects who have specifically consented to this procedure.

Other samples to be collected include nasal secretions and house dust. The nasal secretions will be thoroughly mixed, centrifuged, aliquoted, and frozen (-80ºC) pending shipment to the Diagnostic Virology Laboratory in Madison. House dust will be kept at –20ºC in plastic bags, pending shipment to Dr. Eggleston's laboratory (JHU) where the samples will be sieved, and then divided for analysis of allergens and innate immune stimuli. All specimens will be labeled with a subject ID, and will be free of any personal identifiers.

10.2 Shipping and Repository Storage

Most samples that are collected at the clinical sites will be shipped directly to processing laboratories. However, samples that will need to be stored for at least 3 months (specimens of DNA, plasma, etc), as well as specimens that are generated by laboratories (e.g. supernatant fluids from cytokine secretion assays) that are awaiting further analysis, will be sent to a central storage facility pending analysis. This facility will maintain a computerized inventory of all of the specimens to be analyzed, and will ship the specimens to designated laboratories for analysis upon request. In order to protect subjects, all samples are stored and shipped under code so that neither the child nor his/her parents can be identified individually.

10.3 Analyses

Samples of plasma will be shipped to a central laboratory for analysis of allergen-specific IgG and IgE. Plasma cotinine levels will also be determined as an indicator of passive exposure to tobacco smoke. House dust analysis for allergens and innate immune stimuli will be also be performed at central laboratories. The laboratories at each clinical site will participate in analyzing culture supernatant fluids for cytokines and other immunologic factors. In each case, all of the samples for any one immunologic assay will be performed by a single laboratory to minimize variability in the performance of the assay, and maximize the utilization of expertise at each site. All samples will be processed using professional standards of confidentiality.

11 Data Management

11.1 Overview Of Data Management System

This study will use a distributed Data Management System (DMS). Each research site will have a study computer with Internet access to the DMS. The system will be used to maintain databases of potential participants and enrolled participants. It will facilitate contacting potential participants, scheduling visits, and entering data.

11.2 Data Collection, Data Entry, and Data Quality

Site personnel will record all information required by the protocol onto the designated study forms. The study forms are the source documentation for all data collection. Site coordinators will review the study forms for completeness and accuracy and instruct site personnel to make any required corrections or additions prior to data entry. The original study form will be given to the appropriate personnel at the site for immediate data entry. All original study forms will be filed in the designated, secured area at the investigational site.

The following procedures will be used to improve the accuracy and consistency of data collection. Central training will be held prior to the enrollment of the first participant at any site. Staff will be trained on all aspects of the study protocol. They must perform additional practice sessions and proficiency exams before performing an activity with a study participant. A Manual of Operations (MOP) will document all study procedures and will be distributed to all study personnel. Site visits from the SACCC and NIAID will ensure that procedures, including those regarding data collection, are being followed accurately. The SACCC will review all data collection procedures during site visits, including completion of study forms, data entry of study forms, flow of forms and edit reports, filing of forms and edit reports, audit trails, and security measures for study data.

11.3 Data Entry

Data items from the study forms will be entered into the study database at each investigational site via the Internet using single data entry with electronic verification. Only designated data entry personnel will have access to the data entry and editing routines in the Data Management System, and the DMS and study database as a whole are password protected.

The data entry screens mirror the original paper copy study forms, facilitating data entry ease and accuracy. Electronic verification of all data fields including range checks and omission of data allows for immediate feedback via error messages to the data entry operator. Errors on the study forms are also captured through this electronic verification process. Printable edit reports with the study form errors are given to the site personnel who completed the study form for resolution. An edit is considered resolved either after the correction has been made to the study form, noted on the edit report, and changed in the data entry system or after the questionable data has been reviewed and verified and thus noted on the edit report and in the DMS. All study form and edit report changes and corrections are initialed and dated. The edit reports are filed with the original study form. Quality control audits are done after the edit checks have been resolved.

In addition to the edits that are run at the time of data entry at each investigational site, the SACCC will perform centralized edit checks on logical consistency, cross-database consistency, and table look-up comparisons. The centralized edit check programs will generate edit reports displaying the questionable data fields and entered values, status flags indicating the type of edit, explanations of the specific problems. The centralized edit reports will be sent to the sites for resolution. All corrections will be made to the original study form and in the DMS. The resolution is noted on the report and sent back to the SACCC for final review. This process will assist in identifying and rectifying problems with the forms and data collection procedures. Every effort will be made to minimize the latency between the original collection of the data and the feedback to the site about possible errors.

A data entry error rate is determined by having the SACCC regularly re-enter approximately 5% of the data forms.

11.4 Database Management

Study data will be entered via a login-secured web-based data management system. The data will be transferred via https (ssl encryption) to a central database on the Statistical and Clinical Coordinating Center's (SACCC) network. The database will reside on a dedicated server in a locked server room that only SACCC IT administrators can access. Remote access to the server will be allowed only via encrypted (ssh) login from within the SACCC internal network, and only by SACCC IT developers.

Every evening the database will be copied to tape backup. The last tape of each week will be rotated out and stored for a period of two months. The last tape of each month will be rotated out and stored off site.

Audit trails are automatically generated to monitor changes to the study database. The transaction (edit) file structure includes the staff person making the change, the date executed, the original datum, and the new value. The Project Coordinators, the SACCC, and the project officers are able to evaluate delays in error resolution based upon reports generated from this file. The reports indicate the number of data records that are “complete,” the number that are in the edit/resolution process, and the length of time these data have been in their respective stage of processing.

When the database has been declared complete and accurate, the database is locked. No changes other than clean up will be made to the locked data base.

11.5 Retention Of Documents

Study documents must be maintained at the research center or a local storage facility for at least seven years following the completion of the study. Study documents that must be retained include all Case Report Forms, laboratory reports, IRB approval documentation and related correspondence, and signed informed consent forms.

12 Statistical Methods

12.1 Statistical Analysis Plan

Our analysis strategy is designed to meet the primary and secondary study objectives while ensuring validity and reliability of results. In the following sections, we present strategies for controlling Type I error rates to minimize the possibility of finding spurious results, present details of the anticipated data analysis methods to address the study objectives, and consider other statistical issues pertinent to the study.

12.1.1 Controlling Type I error

In any study with multiple correlated assessments measured repeatedly over time, a carefully planned analysis strategy can hedge the impulse to "dredge the data" thus providing a safeguard against spurious findings and inaccurate p-values. One approach to this problem is to alternate exploratory analyses (used to refine variables, perfect statistical methods, and generate

hypotheses) with confirmatory analyses (used to test a relatively small number of well-defined hypotheses). The type-I error rate is controlled by limiting the number of confirmatory hypothesis tests.[110,111]

Given the data collection schedule proposed for this study, the design is well suited for this "leap-frog" approach. At each of the four data collection points (birth and yearly for 3 years), small sets of well-defined cross-sectional hypotheses about, for example, correlations among immunologic or atopy endpoints with child, family, or environmental characteristics or differences among population subgroups can be tested. To illustrate, the first round of cross-sectional analyses could be designed to confirm relationships suggested in the literature about maternal characteristics and prenatal experience with innate immunity endpoints. This confirmatory analysis using the baseline data would be followed by exploratory analyses. Goals for the exploratory analysis may be, for example, to explore ways to construct meaningful summary scores from multiple correlated immunologic and cytokines assessments, to identify previously undocumented relationships among maternal characteristics, prenatal experience and innate immunity endpoints, or to identify subgroups who theoretically appear at higher risk for future development of atopy or asthma symptoms. The purpose of the exploratory phase is to refine statistical methods and define hypotheses for a second round of confirmatory analyses planned for the 1-year follow-up data. Year 1 data might be used, for example, to test a hypothesized relationship between the cytokine responses at birth and development of antigen-specific IgE levels and then to explore the possibility of other suspected relationships. After three rounds of confirmatory and exploratory analyses on the cross-sectional data, we will have information on definitions of cytokine dysfunction, temporal changes in immune development, and at-risk subgroups that will be used to refine the analyses planned to meet the primary and secondary study objectives.

