RISK FACTORS FOR INCIDENCE AND PERSISTENCE OF
ASTHMA-LIKE SYMPTOMS

by

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A Dissertation Submitted to the Faculty of the
GRADUATE INTERDISCIPLINARY PROGRAM IN EPIDEMIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2003
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ACKNOWLEDGEMENTS

I would like to thank my advisor, Duane L. Sherrill, PhD, for his continued support throughout these years and his valuable advice not only on this endeavor, but also on my general career goals. I am also grateful to all my committee members, Robert Barbee, MD, Robert Erickson, MD, Anabel Hill, PhD, and John Meaney, PhD, for their support and insight. I feel that my research skills and critical thinking have been enormously enriched by working with them. I would like to thank all my colleagues at the Arizona Respiratory Center, the co-authors of the papers appended to this dissertation and my fellow students for making this endeavor a feasible and even enjoyable task. Last but not least, my warmest thanks to Fernando Martinez, MD, Anne Wright, PhD, and Marilyn Halonen, PhD, for allowing me to use for this dissertation the cohorts of the Tucson Children’s Respiratory Study and Infant Immune Study and for making me part of an extraordinary research effort.
DEDICATION

This dissertation is dedicated to my wife, who has been part of this endeavor in any imaginable way.

She has brought to it her patience for my many evenings at work; her strength for taking constant care of our two little "hurricanes"; her insight for giving me a different and, almost invariably, better way to look at things.

Most important, she has brought her brightening love.

With the very same love, to Cristiana.
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ABSTRACT

Asthma represents the most common chronic disease in childhood. Children with asthma are at increased risk for developing long-term irreversible airway obstruction in adult life, the fourth leading cause of death in USA.

Our aims were to:

1 - Determine whether reduced IFNγ production and plasma soluble CD14 (sCD14) levels in early life are significant risk factors for the development of wheezing in the first year of life;

2 - Estimate rates of persistence and remission of childhood wheezing after puberty;

3 - Study risk factors affecting persistence of childhood wheezing after puberty.

We used data from the two large ongoing birth cohorts of the Tucson Infant Immune Study (IIS) and the Tucson Children’s Respiratory Study (CRS).

Among 238 children from IIS, we found the odds of developing recurrent wheezing in the first year of life to be 4.5 times higher for children in the lowest quartile of IFNγ
production at 3 months (p = .0005) and 3.2 times higher for children in the lowest quartile of sCD14 levels at birth (p = .004) as compared with children in the other 3 combined quartiles of IFNγ and sCD14, respectively.

We studied persistence and remission of wheezing after puberty among 732 children from the CRS cohort. We found that 29% of children with infrequent wheezing during childhood experienced persistent wheezing after puberty. In contrast, the proportion of persistent wheezing was much higher (60%) among children meeting the criteria for asthma during childhood. Frequency of wheezing during childhood, obesity, an early onset of puberty, bronchial hyperreactiveness, and skin test sensitization were significant predictors of persistent asthma after puberty. By looking at genetic factors, we also found that the homozygous status for Gly in codon 16 of the β2 Adrenoceptor doubled the risk for persistent wheezing after puberty among boys (RR 2.01, p = .0008) but not girls.

Our findings from two population-based longitudinal cohorts provide the first evidence that altered immunological markers precede the onset of wheezing early in life, challenge the commonly held view that most asthma cases
remit during adolescence, and provide a profile of risk factors for persistence of asthma after puberty.
INTRODUCTION

Explanation of the Problem and Its Context

In western societies, asthma prevalence has been dramatically increasing in the last decades and, at the present time, asthma is the most common chronic disease in childhood. According to surveillance data from the Centers for Disease Control and Prevention, the annual prevalence of self-reported asthma among 5 to 14 years old children has almost doubled between 1980 and 1995 in the United States (from 45 per 1,000 to 82 per 1,000)(1).

Given the high prevalence rates of this disease, it is not surprising that asthma is associated with an important economic burden to society. In 1985, the economic burden of asthma represented over 1% of total health care costs in USA, the largest proportion a single illness accounted for(2). The estimates in 1994 US dollars of the total direct costs of asthma were close to $5,000 millions in two different studies(2, 3), with the estimates for indirect costs varying from $700 to $3,000 millions.

The study of asthma during childhood holds particular interest from both a public health and a scientific perspective. In addition to the high prevalence and
associated costs, in fact, pediatric asthma has been linked to the presence of Chronic Obstructive Pulmonary Disease (COPD) in adulthood(4), a group of severe diseases that represent the fourth leading cause of death in the United States. Moreover, epidemiological longitudinal cohort studies have shown 1) that the inception of asthma is related to the development of the immune system and respiratory tract in the first years of life and it is strongly affected by early life events(5); and 2) that almost 50% of children experience at least one wheezing episode by the age of 6(6). Taken together, this evidence suggests that studies investigating the inception of asthma should focus on infancy and childhood (see Specific Aim 1) and that the identification of patterns of risk factors for the onset and persistence of the disease in childhood may have important public health implications (see Specific Aims 2 and 3).

With respect to the persistence of asthma, the reaching of school-age and the onset of puberty have been traditionally considered two critical time-points to evaluate the course of the disease. In the cohort of the Tucson Children’s Respiratory Study, Martinez et al(6) found that about 60% of the children with wheezing before the age of 3 years were free of symptoms by age 6. These findings suggest that the majority of infants with wheezing have transient conditions
and no increased risk for asthma later in life. However, they also confirm that in a substantial minority of infants wheezing episodes are related to a predisposition to asthma and that the presence of wheezing early in life is indeed a significant risk factor for childhood asthma. While several studies have investigated the outcome at school-age of wheezing in infancy, to date only few population-based longitudinal studies have assessed rates and predictors of persistence/remission of childhood asthma at puberty. This is particularly surprising since it has been long recognized that many children with asthma may outgrow the disease during adolescence(7), a period characterized by rapid hormonal, physical and behavioral changes, all of which might affect the natural course of asthma. However, this observation has been based on "anecdotal" clinical experience rather than unbiased epidemiological data and, to date, it remains unknown what proportion of asthmatic children experience a remission of the disease after the onset of puberty and what factors affect the outcome(8). The identification of rates and predictors of persistent wheezing/asthma at puberty represented Specific Aims 2 and 3 of this dissertation.

The identification of children with asthma in epidemiological studies can be a challenging task. The approach of using only cases with a physician confirmed
diagnosis of the disease is possibly biased by SocioEconomic Status (SES), ethnic and cultural factors. The use of objective measurements, such as bronchial hyperresponsiveness, can fail to capture the complex phenotypic nature of the disease and requires frequent bronchial challenge tests, which can be unacceptable to children and parents. Indeed, the self-reported presence of respiratory symptoms appears to be an approach both reliable on a scientific ground and feasible from a logistic point of view, as it requires simply the use of parent-completed questionnaires. Among the respiratory symptoms, wheezing has been frequently used as a marker for asthma in pediatric populations for several reasons. The presence of wheezing has been shown to strongly correlate with other asthma-related phenotypes, such as atopy and bronchial hyperresponsiveness(9), and to have good reproducibility in epidemiological studies(10). Although children with asthma may present simply with recurrent cough in the absence of any wheezing (the so-called “cough-variant asthma”)(11), these cases are probably rare. Indeed, the vast majority of children with asthma experience wheezing episodes and wheezing has been used as a “clinical hallmark” for asthma in childhood in most epidemiological studies.

The aims of the present study were:
1 - To determine whether specific immunologic markers, i.e. reduced IFNγ production and plasma soluble CD14 (sCD14) levels, in early life are significant predictors for the development of wheezing in the first year of life;

2 - To estimate the rates of persistence and remission of childhood wheezing after the onset of puberty;

3 - To study demographic, clinical and genetic risk factors affecting persistence of childhood wheezing at puberty.

**Background and Literature Review**

**Specific Aim 1.**
Approximately one out of three children wheezes in the first year of life(12) and one out of two children in the first three years of life(6), usually in association with viral infections. Although in most cases wheezing in infancy represents a transient condition that has no apparent effect on later health, a substantial proportion of infants with wheezing, especially if recurrent, appear to be predisposed to asthma(6, 13). Therefore, understanding the risk factors
for early recurrent wheezing may have important public health implications.