One of the primary challenges to be tackled during the exploratory analysis phases will be to find meaningful ways to summarize the large amount of data on cytokine levels. Mononuclear cell cytokine responses to a panel of 12 stimuli (i.e. both non-specific and antigen-specific) will be assessed at birth and annually for three years. At each assessment, ten cytokines representing five different aspects of inflammation and immune function will be measured: Th1 inflammation (IL-12, IFN-α), Th2 inflammation (IL-4, IL-5, IL-13), airway dysfunction (IL-9), anti-inflammatory (IL-10, TGF-α), innate immunity (IL-8, TNF-α). After 36 months, subjects completing all assessments will have a profile of 480 cytokine measurements. With so many correlated assessments, testing the relationship of individual cytokine responses at each time point is impractical, likely to lead to Type I errors, and ignores connections among the cytokine responses that could enhance interpretation of the relationship between immune development and study outcomes (i.e. recurrent wheeze and atopy). Hence, a primary objective of the exploratory analysis will be to create a relatively small number of composite scores to summarize the observed patterns in the cytokine data.

Principal components analysis is a statistical method used to identify a relatively small number of composite “factor” scores, or linear combination of the original variables, that represent the relationships among and account for most of the observed variance in a large collection of correlated variables. Although principal components analysis has its root in the fields of psychology, education, and social science, it has been applied previously in research on asthma and other conditions with immunologic origins.[112-114] To illustrate its use, if a Th1 type

inflammatory response is "turned on," levels of both IL12 and IFN-α may be relatively high while levels of Th2-type cytokines (e.g. IL-4, IL-5, IL-13) may be relatively low. Hence, it may be possible to identify a composite score using assessments on these five correlated cytokines levels that represents the "Th1 response" more precisely and accurately than any individual assessment alone. Increased precision associated with the composite score should also increase power to detect hypothesized relationships. Ultimately, we will use the composite summary scores to define "cytokine dysfunction" and "patterns of cytokine responses". These phenomena are not well characterized in the literature but are essential for testing hypothesized relationships in the primary and secondary analyses.

12.1.2 Data Analysis Plan

12.1.2.1 Primary Study Objective.

The purpose of the primary analysis will be to examine the relationship between recurrent wheeze at 36 months and patterns of cytokine responses observed during the first three years. Using logistic regression, the presence or absence of recurrent wheeze will be modeled as a function of immunologic explanatory variables that describe "cytokine dysfunction" and "patterns of cytokine responses". The explanatory variables, developed during the exploratory analysis phases, will be clearly specified prior to the analysis and may include composite summary scores to describe inflammation or immunologic functions at different time points, individual cytokine levels of unique importance (for cytokines that are independent of composite summary scores), and/or variables for at-risk subgroups defined by, for example, temporal occurrence of immune system developmental events or evidence of cytokine dysfunction. Explanatory variables will be selected for inclusion in the model based on potential importance noted in the literature and hypotheses generated during exploratory analyses. The resulting regression model will provide insight into how timing, type, and sequence of immunologic developments (defined by cytokine responses) impact the risk of recurrent wheeze by age three years.

12.1.2.2 Secondary Study Objectives.

The first of two secondary study objectives will be to identify patterns of immunologic development associated with development of atopy. Atopic children at age 3 will be identified based on levels of total IgE and/or allergen-specific IgE. Specific definitions for "atopy" will be evaluated during the exploratory analysis phase. Subsequently, one analysis will be analogous to that described for the primary analysis using a logistic regression to model the presence or absent of atopy as a function of immunologic explanatory variables describing "cytokine dysfunction" and "patterns of cytokine responses". Additional analyses using linear regression or survival analysis methods will examine the relationship between the age of onset of atopic sensitization (assessed using allergen-specific IgE levels) and immunologic explanatory variables.

An additional secondary study objective is to identify environment exposures that modify immune responses and ultimately influence the development of atopy and recurrent wheeze. Environmental factors include: 1) the frequency and severity of respiratory infections in the first year of life, 2) prenatal and postnatal stress, 3) exposure to microbial products in household dust, 4) exposure to selected allergens in household dust, and 5) household exposure to the pollutants NO_2 and tobacco smoke. For this objective, the two-phase plan is to first identify factors

associated with the risk of atopy and/or recurrent wheeze then assess the influence of these factors on early immune development.

In the first phase, the risk of atopy and recurrent wheeze will be modeled using separate logistic regressions that include main effects and interaction terms for environmental factors. Because early life viral infections are hypothesized to have a key role in how the immune system develops, the importance of timing of viral infections will be considered first. This will be accomplished by estimating a (possibly) time-dependent odds ratio for viral LRI and other viral infection risk factors using generalized additive regression models. This method allows the inclusion of regression parameters that are continuous functions of age in the logistic regression setting. Thus, for example, we will be able to determine how the odds ratio associated with LRI changes as a function of the age at first LRI and season of the year. After accounting for the importance of early viral exposure, the influence of other environmental factors will be examined by extending the models to incorporate terms for other risk factors.

In the next phase, analyses will address how important environmental risk factors for atopy or recurrent wheeze impact early immune development. A focal point for this investigation will be to elucidate the role of viral infections. An ideal statistical model for addressing this question is a multistate Markov transition model with (possibly) time-dependent covariates. Specifically, the cytokine profile at years 1 through 3 will be used to distinguish between "normal" or "dysfunctional" immunologic states (Markov states), then the Markov model can be used to examine how changes between states are associated with variables describing the cytokine profile at birth and viral infection history (as time-dependent covariates). As an alternative, a logistic regression model for immunologic state could be fit using the generalized estimating equations (GEE) approach with an exchangeable (or independent) working correlation matrix to account for the within-subject correlation among repeated measures. We will also explore the possibility of using the composite scores, developed to summarize cytokine response data (described above), to define an immune dysfunction scale on a continuum. The association between the immune dysfunction scale and variables describing the innate immunity and viral infection history could be modeled using either GEE linear model or general linear mixed model (GLMM). Factors identified in the Markov transition model are associated with movement between the two immunologic states, whereas, in the GEE or GLMM models, factors are associated with the overall risk of attaining a dysfunctional immune status. Finally, the impact of other environmental risk factors (exposure to cockroach, LPS, family stress, pollution, etc.) will be examined by augmenting these models with main effect and interaction terms to test for hypothesized associations.

12.1.3 Other Statistical Considerations

12.1.3.1 Multiple comparisons

The rich database resulting from the proposed longitudinal study to follow a cohort of inner-city children from the prenatal period through early childhood will be the first to include information on symptoms of asthma, viral exposures, immunologic responses (IgE, IgG, cytokines), allergen sensitization and exposure, exposure to pollutants, stress, and other environmental exposures. To balance the responsibility to use this database to it fullest potential while guarding against spurious findings, we have proposed a plan to alternate exploratory analyses (used to refine variables, perfect statistical methods, and generate hypotheses) with confirmatory analyses. By

limiting confirmatory analyses to a relatively small set of clearly defined hypotheses motivated by findings in the literature and results of exploratory analyses, we will guard against spurious findings. Even so, given the many analyses planned for this study, some distortion of p-values is unavoidable. As such, commensurate caution will be exercised when interpreting results particularly for unexpected findings.

12.1.3.2 Multiple sites

For all analyses outlined above, data from multiple inner-city sites will be pooled. To minimize between between-site variability, cells and other samples will be collected according to standardized procedures, and central laboratories will be utilized for the analyses. Eliminating this source of variability should improve power to detect hypothesized relationships. Finally, , all statistical models will include terms to account for additional variability among sites.