In addition to known risk factors, such as exposure to other children (14), alterations in the maturation of the immune system very early in life have been hypothesized to influence host susceptibility to viral infection and wheezing. In this regard, several studies have investigated the relation of reduced IFNγ production to early wheezing, but they present important limitations in resolving the temporal relationship between exposure and outcome. These studies were, in fact, carried out either during the acute phase of a viral infection or once the wheezing episodes had already occurred, leaving unclear whether the altered production of IFNγ was a predisposing factor for wheezing or simply a consequence of the accompanying inflammatory process.

Reduced IFNγ production (15) and gene expression (16) were found in infants during the acute phase of severe respiratory syncytial virus (RSV) infection, the most common cause of wheezing in the first year of life. Similarly, IFNγ
has been consistently found to be reduced among children with both atopic(17) and non-atopic(18, 19) wheezing when measured apart from acute infectious episodes. However, to date no evidence has been reported that reduced IFNγ production precedes the inception of the disease.

We speculated that, if a reduced IFNγ production is indeed a significant risk factor for early wheezing, factors affecting the maturation of IFNγ responses early in life might play an important role in the development of this condition as well. IFNγ production shows a rapid maturation in the post-natal period(20). Impairment in this process may facilitate shifting the immune system toward Th2 differentiation of T cells, which may subsequently lead to atopic disease, and/or it may increase the host susceptibility to viral infections. Most likely, the maturation of IFNγ production is affected by both genetic and environmental factors. Exposure to endotoxin (lipopolysaccharide, derived from cell walls of gram-negative bacteria), has been suggested to be one of these environmental factors(20). Indeed, a reduced exposure to microbial burden and endotoxin and the consequent impairment
of the early maturation of the immune system have been hypothesized in the so-called "hygiene hypothesis" (21) to be major determinants of the dramatic increase of allergy and asthma prevalence in modern western societies. In this regard, several studies have shown that lifestyle factors that correlate with endotoxin exposure, such as farming (22) and having indoor dogs (23), may prevent asthma-like symptoms, when they begin very early in life.

A pattern recognition receptor with specificity for endotoxin (CD14) is present both anchored on the membrane (mCD14) of mature myeloid cells and in soluble form (sCD14) in plasma. In this scenario, the CD14-mediated response to endotoxin exposure might play an important role in enhancing the maturation of IFNγ responses and, consequently, in preventing the inception of recurrent wheezing. To date, no study has correlated plasma sCD14 concentrations to IFNγ production and/or onset of wheezing early in life.

Specific Aims 2 and 3.
Despite the commonly held view that many children with asthma outgrow the disease in adolescence, limited
epidemiological data are available for estimating rates and predictors of persistence/remission of asthma at puberty.

A possible explanation for this lack of epidemiological studies is that puberty can start within a relatively wide range of ages and, therefore, longitudinal cohorts of children need to be followed consistently at short intervals in order to identify the onset of puberty reliably.

Clearly, only longitudinal population-based cohort studies may provide unbiased answers to the questions addressed by Specific Aims 2 and 3. Retrospective studies, in fact, are sensitive to recall bias and they cannot resolve the temporal relationship between risk factors and asthma outcome in adolescence. Similarly, longitudinal studies on children selected from "at risk" cohorts or in different clinical settings can reflect the experience of the selected group of children rather than that of the general population and provide distorted rates of asthma persistence and distorted profiles of risk factors. In this regard, it is not surprising that the longitudinal outcome of children with asthma has been generally more favorable among children enrolled in population-based studies than among those selected in clinical settings, who are likely to suffer from more severe forms of the disease(24).
A few longitudinal population-based studies have investigated rates and predictors of persistence of childhood asthma in adolescence, but they present important limitations in the design and/or in the information available on potential risk factors. Nicolai and coworkers(25) found that almost 70% of children with asthma at age 10 reported no acute asthma symptoms during the last 12 months in the follow-up survey completed at age 14, if they had had signs of late puberty (change of voice in boys and menarche in girls). Children with asthma were identified from a large cohort of all fourth-grade schoolchildren in Munich and may be representative of the general population. However, these remission rates were based on a single follow-up survey and cannot reflect the experience of these children throughout adolescence. In a similar cohort from UK, among children with wheeze at age 6-8 years Withers et al(26) reported much lower (45%) rates of remission of wheezing at age 14-16 years. Interestingly, despite the very different remission rates, both these studies failed to find female gender a significant risk factor for the persistence of wheezing at puberty. It is well-known that the positive male/female ratio present among asthma cases in childhood decreases, or even reverses, after the onset of puberty(24). This can be related to higher rates of asthma incidence, persistence, or both, among girls than among boys in adolescence. Indeed, data from the UK cohort(26) support the
hypothesis that increased incident cases among girls account, at least partly, for the reversed gender ratio of asthma prevalence after puberty, since being female was found to be a significant risk factor for late-onset wheeze (wheeze present at age 14-16 but absent at age 6-8) rather than for persistent wheeze.

Several other population-based longitudinal studies with larger sample sizes and longer follow-up periods, have investigated rates and predictors of persistence of childhood asthma in adult life, but whether and to what extent these findings can be generalized to the peculiar period of adolescence remains to be determined. One common finding across these studies was that the frequency and severity of childhood wheezing is among the strongest predictors of persistent asthma in adult life. This finding illustrates once again the importance of using population-based cohorts, rather than "at risk" cohorts or outpatient populations, in order to study the outcome of childhood asthma in adult life.

In a large Australian cohort, only 26% of the subjects with asthma or wheezy breathing by the age of 7 reported current asthma as an adult (age 29-32)(27). Children who had had more than 10 asthma attacks by the age of 7 had a significantly increased risk of reporting current asthma as
an adult, as compared with children with milder forms of the disease. Despite the very long follow-up period, this study was composed of a single follow-up survey. A second cohort study from Australia, the Melbourne Asthma Study(28), had the longest follow-up period (up to 42 years). Using this cohort, Oswald et al(29) and Horak et al(30) found a clear trend for persistent asthma at ages 35 and 42 across increasing levels of severity of asthma-like symptoms at age 7. Among children with mild wheezy bronchitis at age 7, 35% reported wheezing in the last 3 years at age 35 and 34% at age 42, as contrasted with 90% and 89% of children with severe asthma, respectively. Intermediate rates of current asthma in adult life were found in the groups of children with intermediate degrees of severity of asthma at age 7. However, a major limitation of the Melbourne Asthma Study is that the cohort was enriched with severe asthma cases several years after the original enrollment, making it difficult to generalize these findings to the total population of asthmatic children.

The strong association between severity of childhood asthma and persistence of the disease in adult life led us not only to investigate the frequency of childhood wheezing as a potential risk factor for persistent asthma at puberty, but also to hypothesize a major role of the $\beta_2$ Adrenoceptor
(β₂AR) polymorphism at codon 16, which had been associated with severe asthma in previous studies (31-33).

In 1993, Reihsaus et al (32) described nine different single nucleotide polymorphisms in the β₂AR gene, including two polymorphisms in codon 16 and 27 resulting in the amino acid substitutions Arg16→Gly16 and Gln27→Glu27. Shortly later, in vitro studies (34, 35) showed that these polymorphisms had a functional impact on the receptor. Using CHW fibroblast permanently expressing each receptor, Green et al (35) found no differences in affinity for agonists or in coupling to adenylyl cyclase between the Gly16 and Arg16 and between the Gln27 and Glu27 receptors. However, significant differences were found in agonist regulation of the receptor. Particularly, the Gly16 receptor underwent an enhanced down-regulation, possibly by altering the trafficking of the internalized receptor into the degradation pathway. Interestingly, in these experiments the haplotypes Gly16-Gln27 and Gly16-Glu27 showed a similar down-regulation phenotype (35), suggesting that the Gly16 allele was the major determinant in the process. The enhanced down-regulation associated with the Gly16 receptor was subsequently confirmed in primary cell lines of airway smooth muscle (34), in which the β₂AR acts to relax and dilate the airway. This is a protective event against the
asthma-related bronchoobstruction and it is the major mechanism by which β agonists exert their therapeutic effects in asthma treatment.