12.2 Sample Size Estimation

Although the current study proposal calls for four years of follow-up, the diagnosis of asthma is very difficult to make with certainty at the age of four years. At this age, most of the wheezing episodes will be due to viral infections, and only a subset of these children will go on to develop typical allergic asthma later on in childhood. Recognizing the uncertainty of an asthma diagnosis at age three or four, the clinical outcome planned for this proposal is recurrent wheezing. Nevertheless, the long-term goal of the study will be to determine the etiology of inner city asthma. As such, the study will be powered to evaluate risk factors for asthma diagnosed by 5 or 6 years of age; the birth cohort enrollment will reflect this goal. The hypothesis upon which the sample size calculations are based is that 3 year olds with both a history of a viral LRI and evidence of cytokine dysfunction will be at greatest risk for developing asthma by age six. The study has been designed to achieve 90% power to test for an association between cytokine dysfunction at age 3 years and increased risk of asthma at age 6 years while also allowing for the increased risk associated with viral lower respiratory illness (LRI).

Direct information on the relationship between asthma risk and cytokine dysfunction is not available. Thus, for the purpose of sample size estimation, we considered two possible indicators of cytokine dysfunction at age 3 years: IL-13 response and sensitivity to cockroach allergen (CR). The motivation for these selections comes from (1) the epidemiologic evidence of a close relationship between sensitization and exposure to cockroaches and the development of asthma in inner city environments and (2) evidence of Th2-like responses to purified cockroach allergen (Bla g 2). Although “cockroach sensitivity” is not a direct assessment of cytokine dysfunction, it was chosen because it should be highly correlated. IL-13 was selected, because experimental evidence in the murine model links it to IgE synthesis, Th2 differentiation, and asthmagenic effects. For the sample size calculations, we used data from other studies and published reports as well as clinical judgment to postulate the “true” nature of the relationship between asthma risk and cytokine dysfunction.

The protocol development group speculated that the “true” odds ratio (OR) comparing the odds of asthma with and without of cytokine dysfunction would have to be at least 2 to be considered clinically relevant, but, in fact, may be much higher. Evidence suggests that for cockroach sensitivity the odds ratio may be as high a 5.5 (i.e. comparing odds for LRI+, CR+ subjects with LRI-, CR- subjects). The logistic model was the framework chosen to estimate the sample size

required to have sufficient power to detect significance given the "truth" of postulated OR. Although the OR is the parameter that will ultimately be tested, power of the test depends on the asthma risk (i.e. probabilities) and frequency of children in each cell of a table with rows and columns defined by LRI exposure (yes or no) and degree of cytokine dysfunction (i.e. quartiles for IL-13 and presence/absence for cockroach sensitivity). Assumptions used to compute these probabilities under a variety of scenarios are summarized in the table below.

Parameter	Considered range	Best Choice Decision	Source
Assumptions relevant to both IL13 and CR based calculations			
Prevalence of asthma among inner-city 6 yr olds	10% to 20%	20% Sampling among children with parental history of allergies or asthma	ICAS study (unpublished data) and clinical judgment
Percent of inner-city 3 yr olds who have experienced an LRI	45% to 55%	50%	Data from Diane Gold and clinical judgment
Ratio of odds for developing asthma @ 6 yr for LRI+ vs. LRI-	3.5 to 5	3.5	Tucson,[12] data from Diane Gold, clinical judgment
Dropout rate	40%	40%	Experience with studies on inner-city populations
Additional assumptions for IL13 calculations			
Distribution of LRI+ children in groups defined by quartiles of the IL13 distribution	Assume 50% LRI+ in each quartiles	Assume 50% LRI+ in each quartiles	Speculation
OR for developing Asthma: Comparison of highest and lowest quartile	2 to 5	4	Clinical relevance and Castro-Rodriguez[12]
Additional assumptions for CR calculations			
% of inner-city 3 yr olds with cockroach sensitivity	20% to 33%	20%	Columbia study (unpublished data)
Ratio of Risks [1] (CR+ vs. CR- for LRI+) vs. (CR+ vs. CR- for LRI-)	1 to 1.2	1.2	UVa study indicating an interaction between allergy and virus-induced wheeze[115]
OR for developing Asthma: Comparison of LRI+,CR+ vs. LRI-,CR-	5.5	5.5	Clinical relevance and Castro-Rodriguez[12]

[1] $\text{Ratio of relative risks} = \frac{(\text{asthma risk for CR+,LRI+})/(\text{asthma risk for CR−,LRI+})}{(\text{asthma risk for CR+,LRI−})/(\text{asthma risk for CR−,LRI−})}$

Preliminary power estimates for a range of sample sizes were first computed by fitting logistic models to the expected number of asthma cases in each cell of the table defined by LRI exposure (yes or no) and degree of cytokine dysfunction (i.e. quartiles for IL-13 or presence/absence for cockroach sensitivity). Then, for each scenario, the X^2 test statistic from the logistic model was used as a non-centrality parameter to compute power using a normal approximation ($\alpha = 0.05$, two sided). Using this approach, the sample size required for 90% power under the "Best Choice" decisions (see table) was estimated as 534 and 467 for the IL-13 and cockroach sensitivity models, respectively. Based on the preliminary power estimates, the protocol development group made the decision to enroll 500 children with parental history of allergy or asthma. Subsequently, the power estimate was confirmed using a more refined approach by simulating 20,000 data sets under the "Best Choice" decisions for the IL-13 models. For each simulated data file, the test statistic was computed. Power, computed as the number of rejected tests, was confirmed at 91%.

If 30% of the 500 children drop out by age three, 350 children will be available for analyses to meet the primary study objective. If the prevalence of recurrent wheeze among three-year olds is 17% (based on preliminary analysis of the COAST cohort at age 3 [unpublished data]) and the percent with immune dysfunction (defined dichotomously) is 25%, the power to detect a significant difference in the risk of recurrent wheeze among those with and without immune dysfunction is ≥ 80% if the relative risk ≥ 2.0 and ≥ 90% if the relative risk ≥ 2.2. If the prevalence of recurrent wheeze is as low as 15%, power will still be ≥ 90% if the relative risk ≥ 2.4. If the percent of children with immune dysfunction is as low as 12.5%, power will still be ≥90% if the relative risk ≥ 2.5. (These power calculations are based on results of X^2 tests ($\alpha = 0.05$) in 5000 simulated data files.)

13 Study Organization

13.1 Funding

The URECA Study is a project of the Inner City Asthma Consortium (ICAC), which is funded by the National Institute of Allergy and Infectious Diseases.

13.2 Program Staff

Under the terms of the contract, the NIAID/DAIT Program staff will both oversee and participate in activities. These activities include facilitating the development of ICAC protocols, implementing the study, and analyzing and interpreting the data.

13.3 ICAC Structure

The Inner City Asthma Consortium (ICAC) consists of the Administrative Site, ten sub-contracted research sites, and the Statistical and Clinical Coordinating Center.

The Administrative Site is lead by William Busse, MD, as the Principal Investigator. The administrative site is responsible for budgetary issues and overall direction of the Consortium.

Each of the ten study centers represents a non-federal institution. The investigators at these sites are responsible for participating in the Steering Committee and subcommittees and for performing the studies.

The Statistical and Clinical Coordinating Center (SACCC) is lead by Herman Mitchell, Ph.D. as the Principal Investigator. It is located at Rho Federal Systems Division, Inc., in Chapel Hill, NC. The SACCC is responsible for establishing common data collection instruments, for assisting quality control efforts, and for analysis of the data.

The Steering Committee is the principal decision-making body of ICAC. The voting membership of the Steering Committee is composed of the Principal Investigators from each of the ten study centers, one representative from the Program Office, and the Principal Investigator of the SACCC.

13.4 External Scientific Review Board

An External Scientific Review Board will review this protocol. The Board members will be chosen by the NIAID and will represent areas of importance to the consortium goals. Additional Board members may be added for specific protocols. The main purpose of the Board is to review the final protocol for scientific merit. They will make suggestions, changes, or modifications, and the Protocol Writing Committee will respond to each suggestion before the study begins recruitment.

13.5 Data and Safety Monitoring Board

The Data and Safety Monitoring Board (DSMB) is responsible for 1) examining endpoint and toxicity data from ICAC protocols on a regular schedule, 2) making recommendations to ICAC concerning continuation, termination, or other modification of the study based on observed beneficial or adverse effects of any treatments under study, and 3) reviewing the general progress of the studies and assisting in resolving any problems which may arise. The DSMB members and chairperson are appointed by the NIAID and reflect the disciplines and medical specialties necessary to interpret the data from the study. Members include experts in the fields of biostatistics and medical ethics, in addition to clinicians.

14 Adverse Event Collection and Reporting

14.1 Definitions

14.1.1 Adverse Events

The URECA study has been designated as an observational birth cohort study by the ICAC and DAIT. As such, this study involves minimal active intervention outside of the normal standard of care for the participant. Thus, for this study, “reportable” is used to denote “reportable to the IRB/Ethics Committee and the study sponsor (NIAID/DAIT)” and reportable adverse events are limited to any occurrence or worsening of an undesirable or unintended sign (including an abnormal laboratory finding), symptom, or disease in a study subject that is specifically associated with a study procedure that is not part of the normal standard of care for the participant. In addition, all hospitalizations will be evaluated by the study physician to determine their relatedness to the study, and will be monitored by the DAIT medical monitor. Only those related to study procedures will be considered “reportable”as defined above.In the URECA study, reportable adverse events are those related to the blood draws, nasal lavage, allergen skin testing, spirometry, or forced oscillation breathing test not included as part of standard of care for this group of patients.

14.1.2 Serious Adverse Events

For this observational study, an adverse event associated with the blood draws, nasal lavage, allergen skin testing, spirometry, or forced oscillation breathing test that suggests a significant hazard, contraindication, side effect, or precaution may be considered a serious adverse event. This includes, but is not limited to any of the following type of events: death; life threatening event; inpatient hospitalization or prolongation of existing hospitalization; persistent or significant disability or incapacity; congenital anomaly or birth defect; an event requiring intervention to prevent permanent impairment or damage.

In addition, important medical events (if they are felt to be associated with the blood draws, nasal lavage, allergen skin testing, spirometry, or forced oscillation breathing test) that may not result in death, be life threatening, or require hospitalization may be considered a serious adverse event when, based upon appropriate medical judgment, when they jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed above.

For this limited subset of reportable adverse events, Investigators should adhere to the requirements and guidelines for Adverse Event and Serious Adverse Event (SAE) reporting as provided in the following sections. Only SAEs are to be reported following the reporting procedure described below. Throughout the study, the investigator must record reportable adverse events on the appropriate adverse event form, regardless of the severity. The investigator should treat participants with adverse events appropriately and observe them at suitable intervals until the events resolve or stabilize.

- Adverse events may be discovered through:
- observation of the participant;
- questioning the participant;
- unsolicited complaint by the participant; or
- discovery of abnormal clinical laboratory values or abnormal results of other evaluations (radiographs, ultrasound, ECG, etc.).

In questioning the participant the questioning should be conducted in an objective manner.

In the event of an abnormal laboratory value, the test should be repeated until it returns to normal or can be explained and the participant's safety is not at risk. Clinically significant laboratory abnormalities as determined by the investigator must be recorded as adverse events.

14.1.3 Death Not Related To Study Procedures

A death occurring in a study participant not associated with blood draws, nasal lavage, allergen skin testing, spirometry, or forced oscillation breathing test will be reported as a serious adverse event not related to study procedures. The reporting process will follow the SAE reporting process.

14.1.4 Expected Adverse Events

The following adverse events are known risks among patients who are participants in the URECA protocol, who, as part of their participation in this observational study, will undergo blood draws, nasal lavage, allergen skin testing, spirometry, and a forced oscillation breathing

test, and are considered "expected" for the purpose of reporting as described in the preceding section.

Expected Adverse Events Associated with Blood Draws

- Fainting \ Vasovagal events
- Bruising at puncture site larger than 2 cm diameter
- Bleeding from puncture site lasting more than 30 minutes
- Swelling at puncture site larger than 2 cm
- Infection at puncture site or cellulitis
- Rash at site of application of EMLA cream
- Thrombophlebitis

Expected Adverse Events Associated with Nasal Lavage

- Bloody nose

Expected Adverse Events Associated with Allergen Skin Testing

- Prolonged (>24 hours) itching at injection site
- Swelling (> 10 mm) at site of injection lasting more than 24 hours
- Nasal allergic symptoms
- Fainting \ Vasovagal event
- Anaphylaxis

Expected Adverse Events Associated with Spirometry

- Wheezing or bronchoconstriction
- Coughing requiring treatment with bronchodilators

Expected Adverse Events Associated with the Forced Oscillation Breathing Test

- Wheezing or bronchoconstriction
- Coughing requiring treatment with bronchodilators

14.1.5 Unexpected Adverse Events

An unexpected adverse event is any adverse event, the nature or severity of which is not consistent with the current protocol or informed consent document.

14.2 Grading of Adverse Events

14.2.1 Event Severity

Adverse events should be recorded and graded 1 to 5 according to the General Grade Definition provided below:
Grade 1 (Mild): Transient or mild discomforts (< 48 hours), no or minimal medical intervention/therapy required, hospitalization not necessary (non-prescription or single-use prescription therapy may be employed to relieve symptoms, e.g., aspirin for simple headache, acetaminophen for post-surgical pain).

Grade 2 (Moderate): Mild to moderate limitation in activity, some assistance may be needed; no or minimal intervention/therapy required, hospitalization possible.
Grade 3 (Severe): Marked limitation in activity, some assistance usually required; medical intervention/therapy required, hospitalization possible.
Grade 4 (Life-threatening): Extreme limitation in activity, significant assistance required; significant medical/therapy intervention required, hospitalization or hospice care probable.
Grade 5 (Death): Death

14.2.2 Relationship to Procedure

The relationship or attribution between an adverse event and a study procedure (i.e., definitely, probably, possibly, unlikely, or not related) is determined by the site investigator or sub-investigator according to the following criteria. **Only events considered definitely, probably, or possibly related to a study procedure (i.e. blood draw, nasal lavage, allergen skin testing, spirometry, or forced oscillation breathing test) are to be reported as adverse events.**

Unrelated: Another cause of the event is most plausible, and/or a clinically plausible temporal sequence is inconsistent with the onset of the event and the study procedure, and/or a causal relationship is considered biologically implausible. No temporal association or rock solid etiology.
Unlikely Related: Another etiology is identified, and/or the event could be readily reproduced by clinical state or by environmental or other interventions; the event does not reappear or worsen with re-challenge.
Possibly Related: An event that follows a reasonable temporal sequence from timing of study procedure, follows a known or expected response pattern to the study procedure, but that could readily have been produced by a number of other factors.
Probably Related: An event that follows a reasonable temporal sequence from timing of the study procedure. In addition, the relationship may be confirmed by improvement on stopping and reappearance of the event on repeated study procedures.
Definitely Related: There is a reasonable temporal relationship to the study procedure; the event is not readily produced by the subject's clinical state or by environmental or other interventions; the event follows a known pattern of response to the study procedure; the event decreases with de-challenge and recurs with re-challenge.

- The following events show that there is no reasonable possibility that the event may have been caused by the study procedure:
- The event is judged to be due to extraneous causes such as disease or environment or toxic factors;
- The event is judged to be due to the subject's clinical state or other therapy being administered;
- The event is not a known response to study procedure based on clinical or pre-clinical data;
- The event does not reappear or worsen when study procedure is re-administered;
- The event does not follow a temporal sequence from administration of the study procedure.