Based on the rationale provided by these experimental studies, many clinical studies were then conducted to link the β₂AR-16 and β₂AR-27 polymorphisms to several asthma phenotypes. Not surprisingly, one of the major outcomes to be studied in relation to the presence of these polymorphisms was the response to β agonists. If the Gly16 polymorphism, in fact, enhances the down-regulation of the receptor, it should be associated with an increased tachyphylaxis (the clinical manifestation of desensitization) and a reduced response to β agonists. These were indeed the findings of two studies(36, 37) published in 1997. The former(36) was conducted on the same cohort used in the present study, the Tucson Children’s Respiratory Study. At 11 years of age, as compared with homozygotes for Gly16 children homozygous for Arg16 were found to be 5 times and heterozygotes 2 times more likely to respond to albuterol, independent of asthma status, baseline lung function, and ethnicity. Similarly, in the latter study Tan et al(37) found a significantly greater degree of bronchodilator (formoterol) desensitization among adult
asthmatics homozygous for Gly16 than among those homozygous for Arg16.

Although the $\beta_{2}$AR-16 and $\beta_{2}$AR-27 polymorphisms have not been associated with the development of asthma per se, among asthmatics the Gly16 polymorphism has been associated with other specific phenotypes, including nocturnal symptoms(38) and, most importantly, several indicators of disease severity(31-33). In the above-mentioned study by Reihsaus et al(32), a significantly higher proportion of subjects homozygous for Gly16 was found among patients with asthma requiring oral steroids than among those with a milder form of the disease. Similarly, Holloway et al(31) found that patients with at least one admission to hospital with asthma, but not those with mild asthma, were almost twice as likely to be homozygous for Gly16 than controls. In another study by Weir et al(33), the Gly16-Gln27 haplotype was found to be more prevalent in moderate asthmatics (taking > 400 $\mu$g of inhaled beclomethasone or equivalent per day and/or having an FEV1 < 75% of predicted) than in mild asthmatics.

Since severity of childhood asthma has been shown to be strongly linked to the persistence of the disease in adult life and the Gly16 receptor has been found over-represented among patients with severe asthma, we speculated that the
\( \beta_2 \text{AR Gly16 polymorphism} \) might be, in turn, a significant risk factor for persistent asthma at puberty. No previous study has investigated the effects of the \( \beta_2 \text{AR-16} \) and \( \beta_2 \text{AR-27} \) polymorphisms on persistent asthma.

**Explanation of the Dissertation Format**

This dissertation is based on three papers, which are included as Appendices A.1, A.2 and A.3. This subsection explains the relationship of these three papers and my original contribution to the research and production of the papers.

The specific aims of the research presented in this dissertation include the identification of rates and predictors for the onset of wheezing in the first year of life and for the persistence of wheezing after the onset of puberty. We believe there are both epidemiological and clinical reasons for choosing this timeframe, i.e. for studying incident wheezing in infancy and persistent wheezing from childhood through adolescence.

As already mentioned, several population-based studies have shown beyond any reasonable doubt that wheezing is a very
frequent respiratory complaint and one of the most common reasons for seeking medical advice in early life and that a substantial proportion of infants with wheezing will develop asthma later in childhood. This evidence suggests that children with wheezing are very likely to have experienced the onset of the disease in infancy and that cases of wheezing that appear to be incident later in life may indeed represent simply relapses of a pre-existing disease. In this regard, in a large longitudinal cohort Jenkins and coworkers found that two thirds of the subjects with parent reported childhood asthma reported at age 30 that they had not had asthma(27). This indicates that they did not know, had forgotten, or refused to disclose whether they had had asthma. These findings suggest also that results from cross-sectional studies are unreliable for estimating cumulative incidence rates of asthma and information on wheezing in infancy collected later in life is likely to be very sensitive to recall bias. Consequently, only by studying birth cohorts in early life, specifically in the first year(s) of life, can we be certain to identify the real incidence of cases of wheezing and this reasoning provided the rationale for the first paper included in Appendix A.1.

The presence of wheezing in early life has been shown to be a significant risk factor for asthma later in childhood. The natural history of childhood asthma is far from being fully
understood, but asthma can be considered a chronic disease characterized by variable remissions and relapses up to the onset of puberty, when many cases are believed to outgrow the disease. However, to date, it remains unknown what proportion of asthmatic children experience a remission of the disease after the onset of puberty and what factors affect the outcome. Only longitudinal population-based cohort studies, such as the CRS, may provide unbiased answers to these questions, which represent Specific Aims 2 and 3 and are addressed in the papers included as Appendices A.2 and A.3.

The research presented in this dissertation is based on the two large birth cohorts of the Tucson Infant Immune Study and the Tucson Children’s Respiratory Study. In the last three years, I have been extensively working on these two cohorts by designing studies, undertaking analyses and participating on a regular basis to meetings with co-investigators to insure that the overall study objectives were met and new goals developed. I have been working under the supervision of my Advisor (Dr. Sherrill) and that of the PIs of these studies (Dr.s Wright and Martinez) in writing several papers and presenting abstracts at meetings of some of the leading societies for respiratory and allergy research (such as the American Thoracic Society and the European Academy of Allergology and Clinical Immunology).
I was the primary author for each of the papers included in this dissertation. I designed the studies, undertook the analyses, and wrote and prepared the papers for publication. All the co-authors provided editing and critique. I conducted all the statistical analyses, including those using mixed models and population genetics techniques. Appendix A.1 was submitted to the American Journal of Respiratory and Critical Care Medicine (the leading journal for respiratory medicine) on April 9, 2003 and has received a favorable review. Appendix A.2 and Appendix A.3 will be soon submitted.
PRESENT STUDY

The methods, results, and conclusions of this study are presented in detail in the papers appended to this dissertation. The following is a summary of the methods and the most important findings of these papers.

Methods

Study Populations.
The study populations of the research presented in this dissertation are composed of two large birth cohorts from the Tucson Infant Immune Study (IIS) and the Tucson Children's Respiratory Study (CRS). The former is a prospective study of the development of immunologic markers of asthma risk in infancy and childhood. Started in 1996, the recruitment process for this study is still ongoing. Pregnant women planning to obtain care for their newborns through collaborating pediatricians are contacted at 32-35 weeks gestation and, as of May 22, 2003, 421 children have been enrolled in IIS. In contrast, the birth cohort of CRS was enrolled between May 1980 and October 1984. A total of 1,246 children were enrolled by contacting parents planning to use the pediatricians of a large maintenance organization.
in Tucson. The CRS birth cohort is now entering into its third decade.

Both in IIS and CRS, the parents of the enrolled children completed initial questionnaires on health status during pregnancy, smoking history and current and previous history of respiratory diseases.

In IIS, after the child’s birth, periodic questionnaires on child’s health status and environmental exposures were completed at 1, 2, 3, 6, 9, and 12 months. These questionnaires included specific questions about the child’s passive smoke exposure, breastfeeding status and exposure to other children (defined as either the presence of ≥ one older sibling or day care attendance with other children in the first 3 months of life). In this dissertation (Appendix A.1), we used information on infant’s respiratory health that was obtained in the questionnaire completed at 12 months of age (mean age ± SD: 1.06 ± 0.13 years). Parents were asked if the infant’s chest had ever sounded wheezy or whistling and, if yes, how often on a 5-point scale from (1) “Very rarely” to (5) “On most days”. A ranking of ‘1’ on this scale was defined as infrequent wheezing, while a ranking of ‘2’ or more was defined as recurrent wheezing. Age at the first episode of wheeze was also obtained.
In CRS, parents completed a series of questionnaires regarding their child’s health status and exposures at different ages: YR2 survey (mean age ± SD, 1.62 ± 0.3 years), YR3 (2.93 ± 0.5), YR6 (6.27 ± 0.9), YR8 (8.62 ± 0.7), YR11 (10.90 ± 0.7), YR13 (13.47 ± 0.6), and YR16 (16.55 ± 0.5). In each of these questionnaires, they were asked whether their child had experienced any wheezing episodes during the previous 12 months. Wheezing was defined as infrequent (≤ 3 wheezing episodes during the previous year) or frequent (> 3 wheezing episodes). In YR13 and YR16 questionnaires, parents were also asked whether and when their child’s puberty started. Specific examples of signs identifying onset of puberty were provided in order to reduce the risk of misclassification. The prepubertal period was defined as that between age 6 and the onset of puberty and the follow-up period as that between the onset of puberty and up to age 16.

Specific Techniques

In IIS, blood specimens were obtained from children at birth (from the umbilical cord) and 3 months of age. Soluble CD14 (sCD14) and IgE levels were measured in plasma using commercially available assays. IFNγ production from
peripheral or cord blood mononuclear cells (PBMCs) was measured after stimulation with 10 μg/ml Concanavalin A (Pharmacia, Piscataway, NJ), and 10 ng/ml phorbol myristate acetate.

In CRS, skin prick tests for 6 of the most common aeroallergens in the Tucson area (i.e., Alternaria alternata, Bermuda grass, olive, careless weed, mesquite, and mulberry) were performed at ages 6, 11, and 16. A wheal reaction at least 3 mm larger than the control wheal was recorded as positive. Spirometric tests were also performed at age 6, 11, and 16. At the time of each spirometric test, the child’s weight and height were measured by the study nurses. At age 11, methacholine challenge tests were also performed. Bronchial hyperresponsiveness was defined as a methacholine dose-response slope (DRS) below the 10th percentile of the methacholine-DRS distribution for a healthy reference subgroup, as described previously(39). 479 children were also genotyped for polymorphisms at both codon 16 and codon 27 of the β2AR using a combination of primer-induced restriction site assay and restriction fragment assay. Details of these molecular techniques have been provided in a previous publication(36) and they will not be repeated here.
Study Design and Statistical Analyses.

In the research presented in Appendix A.1, wheezing (infrequent and recurrent) by 1 year of age represented the main outcome of interest. IFNγ production and sCD14 levels at birth and 3 months of age represented the main independent variables. The odds of developing infrequent and recurrent wheezing were compared between children in the lowest quartiles of IFNγ and sCD14 levels (low levels) and children in the other 3 combined quartiles of IFNγ and sCD14, respectively, (medium-high levels) at each point time. Using information on the age of onset of the first wheezing episode, the survival curves (time to the first episode of wheezing) between children with low and medium-high levels of IFNγ and sCD14 at birth and 3 months were compared using log-rank tests.

In Appendix A.2, based on the presence of wheezing and/or asthma in the pre-pubertal period and its outcome in the follow-up period we identified 5 major groups: "No Wheezing" (n = 369); "Remitting Wheezing" (n = 89); "Unremitting Wheezing" (n = 37); "Remitting Asthma" (n = 64); and "Unremitting Asthma" (n = 94). Detailed information on the criteria used for identifying these groups can be found in Appendix A.2 (methods section and Table II). Statistical comparisons were performed across the 5 groups and,
specifically, between Remitting and Unremitting groups in order to identify risk factors for persistence of wheezing and asthma after the onset of puberty. In Appendix A.3, because of the limited number of subjects genotyped, the two "Unremitting" groups were combined ("Persistent Wheezing") and contrasted with the two "Remitting groups" combined ("Remitting Wheezing"). The $\beta_2$AR-16 and $\beta_2$AR-27 genotype and haplotype frequencies were compared between the "Persistent" and "Remitting Wheezing" groups. Stratification according to the Mantel-Haenszel method was used to adjust for ethnicity.

Mixed models (40) (i.e., Generalized Estimating Equations and Random Effects Models) were used to analyze intra-subject repeated observations, such as IgE levels (Appendix A.1), skin prick tests results and Body Mass Index measurements (Appendix A.2). Multinomial logistic regression (Appendix A.1) and logistic regression (Appendix A.2) models were used to control for confounding and test for interaction.

**Results**

**Specific Aim 1.**
As shown in Table II of Appendix A.1, the odds of developing recurrent wheezing by 1 year of age were up to 4.5 times
higher for children in the lowest quartile of IFNγ production at 3 months ($p = .0005$) and 3.2 times higher for children in the lowest quartile of sCD14 levels at birth ($p = .004$) as compared with children in the other 3 combined quartiles of IFNγ and sCD14, respectively. Findings were confirmed in multivariate models including both immunological markers to predict recurrent wheezing (Table III).

**Specific Aim 2.**
Findings from research presented in Appendix A.2 show that less than 30% of children with infrequent wheezing during childhood kept wheezing after the onset of puberty. However, this proportion increased up to 60% when we selected children meeting the criteria for presence of asthma in childhood (either frequent wheezing or a physician confirmed diagnosis plus any wheezing).

**Specific Aim 3.**
Table VI in Appendix A.2 provides a profile of independent risk factors for the persistence of wheezing and asthma after the onset of puberty. In addition to the frequency of wheezing episodes and to the presence of skin sensitization during childhood, being overweight at age 11 increased significantly the odds of experiencing persistent wheezing
(OR 3.6, 1.1 - 12.2) and asthma (4.6, 1.1 - 18.7) after the onset of puberty. Surprisingly, we also found a 30% to 40% protective effect against persistent wheezing/asthma associated with every 1-year increase of the age at onset of puberty. The β^AR-16 polymorphism was also found to be strongly associated with persistent wheezing (Appendix A.3). Boys, but not girls, homozygous for Gly at codon 16 were twice more likely to experience persistent wheezing at puberty than carriers of the other genotypes (RR 2.0, 1.3 - 3.1). This finding was confirmed after adjusting for ethnicity and after selecting only children with both parents Caucasian.

**Limitations**

There are some limitations to the studies presented in this dissertation. A variable proportion of children included in the study of Appendix A.1 had missing measurements of sCD14 levels and IFNγ production either at birth or 3 months. This could lead to selection bias if the missing observations were not at random based on the outcome. Indeed, we found homogeneous rates of wheezing incidence between children with and without missing measurements. This observation argues against the presence of a selection bias. It should
be also acknowledged that the temporal relationship between IFNγ / sCD14 measurements at 3 months and wheezing in the first year of life remains unsolved, as the onset of the first wheezing episode was recalled by the parents when the child was 1 year old and recall bias might be present.

In the research presented in Appendices A.2 and A.3, the age of onset of puberty was estimated based on parental report. We tried to minimize the risk of misclassification by providing parents with very specific examples of signs identifying the onset of puberty. However, a certain degree of misclassification is likely to have occurred and it might have affected our estimates of persistence and remission of asthma at puberty (Specific Aim 2 and Appendix A.2).

Moreover, since the questionnaires were administered every 2-3 years and in each questionnaire participants were asked about the presence of wheezing episodes in the last 12 months, information on the presence of wheezing during several “time windows” was disregarded in this approach. For example, a subject completing surveys at age 12 and 14 would have reported on the presence of wheezing episodes between age 13 and 14, but information on the presence of wheezing between age 12 and 13 would have been disregarded. This could have provoked an under-estimation of the rates of period prevalence for wheezing in adolescence. However, the
approach of limiting information on the presence of wheezing only to the last year is frequently used in epidemiological studies as it increases the reliability of self-reports and minimizes any recall bias. Finally, the small sample size of some of the "Remitting" and "Unremitting" groups did not provide sufficient statistical power to test the interaction between gender and other risk factors in predicting persistence of asthma after puberty (Appendix A.2) and to test the effect of the $\beta_2$AR-16 polymorphism on persistent wheezing within different ethnic groups (Appendix A.3). These can be important limitations, since it is biologically plausible that the patterns of risk factors may differ between the two genders (Appendix A.2) and since the distribution by $\beta_2$AR haplotypes appears to be heterogeneous across ethnic groups (Appendix A.3).