The following events show there is a reasonable possibility that the event may have been caused by the study procedure:

- The event follows a temporal sequence from onset of study procedure;
- The event is a known response to the study procedure based on clinical or pre-clinical data;
- The event could not be explained by the known characteristics of the subject's clinical state, environmental or toxic factors, or other therapy administered to the subject;
- The event disappears or decreases upon cessation or reduction of the dose of study procedure;
- The event reappears or worsens when study procedure is re-administered.

14.3 Reporting of Adverse Events to the SACCC

14.3.1 Reporting Procedure

All adverse events occurring during the study that are considered to be related to the blood draws, nasal lavage, allergen skin testing, spirometry, or forced oscillation breathing test will be reported to the SACCC. The SACC reports to the NIAID Medical Monitor who is responsible for passing the information on to the DSMB. Serious events will be followed until resolved or considered stable.

14.3.2 Serious Adverse Event Reporting

All serious adverse events (SAE) and grade 3 or higher unexpected adverse events as defined above (if they are considered to be related to the blood draws, nasal lavage, allergen skin testing, spirometry, or forced oscillation breathing test) must be immediately reported to the SACCC via email as described in the study Manual of Operations. In addition to the email, the site must complete an SAE form and transmit it to the SACCC. The instructions on how to complete the SAE form and the SACCC fax number can be found in the study Manual of Operations. If the event is a fatal or life-threatening event, the SAE form must be completed and faxed to the SACCC within one business day. If the event is not fatal or life threatening, then the SAE form must be faxed to the SACCC within three business days. The SACCC will review the SAE form and may query the study site for omitted or inconsistent SAE information.

If the SAE or grade 3 or higher unexpected adverse event, which is related to a study procedure, is proven to be fatal or life-threatening, a safety report will be sent to the NIAID and DSMB within 7 days. The SACCC will continue to query the study site until resolution or stabilization of the event. The SACCC will generate follow-up reports.

14.3.3 Adverse Event Reporting

Adverse events not meeting the definition of serious as defined above and less than grade 3 will be recorded on the appropriate case report form and sent to the SACCC for incorporation into periodic reporting to the NIAID and DSMB.

14.3.4 Reporting to the IRB

The Principal Investigator (or delegate) must report adverse events and serious adverse events to their local ethics review committee (or IRB) promptly in accordance with local regulations or policies, in addition to providing the information to the SACCC.

14.4 Safety Monitoring

14.4.1 Site Review

The site investigator must apply his/her clinical judgment as to whether an adverse event is of sufficient severity to require that the subject immediately be removed from further treatment under the protocol. The investigator must institute any necessary medical therapy to protect a subject from any immediate dangers. Subsequent review by the DAIT Medical Monitor, DSMB, ethics review committee or IRB, the NIAID or relevant local regulatory authorities may also suspend further study activities at a site.

14.4.2 Routine SACCC Review

The SACCC will generate and review reports that compile all newly submitted adverse events. Items that are considered questionable, inconsistent or unexplained (including use of medications for what may be an unreported adverse event) will be referred to the site Nurse Coordinator for confirmation and response. Subsequent review is performed as appropriate by the DAIT Medical Monitor and the DSMB.

14.4.3 Medical Monitor Review

The DAIT Medical Monitor will review all serious adverse events and grade 3 or higher unexpected adverse events immediately upon notification by the SACCC. The Medical Monitor will review reports prepared by the SACCC of all adverse events and laboratory findings at least monthly.

14.4.4 DSMB Review

The DSMB will review any events as requested by the Investigators, SACCC, or DAIT Medical Monitor. They will review a listing of all adverse events and laboratory findings approximately twice per year. Further, the DSMB will be informed of expedited SAEs at the same time as local IRBs.

15 Quality Assurance

15.1 Training

Training will be conducted centrally and/or at each site prior to beginning recruitment. The training will include lecture, demonstration, and practice components to insure that all staff members are fully trained in all aspects of the study protocol. After the training sessions, staff will complete certification exams (written and/or practical) to demonstrate acceptable levels of knowledge regarding each study component they will be involved in performing. Details of the certification exams are provided in the Manual of Operations.

15.2 Quality Control Procedures

The study investigator and study coordinator will be responsible for insuring that all procedures are performed according to the protocol. The investigator or study coordinator will oversee all activities at Visits 1 and 2 for each of the first three participants enrolled. Feedback from these sessions will be given to the staff members. If necessary, extra training will be provided, and the staff member will be required to perform two additional practice sessions. Periodic reviews (every three to six months) of procedures will be conducted by the study coordinator according to an individual schedule for each staff member that is based on the activities he/she is responsible for conducting. Details of the quality control plan are provided in the Manual of Operations.

15.3 Operations Manuals

A study Manual of Operations will be given to all investigators and staff members. This manual will describe in detail how to perform each study procedure or activity.

15.4 Monitoring And Site Visits

Representatives from the NIAID and the SACCC will visit each site after the beginning of the study, and yearly thereafter to check the adherence to the protocol and to Good Clinical Practice, and the progress of enrollment. In addition, the SACCC will review all data collection procedures, including completion of study forms, data entry of study forms, flow of forms and edit reports, filing of forms and edit reports, audit trails, and security measures for study data. Key trial personnel must be available to assist the visitors during these visits.

No information in these records about the identity of the subjects will leave the study center.

16 Other Administrative Procedures

16.1 Disclosure And Confidentiality

Each participating investigator agrees to keep all information provided by Rho, and NIAID in strict confidence and to request similar confidentiality from his/her staff and the IRB. Study documents NIAID or Rho (i.e., protocols, study forms, Manuals of Operation, and other study materials) will be stored appropriately to ensure their confidentiality.

Data on participants collected on study forms during the trial will be documented in an anonymous fashion. The participant will only be identified by the participant number, and by his/her initials if also required. If, as an exception, it is necessary for safety or regulatory reasons to identify the participant, Rho and the investigator are required to keep this information confidential.

16.2 Institutional Review Board

Before implementing this study, the protocol, the proposed informed consent form, and other information to be given to participants, must be reviewed by a properly constituted Institutional Review Board (IRB) at each investigational site. A signed and dated statement that the most current version of the protocol and informed consent have been approved by the IRB must be given to the SACCC before study initiation. The name and occupation of the chairperson and the

members of the IRB must be supplied to the SACCC. This committee must approve any amendments to the protocol, other than administrative ones.

16.3 Informed Consent

The investigator must explain to each participant (or legally authorized representative) the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved and any discomfort it may entail. Each subject must be informed that participation in the study is voluntary and that he/she may withdraw from the study at any time and that withdrawal of consent will not affect his/her subsequent medical treatment or relationship with the treating physician.

This informed consent should be given by means of a standard written statement, written in non-technical language. The subject should read and consider the statement before signing and dating it and should be given a copy of the signed document. If written consent is not possible, oral consent can be obtained if witnessed by a signed statement from one or more persons not involved in the study, mentioning why the participant was unable to sign the form. No participant can enter the study before his/her informed consent has been obtained.

The informed consent form is considered part of the protocol and must be submitted by the investigator with the protocol for IRB approval. A proposed informed consent form, which complies with regulatory requirements and is considered appropriate for the study, will be developed. Each investigational site may change the proposed consent in accordance with requirements of their local IRB. A copy of the approved version must be provided to Rho after IRB approval.