Conclusions

The research presented in this dissertation provides new insight for our understanding of the inception and persistence of the wheezing illness.
Specific Aim 1.
To our knowledge, our findings on the relationship between reduced sCD14 levels at birth, reduced IFNγ production at 3 months and increased risk of recurrent wheezing by 1 year of age represent the first evidence that altered immunological markers precede the inception of the disease. Results of this study are consistent with the so-called "hygiene hypothesis"(21), according to which the reduced exposure to microbial burden and endotoxin associated with westernized lifestyle would be responsible for an impaired maturation of the immune system in early life and, in turn, for the dramatic increase of asthma and allergy prevalence over the last decades. Our findings support this scenario in several ways. First, they confirm that the maturation of IFNγ production begins very early in life, as we found mitogen-induced IFNγ expression much higher at 3 months than at birth. They also suggest that sCD14 levels are able to affect this maturation as early as in the first 3 months of life, possibly by mediating the exposure to bacterial components. Finally, they show that the risk for recurrent wheezing in the first year of life is increased among children with reduced levels of sCD14 at birth and/or reduced production of IFNγ at 3 months.
The fact that sCD14 levels at birth and IFN\(\gamma\) production at 3 months were both independently associated with recurrent wheezing in the multivariate analysis suggests that: 1) sCD14 levels may affect the risk for wheezing through alternative pathways, independent of IFN\(\gamma\) maturation, (for instance, by mediating the innate immune response to wheezing-inducing pathogens\(^{41}\)); 2) the maturation of IFN\(\gamma\) responses is affected by other genetic and environmental factors, independent of sCD14 levels.

Several mechanisms might explain the association between an impaired IFN\(\gamma\) production and recurrent wheezing early in life. On the one hand, low IFN\(\gamma\) production may induce an increased susceptibility to developing severe reactions to viral infections, the major trigger for wheezing episodes in the first year of life. Recent findings\(^{42}\) suggest that, at least in mice, IFN\(\gamma\) might play a role in limiting viral replication and inflammatory responses in RSV infection. Alternatively, impaired IFN\(\gamma\) production may facilitate skewing toward Th2 differentiation of T cells that may subsequently lead to atopic wheezing.
Specific Aims 2 and 3.

Given the commonly held view that most children with asthma outgrow their disease after the onset of puberty, findings from the study presented in Appendix A.2 may appear surprising. In this study, only a minority (40%) of children with asthma in the pre-pubertal period experienced complete remission of the disease after the onset of puberty. Furthermore, we found that the earlier the onset of puberty the higher the risk for persistent asthma in adolescence. This association was present according to a dose-response rather than threshold relationship. In the multivariate analysis, the odds for persistent asthma decreased by 40% for every increase by 1 year in the age at onset of puberty. This finding can be interpreted in several ways. Early onset of puberty and asthma persistence may be affected by common risk factors, such as dietary or psychological influences or exposure to some endocrine disrupters (43). Interestingly, at the population level, in the last decades the decrease of mean age at puberty and the increase of asthma prevalence have shown to some extent similar geographical and temporal trends. Alternatively, the relation between early onset of puberty and persistent asthma may be a real biological phenomenon. It is well-known that the course of asthma can drastically change during pregnancy and female hormones have been shown to increase the production of Th2-like cytokines from peripheral blood mononuclear cells (44). Leptin
represents a potential candidate molecule to explain the
link between early onset of puberty and persistent asthma.
It has been, in fact, proposed as one of the signals
controlling sexual maturation(45) and, at the same time,
leptin receptors have been shown to be present in airway and
lung cells and possibly to be involved in the peripheral
regulation of respiratory function(46).

In addition to an early onset of puberty, we found the pre­
pubertal presence of frequent wheezing, bronchial
hyperresponsiveness, allergic skin sensitization, and
obesity to be significant risk factors for persistence of
asthma after the onset of puberty. In recent years, several
studies have shown that obesity is associated cross­
sectionally(47, 48) with the presence and longitudinally(49­
51) with the incidence of asthma symptoms, particularly
among females. In this study, we show that obesity is also a
risk factor for persistence of asthma, consistent with
previous reports that weight reduction in obese patients
with asthma improves lung function and symptoms(52, 53).
These findings expand our understanding of the relationship
between obesity and asthma, but they are limited in
providing evidence of any interaction by gender due to the
reduced sample size.
Importantly, although onset of puberty and pre-pubertal BMI were strongly and inversely correlated, they both remained significantly associated with persistent asthma in the multivariate analysis. Further research will be required to dissect the complex interactions between the developmental processes of body growth and sexual maturation and their impact on the natural history of childhood asthma.

Our findings present also the first evidence for a significant effect of genetic variation on the natural history of asthma. In one of the appended studies (Appendix A.3), subjects homozygous for Glycine at codon 16 of the $\beta_2$ Adrenoceptor were found to be at increased risk for persistence of wheezing and asthma after the onset of puberty than carriers of different genotypes. When analyses were stratified by gender, this association was present only among boys but not girls. These findings are consistent with previous in vitro studies. It has been long known that, although the polymorphism at codon 16 of the $\beta_2$ Adrenoceptor does not alter the affinity of the receptor for the agonists, the Gly16 receptor undergoes an enhanced down-regulation as compared with the Arg16 receptor (34, 35). Furthermore, it has been shown that adrenergic receptors are regulated by sex hormones, at least in animal models (54-56). Based on this evidence, it is plausible to hypothesize that
a differential functional regulation of adrenergic receptors by male and female sex hormones might be involved in explaining the interaction by gender in the association between Gly16 polymorphism and persistence of asthma at puberty. This would be consistent also with the greater sensitivity of the $\beta_2$-Adrenoceptor reported in women as compared with men\cite{57, 58}. Experimental studies will be required to test whether this hypothesis holds true.

We believe that the study populations used in this research represent one of its major strengths. The specific aims of this dissertation, in fact, can be addressed only by using large longitudinal population-based cohort studies, such as the IIS and CRS, for the following reasons:
1. The identification of risk factors requires the use of longitudinal data, since the temporal relationship between exposure (i.e., risk factor) and outcome may be ambiguous and sensitive to recall bias in retrospective studies.
2. The study cohorts need to be population-based, since studies on children selected from "at risk" cohorts or in clinical settings can provide inflated rates of asthma incidence and/or persistence and distorted profiles of risk factors. They, in fact, reflect the experience of the
selected group of children rather than that of the general population.

3. Only large cohorts provide the sufficient statistical power for estimating reliably rates of asthma incidence and persistence and for reducing the risk of Type II error (i.e., erroneously failing to reject the null hypothesis) in detecting the effects of potential risk factors.

In conclusion, our findings from two population-based longitudinal cohorts provide the first evidence that altered immunological markers precede the onset of wheezing early in life, challenge the commonly held view that most asthma cases remit during adolescence, and provide a profile of demographic, clinical and genetic risk factors for persistence of asthma after the onset of puberty.
REFERENCES


Peak flow variability, methacholine responsiveness and atopy as markers for detecting different wheezing phenotypes in childhood. Thorax 52(11):946-52.


APPENDIX A.1

Reduced IFNγ production and sCD14 levels in early life predict recurrent wheezing by 1 year of age

Running Title: IFNγ production and recurrent wheezing

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Submitted to the American Journal of Respiratory and Critical Care Medicine
ABSTRACT

It is unknown whether reduced production of IFNγ in early life, prior to any lower respiratory tract illness, is a risk factor for recurrent wheezing in infancy. Two hundred and thirty-eight non-selected infants were followed prospectively from birth to 1 year of age. At birth and at 3 months of age, IFNγ production from stimulated (ConA/PMA) peripheral blood mononuclear cells and soluble CD14 (sCD14) levels in plasma were measured. The odds of developing recurrent wheezing (assessed by questionnaire) in the first year of life were up to 4.5 times higher for children in the lowest quartile of IFNγ production at 3 months (p = .0005) and 3.2 times higher for children in the lowest quartile of sCD14 levels at birth (p = .004) as compared with children in the other 3 combined quartiles of IFNγ and sCD14, respectively. Findings were confirmed in the multivariate analysis. IFNγ production at 3 months and sCD14 levels at birth were correlated (r = .188, p = .031). Our findings from a longitudinal cohort suggest that impaired IFNγ production at 3 months and reduced plasma sCD14 levels at birth significantly increase the risk of developing recurrent wheezing in the first year of life.
INTRODUCTION

Approximately one out of three children wheezes in the first year of life, usually in association with viral infections(12). In most cases, wheezing in infancy represents a transient condition that has no apparent effect on later health(6). However, for a subgroup of infants who wheeze repeatedly, this condition is associated with the subsequent development of asthma(13) and with long-term, possibly permanent reductions in lung function. In fact, for children with the most severe forms of asthma, the onset of respiratory symptoms usually occurs very early in life(59). Therefore, understanding the risk factors for early recurrent wheezing may have important implications for both researchers and clinicians.

Although environmental risk factors for infection, such as exposure to other children, are primary risks for early wheezing, alterations in the maturation of the immune system
also appear to influence host susceptibility. When measured apart from acute infectious episodes, production of IFNγ has been consistently found to be reduced among children with both atopic(17) and non-atopic(18, 19) wheezing. Studies of infants during the acute phase of respiratory syncytial virus (RSV) infection, the most common cause of wheezing in the first year of life, also reported suppressed production of IFNγ from stimulated peripheral blood mononuclear cells (PBMC)(15). In addition, an inverse relation between IFNγ expression, as measured by the levels of messenger RNA, and the severity of the disease was found(16). However, given the cross-sectional nature of all these studies, it is unclear whether the altered production of IFNγ is a predisposing factor for wheezing, or is simply a consequence of the accompanying inflammatory process(60).

If a relation between low IFNγ production and early wheezing exists, the factors that affect the maturation of IFNγ responses early in life might play an important role in the development of this condition as well. CD14, a sentinel molecule of the constitutive innate immune system that acts as a pattern recognition receptor for endotoxin (lipopolysaccharide, derived from cell walls of gram-negative bacteria), is one such factor. IFNγ production has
been shown to correlate positively with the level of exposure to endotoxin among infants(61) and with plasma soluble CD14 levels themselves among 11-year old children(62). Taken together, this evidence suggests that early in life, the CD14-mediated response to LPS exposure may play an important role in enhancing the maturation of IFN\(\gamma\) production(20), and, consequently, preventing the inception of recurrent wheezing.

The aim of our study was to determine, in a birth cohort followed longitudinally, whether impaired IFN\(\gamma\) production and/or reduced plasma soluble CD14 levels in early life are significant risk factors for the development of recurrent wheezing in the first year of life.

**METHODS**

The Infant Immune Study (IIS) is a prospective study of the development of immunologic markers of asthma risk in childhood. Pregnant women obtaining care for their newborns through collaborating pediatricians were contacted at 32-35 weeks gestation. Questionnaires regarding health during pregnancy, parental respiratory health and demographic
characteristics were obtained prior to the infant's birth. At 1, 2, 3, 6, 9 and 12 months of age, parents completed questionnaires assessing the child's passive smoke exposure, breastfeeding status and exposure to other children (defined as either the presence of one older sibling or day care attendance with other children in the first 3 months of life). Ethnic background of both parents were recorded and a specific variable for ethnicity was created; both parents Caucasian, one parent Caucasian and one parent Hispanic, both parents Hispanic and Other ethnic background.

Consistent with the ethnic distribution in the Tucson area, in the group with other ethnic background we had a few children with parents who defined their ethnicity as African American (3 mothers and 10 fathers), Asian American (6 mothers and 4 fathers) or Native American (2 fathers).

Data about the infant's respiratory health was obtained from parents in a questionnaire completed at 12 months of age (mean age ± SD: 1.06 ± 0.13 years). Parents were asked if the infant's chest had ever sounded wheezy or whistling and, if yes, how often on a 5-point scale from (1) "Very rarely" to (5) "On most days". A ranking of '1' on this scale was defined as infrequent wheezing, while a ranking of '2' or more was defined as recurrent wheezing. Age at the first episode of wheeze was also obtained.
Blood specimens were obtained twice in the infant's first year of life: at birth (from the umbilical cord), and by venipuncture at 3 months of age (mean age: 2.9 ± 1.3 months). Soluble CD14 (sCD14) levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit supplied by R & D Systems Inc. (Minneapolis, MN). Total serum IgE were measured with the Pharmacia AutoCAP FEIA assay.

Lymphocyte stimulations were performed on peripheral or cord blood mononuclear cells (PBMCs) separated by standard density gradient centrifugation. Cells were stimulated with 10 ug/ml Concanavalin A (Pharmacia, Piscataway, NJ), and 10 ng/ml phorbol myristate acetate (Sigma Chemical Co., St. Louis, MO). Supernatant fluids from these cultures and unstimulated control tubes were harvested after 18-24 hr and stored at -70° C for later testing for cytokines. The supernatants were assayed for IFNγ production using a commercially available kit (Genzyme, Minneapolis MN).

The study was approved by the University of Arizona Institutional Review Board. Informed consent was obtained from the mothers at enrollment.
Statistical Analyses

χ² test and standard parametric procedures (t test, ANOVA, Pearson correlation coefficient) were used. Values of IgE, sCD14 and IFNγ production at birth and 3 months were log-transformed due to the right-skewness of their distributions. Normalization of the data following transformation was satisfactory. The means ± SD (and skewness statistics) of sCD14 and IFNγ levels before and after log-transformation were as follows: 0.81 ± 0.5 µg/ml (4.1) and -0.14 ± 0.2 µg/ml (0.5) for sCD14 at birth; 1.63 ± 0.7 µg/ml (3.3) and 0.18 ± 0.2 µg/ml (0.4) for sCD14 at 3 months; 947 ± 1967 pg/ml (4.9) and 2.5 ± 0.6 pg/ml (0.1) for IFNγ at birth; 1209 ± 2759 pg/ml (10.2) and 2.8 ± 0.6 pg/ml (-0.9) for IFNγ at 3 months. In order to adjust for the correlation between measures obtained at birth and 3 months in the same subjects random effects models (40) were used to compare IgE levels between children with and without wheezing.

Based on our working hypothesis, levels of IFNγ and sCD14 were categorized into the lowest quartile vs. the other three quartiles combined (medium-high levels). Before grouping the intermediate and the highest quartiles of IFNγ
and of sCD14 levels into the "medium-high levels" groups, we compared their proportions of wheezing and did not find any significant difference. Log-rank tests were used to compare the survival curves (time to the first episode of wheezing) between children with low and medium-high levels of IFNγ and sCD14 at birth and 3 months. The relation of low IFNγ production and low sCD14 levels to wheezing was tested for potential confounding in multinomial logistic regression models, which determined odds ratios (OR) for both infrequent and recurrent wheezing in the same multivariate model. In order to include individuals with missing data, a missing category was created for categorical variables, while missing IgE values were replaced with the mean value.

RESULTS

Two hundred and thirty-eight infants who had the 1-year questionnaire completed and sCD14 levels and/or IFNγ production measured were included in this study. Of these infants, 94 (39.5%) experienced wheezing episodes during the first year of life and 41 of these (17.2%) experienced recurrent wheezing.
We compared the characteristics of infants with no wheezing, infrequent wheezing and recurrent wheezing (Table I). The distribution by gender and ethnicity was similar in the 3 groups. Both young maternal age and low parental education level tended to increase the risk for wheezing, but these associations did not reach statistical significance. Risk factors for wheezing included lack of breastfeeding for at least 3 months and early exposure to other children. As expected, a physician diagnosis of LRI was much more common among children in the wheezing groups than among children with no wheezing (31.9% versus 7.6%, p < .0001). IgE levels at 3 months but not at birth were significantly higher among wheezers than non-wheezers (p < .05).

The proportion of children who did and did not develop infrequent and recurrent wheezing within the groups of low and medium-high IFNy production and sCD14 levels are shown in Table II. The odds of developing recurrent (but not infrequent) wheezing were up to 4.5 times higher for children in the lowest quartile of IFNy production at 3 months (p = .0005) and 3.2 times higher for children in the lowest quartile of sCD14 levels at birth (p = .004) as compared with children with medium-high levels of IFNy and sCD14, respectively. In contrast, low IFNy production at birth and low sCD14 levels at 3 months showed no relation
with wheezing in the first year. The increased risk for wheezing appeared to be specific for individuals in the lowest quartiles of IFNγ production at 3 months and sCD14 levels at birth, as the proportion of subjects with wheezing did not differ significantly among the intermediate quartiles and the highest quartile for IFNγ at 3 months nor for sCD14 at birth (data not shown).