16.4 Ethics And Good Clinical Practice

- This study will be conducted according to Good Clinical Practice and applicable federal regulations:
- Good Clinical Practice: Consolidated Guideline (ICH E6) as published in the Federal Register, Vol. 63, No. 111, June 10, 1998, page 31790
- 21CFR50 (Protection of Human Subjects)
- 21CFR56 (Institutional Review Boards)
- 21CFR312 Subpart D (Responsibilities of Sponsors and Investigators)
- 45CFR46 (Protection of Human Subjects)

16.5 Protocol Amendment Policy

Any change or addition to this protocol requires a written protocol amendment that must be approved by the ICAC Steering Committee and the investigator before implementation. Amendments significantly affecting the safety of participants, the scope of the investigation or the scientific quality of the study, require additional approval by the IRB of all investigational sites. Examples of amendments requiring such approval are:

- a significant change in the study design (e.g. addition or deletion of a control group);
- an increase in the number of invasive procedures to which subjects are exposed;
- an addition or deletion of a test procedure for safety monitoring.

These requirements for approval should in no way prevent any immediate action from being taken by the investigator in the interests of preserving the safety of all subjects included in the trial. If an immediate change to the protocol is felt to be necessary by the investigator and is implemented by him/her for safety reasons, the Steering Committee should be notified and the IRB at the investigational site should be informed within 10 working days.

Amendments affecting only administrative aspects of the study do not require formal protocol amendments or IRB approval but the IRB of each center must be kept informed of such administrative changes.

Once approved by the ICAC Steering Committee and the NIAID, amendments to the protocol are sent to the sites in the form of a revised protocol with changes tracked, along with an accompanying list of specific changes to the new protocol version.

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Appendix 1: Urban Environment and Childhood Asthma (URECA) Weight Study

A1.1 Hypotheses

1. Accelerated growth during infancy and early childhood will be a significant predictor of asthma symptoms (recurrent wheeze).

2. An earlier onset of overweight will be associated with a higher risk of developing asthma (recurrent wheeze).

3. Persistent overweight, but not transient overweight, will be associated with recurrent wheeze.

4. Levels of plasma leptin, adiponectin, CRP, and other hormones related to obesity in cord blood and at 1 year of age will be predictive of recurrent wheeze.

5. Overweight will be associated with an allergic cytokine profile (without necessarily overt symptoms of atopy).

6. High levels of plasma leptin, adiponectin, CRP, and other hormones related to obesity in cord blood and at 1 year of age will be associated with an allergic cytokine profile.

A1.2 Background and Rationale

There has been a parallel increase in prevalence of asthma and obesity (Flegal et al, 2002; Mannino et al, 2002; Ogden et al, 2002). Mounting evidence points to obesity as a risk factor for asthma. A number of cross-sectional and longitudinal studies have shown that excess weight increases the risk for the development of asthma in children and adults (Camargo et al, 1999; Castro-Rodriguez et al, 2001; Gold et al, 2003; Luder et al, 1998). Other studies have shown that rapid weight gain early in childhood may be related to development of asthma (Rona, 2005). An association between obesity and airway hyperresponsiveness has been observed in some studies. There are few data on the effect of obesity on the health status of patients with asthma. Obese asthmatic subjects studied after weight loss demonstrated decreased severity and symptoms of asthma (Dixon et al, 1999; Stenius-Aarniala et al, 2000) . In a study of inner city children with asthma, obese children had more symptoms and reported higher medication use than non-obese children but no difference in peak flow rates (Belamarich et al, 2000).

Weight gain resulting in obesity may directly affect the asthma phenotype and may be mediated by mechanical properties of the respiratory system or by modulation of inflammation in the airway. The obese state is characterized by low-grade systemic inflammation (Cottam et al, 2004). Inflammatory markers, such as C-reactive protein (CRP), TNF-α and IL-6, are increased in obese individuals compared with lean subjects, although not to the same extent observed in classic inflammatory conditions (Ford, 2003).

Adipokines are proteins produced mainly by adipocytes. Leptin, an adipocyte derived hormone, is increased in obesity and positively correlates with body fat mass (Maffei et al, 1995). Leptin stimulates the release of proinflammatory cytokines, such as C-reactive protein, IL-6, and TNF-α, and has the capacity to increase airway inflammation. Leptin upregulates cytokine production

in monocytes and the expression of adhesion molecules in endothelial cells. Leptin levels are increased during allergic reactions in the airways in mice, raising the possibility that obesity may modulate the allergic response. Administration of leptin increases airway hyperresponsiveness and Th-2 cytokine production in ovalbumin-sensitized mice (Shore et al, 2005). High leptin levels have been observed in asthmatic children compared with a control group with similar body mass index.

Adipocytes are the most important source of adiponectin but serum levels do not increase with obesity. Adiponectin appears to have anti-inflammatory activities (Wulster-Radcliffe et al, 2004). It inhibits IL-6 production and reduces nuclear factor κB (NF-κB) signaling and induction of endothelial adhesion molecules by TNF-α (Ouchi et al, 1999).

Resistin, adipsin and visfatin are other adipokines. These have been less well studied in relation to the inflammatory response.

The increased chest wall and abdominal mass in obese individuals reduces functional residual capacity (Hakala et al, 2000). The reduced lung volume reduces airway diameter. The result is unloading of airway smooth muscle that allows it to shorten excessively when activated. In addition, lower tidal volumes in obese individuals may compromise the bronchodilating effect of tidal strains.

To understand the relationship between obesity and asthma, measuring fat distribution is important. There are several measures to assess obesity. In studies of asthma, body mass index has been used most frequently. This measure is simple for screening and widely used in epidemiologic studies. Reference values are published. However, it is not the best measure of body fat and does not account for the proportion of muscle mass. The skin fold thickness does measure the proportion of fat but its usefulness is limited by large intra-observer variability. Bioelectrical impedance analysis (BIA) is a good simple measure of percent body fat and regional body fat.

Children living in inner cities have greater asthma morbidity and a higher rate of obesity than the general population. They are exposed to multiple indoor allergens and pollutants that promote inflammation and contribute to the severity of asthma. In the previous Inner City Asthma Study (ICAS), we demonstrated that 1) the adverse effect of outdoor pollutants on asthma morbidity was greater in overweight children compared to normal weight children and 2) the beneficial effect of reduction of cockroach allergen on asthma morbidity was greater in overweight children compared to normal weight children with asthma.

These studies suggest a link between obesity and asthma, but there is a paucity of information on the role of obesity and adipose tissue in modulating asthma symptoms. The URECA cohort provides an opportunity to examine the relationship between markers of inflammation, symptoms, pulmonary function and adiposity, along with the timing of the development of overweight and the development of asthma.

A1.3 Study Overview

A1.3.1 Overall Study Design

To address the primary objectives of the URECA Weight Study Protocol, all the participants enrolled in the URECA Protocol who provide consent for the Weight Study Protocol will be studied.

A1.3.2 Study Population

A1.3.2.1 Population Description

To address the objectives of the Weight Study, children age 0-3 with a history of parental asthma or allergy will be studied.

A1.3.2.2 Inclusion Criteria

Participants to be included in the URECA Weight Study are those who qualify for the general URECA Protocol.

A1.3.2.3 Exclusion Criteria

Participants to be excluded are those who fail to qualify for the general URECA Protocol.

A1.3.3 Recruitment

All URECA participants will be invited to participate in the Weight Study Protocol. If caretakers of participants consent to the general URECA study but do not wish to participate in the Weight Study, they will not be penalized. There will be no over-recruiting at the sites for the purpose of increasing sample size for the Weight Study.

A1.4 Study design

Participants who consent to the URECA Weight Study Protocol will undergo procedures in the general URECA Protocol plus the following procedures:

1. Additional weight and length measurements at approximately 3 months of age.
2. Bioelectrical Impedance Analysis (BIA) at each yearly clinic visit.

A1.5 Special Study Assessments

A1.5.1 3-Month Weight and Length Measurements

At the time of the 3-Month Home Visit or the Pollution Monitoring Pick-Up Visit (which is two weeks after the 3-Month Home Visit), the URECA participant will be weighed in the home, using a portable scale, and his or her length will be measured using a folding length mat.