In figure 1, the time to first wheezing episode associated with low versus medium-high levels of IFNγ at 3 months and sCD14 at birth are shown in separate survival curves. Wheezing occurred significantly earlier for subjects with low IFNγ production at 3 months as compared with subjects with medium-high levels (p = .0007). Survival curves of these two groups diverged. Similarly, children with low sCD14 levels at birth showed a survival curve significantly steeper than that of children with medium-high sCD14 levels (p = .019). In contrast, neither sCD14 levels at 3 months nor IFNγ production at birth had any predictive value for the age of onset of wheezing (data not shown).

We found a significant increase from birth to 3 months of both IFNγ production (geometric means: from 336 to 588 pg/ml) and sCD14 levels (from 0.73 to 1.52 μg/ml). Of note,
a correlation matrix including IFNγ and sCD14 at birth and 3 months showed that only IFNγ production at 3 months and sCD14 levels at birth were significantly correlated \( (r = .188, p = .031) \). IFNγ production at birth and 3 months also tended to correlate \( (r = .224, p = .073) \), but there was no correlation between sCD14 levels at birth and 3 months. Infants with low sCD14 levels at birth showed a reduced maturation of IFNγ production as compared with infants with medium-high sCD14 levels, although the association did not reach statistical significance. Geometric means for IFNγ production increased from 338 pg/ml at birth to 667 pg/ml at 3 months in the latter group, but only from 360 to 461 pg/ml for those in the lowest quartile of sCD14 levels at birth.

After adjusting for gender, ethnicity, early exposure to other children, maternal age, IgE levels and sCD14 levels at birth, the risk for recurrent wheezing among infants with low IFNγ production at 3 months remained significant \( (OR 4.7, p = .002) \) (Table III). Similarly, having low sCD14 levels at birth increased by almost 3 times the odds of developing recurrent (but not infrequent) wheezing. In contrast, early exposure to other children, young maternal age and high IgE levels at 3 months were significant risk factors for infrequent but not recurrent wheezing. All the
associations remained significant when the model was re-tested without substituting missing IgE levels. In this model, IgE levels showed even higher OR for infrequent wheezing (2.8, p = .013).

**DISCUSSION**

This study provides the first evidence that reduced IFNy production in early life is associated with an increased risk of developing recurrent wheezing by 1 year of age. This observation was specific for recurrent wheezing. Both low IFNy production at 3 months and low sCD14 levels at birth were associated with early onset of wheeze and they correlated significantly and directly with each other.

Several previous studies have shown that altered IFNy responses are associated with wheezing during childhood. Among 5 to 15 years old children, Leech and coworkers(19) found that both CD4 and CD8 positive T-cells from non-atopic wheezers produced significantly less IFNy than those from non-wheezing controls. Similarly, Koning et al(18) reported reduced IFNy responses by non-specifically stimulated T-
cells in non-atopic preschool children with recurrent wheezing as compared with healthy subjects.

Studies on cytokine production during the acute phase of lower respiratory illness have reported apparently conflicting findings with respect to IFNγ. van Schaik et al(63) found higher concentrations of IFNγ in the respiratory secretions of children with virus-induced wheezing than in those of children with uncomplicated upper respiratory infection. In contrast, Román et al(15) reported suppressed IFNγ production from stimulated PBMC of infants with acute RSV infection. Although such differences might relate to tissue-specific responses, Aberle et al(16) found higher IFNγ expression in PBMC of infants with RSV illness than in those of healthy controls, but only in the presence of a moderate rather than severe form of the disease. In fact, within the group of diseased children, IFNγ expression correlated inversely with the severity of the disease. Apart from the methodological differences that make these findings difficult to compare, it is of note that these studies were all cross-sectional in nature, leaving the temporal relationship between the alteration of IFNγ responses and the onset of wheezing unknown.
Maturation of IFNγ production in early life is likely to be affected by both genetic and environmental factors. We hypothesized sCD14 to be among these factors. Given its pivotal role in innate immunity as a 'sentinel molecule', serum sCD14 levels have been investigated in several disease states, including septic shock(64), infectious and autoimmune diseases(65, 66) as well as asthma. Interestingly, elevated serum sCD14 levels have been postulated to play a predisposing role in asthma exacerbations(67) but, at the same time, a protective role in asthma inception(20). This protective effect is believed to be exerted during a critical period early in life, when the development of the immune system requires environmental signals that trigger precoded maturational mechanisms. Such environmental signals include exposure to microbial endotoxin(68) which, in turn, involves processes mediated in part by the receptor CD14. Therefore, it is plausible to hypothesize that elevated sCD14 levels might be associated with the postnatal maturation of IFNγ production.

Our findings support this scenario in several ways. They confirm that the maturation of IFNγ production begins very early in life, as we found mitogen-induced IFNγ expression much higher at 3 months than at birth. They also suggest that sCD14 levels are able to affect this maturation as
early as in the first 3 months of life, since sCD14 levels at birth correlated positively and significantly with IFN-γ production at 3 months. Indeed, previous studies have shown that lifestyle factors which correlate with endotoxin exposure, such as farming(22) and having indoor dogs(23), may prevent asthma-like symptoms, when they begin very early in life. The levels of the exposure might be relevant too, as suggested by a recently reported non-linear U-shaped relationship between levels of house dust endotoxin exposure and risk of repeated wheezing among infants at risk for asthma and allergy(69).

Soluble CD14 levels at birth and IFN-γ production at 3 months correlated directly and significantly and, apart from the effect of other confounders, adjusting low sCD14 levels and IFN-γ production for each other in multivariate analyses reduced the magnitude of their association with recurrent wheezing by approximately 10% (data not shown), possibly because of the existence of a common pathway. However, each still showed consistently high and significant odds ratios for recurrent wheezing in the multivariate analysis, suggesting the existence of additional independent pathways. This is not surprising since it is very likely that other genetic and environmental factors may influence the maturation of IFN-γ responses, independent of sCD14 levels.
Similarly, sCD14 levels themselves may affect the risk for wheezing through mechanisms which are independent of IFNγ maturation, e.g. by mediating the innate immune response to wheezing-inducing pathogens, as recently reported for RSV(41).

There are several potential mechanisms through which impaired IFNγ production might be associated with recurrent wheezing early in life. On the one hand, low IFNγ production may reflect an increased susceptibility to developing severe reactions to viral infections, the major trigger for wheezing episodes in the first year of life. Reduced IFNγ production may reflect either decreased maturation of CD8+ T cells or decreased numbers of NK cells or Th1 cells. These deficits may, in turn, affect macrophage responses to viruses, leading to more invasive infections and decreased viral clearance from the lung. Recent findings(42) suggest that, at least in mice, IFNγ might play a role in limiting viral replication and inflammatory responses in RSV infection. Alternatively, impaired IFNγ production may facilitate shifting the immune system toward Th2 differentiation of T cells which may subsequently lead to atopic wheezing. Reduced production of IFNγ at birth has already been shown to be a hallmark of newborns at high risk
of allergy(70) and a significant risk factor for the subsequent development of atopy(71). Consistent with this interpretation, we have already shown in a different birth cohort that IFNγ production at 9 months was inversely related to skin test reactivity at the age of 6 years(72).

Whether the mechanisms leading to low IFNγ production very early in life are different among subjects who will develop atopy- versus non atopy-related wheezing is unknown. It is possible that an alteration of IFNγ production early in life may induce an increased susceptibility to viral wheezing through the same mechanisms, whether or not the infant is predisposed to becoming atopic. Additional genetic and developmental factors would then determine which subjects will go on to develop Th2 mediated responses, persistent wheezing and, in turn, atopic asthma. While there is a lack of experimental and epidemiological data that directly test this hypothesis, indirect evidence is provided by the fact that secular trends show a similar increase for both atopic and non-atopic wheezing, suggesting the existence of common underlying mechanisms(73).