A1.5.2 Bioelectrical Impedance Analysis

Bioelectrical impedance will be used to assess body composition including fat mass, lean mass, and total body water. A Quantum II BIA Analyzer (RJL Systems: Clinton Township, MI) will be used to measure whole body resistance and reactance. These measurements and anthropomorphic data are then entered into the LeanBody software to obtain 2-compartment

body composition. The technique involves attaching surface electrodes to various locations on the arm and foot. These measurements will be done at each annual clinic visit (at approximately 12, 24, and 36 months of age).

A1.6 Laboratory Samples

A1.6.1 Sample Collection, Storage at Site and Shipping

Blood: Plasma samples from all sites are being shipped to a central storage area under the general URECA protocol. For the Weight Study Protocol, a plasma aliquot will be shipped from central storage to the University of Washington for analysis of inflammatory markers. Outcome data will be sent to Rho for analysis.

A1.7 Statistical Methods

A1.7.1 Sample Size

All URECA participants will be invited to participate in the Weight Study Protocol. Since the URECA study was not designed to test the scientific hypotheses posed by the Weight Study Protocol, all analyses are considered exploratory and all available data will be used.

A1.7.2 Variable Definitions

A1.7.2.1 Weight and body composition

- **Overweight versus typical weight groups**:
  Length and weight for the URECA Weight study will be measured at birth, at approximately 3 months, and at 12, 24 and 36 months.
  At risk of overweight is defined as a BMI-for-age between the 85th and the 95th percentiles.
  Overweight is defined as a BMI-for-age at or above the 95th percentile
- **% total body fat**: % total body fat will be measured using BIA. This procedure will be conducted at each annual clinic visit.
- **Plasma leptin level**: Plasma levels of leptin will be measured at birth and at each annual clinic visit, and will be used on a continuous scale.
- **Change in body mass (longitudinal):** The percent change in BMI-for-age percentile measured at the various time points.
- **Accelerated growth:** Increase in BMI-for-age percentile of > 1 standard deviation.
- **Persistent overweight:** Overweight in the first year of life that persists into the second and third years.

A1.7.2.2 Morbidity endpoints

- **Recurrent Wheeze:** Recurrent wheezing is defined as at least two episodes of wheezing in the first three years of life and one of these episodes of wheezing must have occurred during the third year.
- **Allergic Cytokine Profile:** The cytokine profile variables will be developed during the exploratory analysis phases. They will be clearly specified prior to the analysis and may include composite summary scores to describe inflammation or immunologic functions at different time points, individual cytokine levels of unique importance (for cytokines that

are independent of composite summary scores), and/or variables for at-risk subgroups defined by, for example, temporal occurrence of immune system developmental events or evidence of cytokine dysfunction.

A1.7.2.3 Inflammatory marker endpoints

Inflammatory markers will be assessed in cord blood at birth, and in peripheral blood at each annual clinic visit. These inflammatory markers may include: C-reactive protein, IL-6, TNF-α, leptin, adiponectin, or others. Transformations of marker variables will be investigated.

A1.7.2.4 Questionnaire assessments

Diet information already being collected by the general URECA protocol, including breastfeeding duration, food sensitivities, and supplemental vitamin intake, will be incorporated as potential confounders into analyses for the Weight Study Protocol.

A1.7.3 Analyses

Since the Weight Study Protocol is an ancillary study using subjects enrolled in the URECA trial, all analyses are exploratory. P-values presented to assess the evidence supporting the scientific hypotheses will not be adjusted for multiple comparisons and should be interpreted cautiously.

A1.7.3.1 Hypothesis 1

Hypothesis 1) Accelerated growth during infancy and early childhood is a significant predictor of asthma symptoms (recurrent wheeze).

Recurrent wheeze also will be modeled as an effect of BMI-for-age percentile in a logistic regression model. Covariates will include site, gender, and ethnicity. BMI slope, 95% CI and p-values will be reported.

The rate of growth in the first 3 months and months 4-12 of the first year of the infant's life will be calculated. Accelerated growth will be defined as a change in the BMI-for-age percentile of more than one standard deviation, or another cut-off value that may appear to be important in the analyses described above. Recurrent wheeze for accelerated growth and normal growth infants will be estimated and compared using separate logistic regression models for the first 3 months and months 4-12 with site, gender and ethnicity as covariates.

A1.7.3.2 Hypothesis 2

Hypothesis 2) An earlier onset of overweight will be associated with a higher risk of developing asthma (recurrent wheeze).

The risk of developing recurrent wheeze by age 3 years will be modeled as an effect of the time of onset of overweight using logistic regression modeling with site, gender, and ethnicity as covariates. Overweight will be assessed as a dichotomous variable at 3 months, and 1, 2, and 3 years of age.

A1.7.3.3 Hypothesis 3

Hypothesis 3) Persistent overweight, but not transient overweight, will be associated with recurrent wheeze.

The risk of developing recurrent wheeze will be modeled as an effect of persistent overweight (overweight developing in the first year and persisting until 3 years, vs. overweight in the first year which does not last through the following 2 years) using logistic regression modeling with site, gender, and ethnicity as covariates.

A1.7.3.4 Hypothesis 4

Hypothesis 4) High levels of plasma leptin, adiponectin, and CRP in cord blood and at 1 year of age will be predictive of recurrent wheeze.

The risk of developing recurrent wheeze will be examined as an effect of the levels of leptin, adiponectin and CRP, in separate logistic regression models with site, gender, and ethnicity as covariates. Appropriate parameterization of plasma leptin, adiponectin, and CRP levels in these models will be explored.

A1.7.3.5 Hypothesis 5

Hypothesis 5) Obesity will be associated with an allergic cytokine profile (without necessarily overt symptoms of atopy).

The allergic cytokine profile will be defined in exploratory analyses for the general URECA protocol as variables that describe "cytokine dysfunction" and "patterns of cytokine responses." These responses will be modeled as an effect of overweight in appropriate generalized linear regression models with site, gender, and ethnicity as covariates.

A1.7.3.6 Hypothesis 6

Hypothesis 6) High levels of plasma leptin, adiponectin, and CRP in cord blood and at 1 year of age will be associated with an allergic cytokine profile.

The relationship of the allergic cytokine profile as described above and levels of leptin, adiponectin and CRP will be examined using appropriate generalized linear regression models with site, gender, and ethnicity as covariates. Data plots will be used to better understand the relationship between cytokine levels and inflammatory marker levels. Appropriate parameterization of plasma leptin, adiponectin, and CRP levels in these models will be explored.

A1.8 References

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Appendix 2: Urban Environment and Childhood Asthma (URECA) Non-Allergic Families

A1.1 Hypotheses

1. Children without a parental history of allergies and asthma, but with environmental exposures similar to the main URECA cohort, will have a different cytokine profile at birth and later in life.

A2.2 Background and Rationale

The URECA study was initially designed to restrict enrollment to children of atopic parents: this is the group with the greatest risk for asthma, and focusing on a high-risk group helped to keep the sample size manageable.

There are distinct advantages, however, to including children of non-atopic parents in the URECA study. First, several scientific studies indicate that children of atopic parents can have skewed immunologic responses in early childhood, even in the absence of clinical manifestations of atopy.[1-3] It is possible that this skewing reflects an increased likelihood for allergy and asthma later on in childhood. In addition, it is possible that children of atopic vs. non-atopic parents differ in other ways, including environmental exposures to microbes or allergens, the number and severity of viral respiratory infections, or exposure to indoor pollutants. Knowing how family history alters immune development, lifestyle, and early life environmental exposures would be an important advantage to the URECA study: this information would yield insights as to whether risk factors and concepts that are related to immune development, allergies, and asthma apply only to children of atopic families, or are likely to be relevant to the general population.

It is for these reasons that we now propose to enroll a limited number of children of non-atopic parents upon which to base these comparisons. It should be noted that even children of non-atopic parents are at risk for allergy and asthma, and, in fact, the majority of children with allergy and asthma are born to families without a first degree affected relative. Children from non-atopic families who grow up within the catchment area defined by the URECA entry criteria are also at increased risk for atopic diseases on the basis of ethnicity, socioeconomic status, and environmental influences that are incompletely understood.

Based on this information, **we hypothesize that children of non-atopic (vs. atopic) parents will have distinct patterns of immune development (cytokine response profiles of peripheral blood cells) in early childhood.** We propose that the reason for this difference is that a greater percentage of children of atopic parents will develop allergies, asthma, or atopic dermatitis; in other words, the immunologic abnormalities precede the clinical expression of atopy.

A2.3 Study Overview

A2.3.1 Overall Study Design

To address the primary objectives of the URECA Non-Allergic Comparison Group Protocol, 50-60 participants will be enrolled that do not meet the parental allergy/asthma criterion of the main URECA protocol. These participants will complete the entire URECA protocol.

A2.3.2 Study Population

A2.3.2.1 Population Description

To address the objectives of the Non-Allergic Comparison Group Protocol, children age 0-3 *without* a history of parental asthma or allergy, but who meet all the other eligibility criteria of the URECA protocol, will be studied.

A2.3.2.2 Inclusion Criteria

Participants to be included in the URECA Non-Allergic Comparison Group are those who qualify for the general URECA protocol, with the exception of having a parental history of asthma, allergic rhinitis (hay fever), or eczema (atopic dermatitis).

- Planning to deliver at the study hospital.
- All study subjects will reside in census tracts with at least 20% of the residents with income below the poverty level.
- Gestational age at delivery of ≥34 weeks
- A suitable cord blood specimen must be obtained and processed to establish baseline cytokine secretion data.

A2.3.2.3 Exclusion Criteria

Participants to be excluded are those who fail to qualify for the general URECA Protocol.

- Respiratory distress requiring intubation and ventilation for four or more hours.
- Respiratory distress requiring either supplemental oxygen or CPAP for four or more days.
- Pneumonia requiring antibiotic treatment for one week or more.
- Significant congenital anomalies.
- Maternal HIV infection at time of delivery.
- Plans for the family to move out of the geographic area during the period of the study.
- Does not consent to all aspects of the study.

- Does not have access to a phone.
- Does not speak English (or Spanish at sites with Spanish-speaking staff).
- Administration of palivizumab (Synagis) for RSV prophylaxis.

A2.3.3 Recruitment

During the recruitment phase, pregnant women who are screened for the URECA study, but fail to qualify because of not meeting the allergy criteria, will be invited to participate in the Non-Allergic Comparison Group Protocol.

A2.4 Study design

Participants who consent to the URECA Non-Allergic Comparison Group Protocol will undergo all the same procedures as in the general URECA Protocol.

A2.5 Statistical Methods

A2.5.1 Sample Size

The chosen sample size should provide enough power to compare differences in cytokine levels in cord blood and at ages 1, 2 and 3. The sample size of 50-60 children 0-3 years of age was calculated using information that PHA-induced IFN-γ may be as much as 50% lower among children with a parental history of allergies.[1]

In contrast to the usual t test on normal data, the hypotheses with lognormal data are defined in terms of geometric means rather than arithmetic means. The test assumes an equal coefficient of variation in the two groups, an alpha level of 0.05, and a fixed sample size for the main URECA group at 500 individuals. The null hypothesis is for the geometric mean ratio to be equal to 1. The coefficient of variation of 0.86 was calculated using data for PHA-induced INF-γ from the first 100 cord blood samples in URECA. Larger coefficients are also tested. In the first panel below, a power of >80% can be achieved with a non-allergic sample of 30 individuals. To allow for study attrition (which we anticipate may be higher in this group than in the main URECA study) we plan to recruit up to 60 participants.

Table 1: Sample size for coefficient of variation and percent change.

Geo Mean Ratio=1.5 CV=0.85

N1	Power
10	0.405
20	0.673
30	0.831
40	0.916
50	0.959
60	0.980
70	0.990
80	0.995
90	0.998
100	0.999
110	0.999

Geo Mean Ratio=1.5 CV=1.00

N1	Power
10	0.331
20	0.568
30	0.734
40	0.841
50	0.906
60	0.945
70	0.968
80	0.981
90	0.989
100	0.993
110	0.996

Geo Mean Ratio=1.5 CV=1.25

N1	Power
10	0.257
20	0.448
30	0.602
40	0.719
50	0.803
60	0.863
70	0.905
80	0.934
90	0.954
100	0.968
110	0.977

Geo Mean Ratio=2.0 CV=0.85

N1	Power
10	0.836
20	0.984
30	0.999
40	1.000
50	1.000
60	1.000
70	1.000
80	1.000
90	1.000
100	1.000
110	1.000

Geo Mean Ratio=2.0 CV=1.00

N1	Power
10	0.740
20	0.954
30	0.993
40	0.999
50	1.000
60	1.000
70	1.000
80	1.000
90	1.000
100	1.000
110	1.000

Geo Mean Ratio=2.0 CV=1.25

N1	Power
10	0.608
20	0.879
30	0.967
40	0.991
50	0.998
60	0.999
70	1.000
80	1.000
90	1.000
100	1.000
110	1.000

A2.7.2 Variable Definitions

A2.7.2.1 Morbidity endpoints

- **Allergic Cytokine Profile:** The cytokine profile variables will be developed during the exploratory analysis phases. They will be clearly specified prior to the analysis and may include composite summary scores to describe inflammation or immunologic functions at different time points, individual cytokine levels of unique importance (for cytokines that are independent of composite summary scores), and/or variables for at-risk subgroups defined by, for example, temporal occurrence of immune system developmental events or evidence of cytokine dysfunction.

A2.7.2.2 Questionnaire assessments

All information being collected by the general URECA protocol, will be collected for the Non-Allergic Comparison Group Protocol as well.

A2.7.3 Analyses

Since the Non-Allergic Comparison Group Protocol is an ancillary study with a small group of subjects, all analyses are exploratory. P-values presented to assess the evidence supporting the scientific hypotheses will not be adjusted for multiple comparisons and should be interpreted cautiously.

A2.7.3.1 Hypothesis 1

Hypothesis 1) Children without a parental history of allergies and asthma will have a different cytokine profile at birth and later in life.

The allergic cytokine profile will be defined in exploratory analyses for the general URECA protocol as variables that describe "cytokine dysfunction" and "patterns of cytokine responses." These responses will be modeled as an effect of parental history of asthma or allergy in appropriate generalized linear regression models with site, gender, and ethnicity as covariates.

A2.7 References

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Appendix 3: Reference Sample for Viral Infections

The number of calls received from mothers for respiratory illnesses was lower than expected in the first winter of the study (2005-2006). In order to help determine the number of respiratory illnesses that may have been missed, a small Quality Control study may be performed each year.

A sample of participants from each site will be contacted every two weeks during the peak respiratory illness season to determine a reference rate for respiratory infections in the study population. Twenty volunteers from each site will be invited to serve as the reference group. The participants will be reimbursed $5 for each successful telephone call. Each participant would be called approximately six times during this window for a total reimbursement of up to $30 per participant. The participating families would be called every other week during the peak of the cold/flu season to ask if their child is having a cold or respiratory illness. Each site's reference group would be divided into two groups of 10 and called on an alternating schedule. Hence, the first 10 participants would be called one week and the second 10 participants would be called the following week.

If the child is sick with a cold or respiratory illness, a scorecard (Form 50) will be filled out and the respiratory illness procedures will be followed as in the main URECA protocol (see Manual of Operations for more details).