While the focus of this study was recurrent wheezing, our findings provide additional evidence for specific risk factors for infrequent wheezing in the first year of life as
well. Early exposure to other children increased the odds for infrequent wheezing by more than 5 times. An increased risk for wheezing associated with day care attendance among preschool children, probably related to an increased exposure to infections, has been shown by previous studies (14, 74). In the multivariate analysis, we found young maternal age to be another significant risk factor for infrequent wheezing. This association has been already described (75, 76) and is likely to be related to both biological and socioeconomic factors. Finally, IgE levels at 3 months of age were also directly associated with the odds for infrequent but not recurrent wheezing. Interestingly, IgE levels were significantly higher only among subjects with the onset of wheezing after 6 months of age (data not shown), consistent with our previous report of an inverse relationship between cord IgE levels and LRI events occurring before but not after 6 months of age (77). High IgE levels might be a marker for a process that delays the development of recurrent wheezing for infants, who may still be at increased risk for the development of repeated wheezing and asthma later in life.

There are some limitations to our study. Among the 238 subjects included in the study, a variable proportion had missing measurements of sCD14 levels and IFNγ production
either at birth or at 3 months, leading to some possible selection bias. Although we cannot exclude such a bias, it appears unlikely since the proportion of children with wheezing was very homogeneous (approximately 40%) across all the groups with missing and non-missing values. Furthermore, the low positive response rates (49.7%) of the Tucson Infant Immune Study may jeopardize the generalizability of our findings. Such low response rates are not surprising given the level of commitment required of participants (questionnaires completed almost on a monthly basis, blood samples drawn frequently, etc). Since we did not obtain environmental samples, we could not investigate whether the level of exposure to microbial burden might modulate the relation between sCD14, IFNγ responses and wheezing.

In conclusion, our findings from a longitudinal cohort suggest that impaired IFNγ production at 3 months and reduced plasma levels of sCD14 at birth increase significantly the risk of developing recurrent wheezing in the first year of life.
Acknowledgements.

The authors thank the study nurses, Jody Mallie and Heidi Erickson; Gina Tebow and Mary Craven for laboratory studies; and Bruce Saul for assistance with data analysis.

REFERENCES

5. Hoekstra, M. O., Y. Hoekstra, D. De Reus, B. Rutgers, J. Gerritsen, and H. F. Kauffman. 1997. Interleukin-4,


Table I. Comparison between infants with no wheezing, infrequent wheezing and recurrent wheezing.

<table>
<thead>
<tr>
<th></th>
<th>NO Wheezing (n = 144)</th>
<th>ANY wheezing</th>
<th>INFREQUENT (n = 53)</th>
<th>RECURRENT (n = 41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male): %</td>
<td>48.6%</td>
<td>45.3%</td>
<td>53.7%</td>
<td></td>
</tr>
<tr>
<td>Parental Ethnicity: %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both parents non-Hispanic Caucasian</td>
<td>60.4%</td>
<td>60.4%</td>
<td>48.8%</td>
<td></td>
</tr>
<tr>
<td>Caucasian / Hispanic</td>
<td>13.2%</td>
<td>17.0%</td>
<td>12.2%</td>
<td></td>
</tr>
<tr>
<td>Hispanic / Hispanic</td>
<td>13.2%</td>
<td>9.4%</td>
<td>17.1%</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>13.2%</td>
<td>13.2%</td>
<td>22.0%</td>
<td></td>
</tr>
<tr>
<td>Maternal age in years: mean ± SEM</td>
<td>29.5 ± 0.5</td>
<td>27.4 ± 0.7</td>
<td>28.7 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Paternal age in years: mean ± SEM</td>
<td>31.6 ± 0.6</td>
<td>29.9 ± 0.8</td>
<td>31.0 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Maternal education in years: mean ± SEM</td>
<td>15.2 ± 0.2</td>
<td>14.9 ± 0.4</td>
<td>14.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Paternal education in years: mean ± SEM</td>
<td>15.0 ± 0.3</td>
<td>14.8 ± 0.3</td>
<td>14.5 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Ever maternal asthma: %</td>
<td>20.9%</td>
<td>17.6%</td>
<td>25.0%</td>
<td></td>
</tr>
<tr>
<td>Ever paternal asthma: %</td>
<td>15.9%</td>
<td>12.8%</td>
<td>25.7%</td>
<td></td>
</tr>
<tr>
<td>Breastfed at least 3 months: %*</td>
<td>66.2%</td>
<td>51.9%</td>
<td>53.8%</td>
<td></td>
</tr>
<tr>
<td>At least one parent smoker: %</td>
<td>22.9%</td>
<td>28.3%</td>
<td>31.7%</td>
<td></td>
</tr>
<tr>
<td>Early exposure to other children: % § +</td>
<td>61.7%</td>
<td>84.3%</td>
<td>75.7%</td>
<td></td>
</tr>
<tr>
<td>Lower Respiratory Illness 1st year: %¶†</td>
<td>7.6%</td>
<td>28.3%</td>
<td>36.6%</td>
<td></td>
</tr>
<tr>
<td>IgE levels in IU/ml: geom. mean (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At birth (n = 206)</td>
<td>0.21 (0.17, 0.26)</td>
<td>0.21 (0.16, 0.28)</td>
<td>0.32 (0.20, 0.53)</td>
<td></td>
</tr>
<tr>
<td>At 3 months (n = 190) *</td>
<td>1.04 (0.84, 1.28)</td>
<td>1.54 (1.13, 2.10)</td>
<td>1.54 (0.91, 2.60)</td>
<td></td>
</tr>
</tbody>
</table>

Statistical comparisons were performed both across the 3 groups and between children with no wheezing and any wheezing.
* No wheezing versus any wheezing: \( p < .05 \)
§ No wheezing versus any wheezing: \( p < .005 \)
† Chi square test across the 3 groups: \( p < .01 \)
¶ No wheezing versus any wheezing: \( p < .0001 \)
† Chi square test across the 3 groups: \( p < .0001 \)
Table II. Proportion of children with no wheezing, infrequent wheezing and recurrent wheezing in the groups with low and medium-high IFNγ production and sCD14 levels.

<table>
<thead>
<tr>
<th></th>
<th>NO Wheezing</th>
<th>ANY wheezing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>INFREQUENT</td>
<td>RECURRENT</td>
</tr>
<tr>
<td></td>
<td>% (n)</td>
<td>% (n)</td>
</tr>
<tr>
<td>IFNγ production at birth (n = 103):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>72.0% (18)</td>
<td>12.0% (3)</td>
</tr>
<tr>
<td>Medium-High</td>
<td>57.7% (45)</td>
<td>23.1% (18)</td>
</tr>
<tr>
<td>* OR (95% CI)</td>
<td>1.00</td>
<td>0.42 (0.11 - 1.59)</td>
</tr>
<tr>
<td>IFNγ production at 3 months (n = 163): *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>40.0% (16)</td>
<td>25.0% (10)</td>
</tr>
<tr>
<td>Medium-High</td>
<td>67.5% (83)</td>
<td>19.5% (24)</td>
</tr>
<tr>
<td>* OR (95% CI)</td>
<td>1.00</td>
<td>2.16 (0.87 - 5.38)</td>
</tr>
<tr>
<td>sCD14 levels at birth (n = 206): ^</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>47.1% (24)</td>
<td>23.5% (12)</td>
</tr>
<tr>
<td>Medium-High</td>
<td>65.2% (101)</td>
<td>21.9% (34)</td>
</tr>
<tr>
<td>^ OR (95% CI)</td>
<td>1.00</td>
<td>1.48 (0.67 - 3.29)</td>
</tr>
<tr>
<td>sCD14 levels at 3 months (n = 186):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>69.6% (32)</td>
<td>13.0% (6)</td>
</tr>
<tr>
<td>Medium-High</td>
<td>58.6% (82)</td>
<td>22.1% (31)</td>
</tr>
<tr>
<td>* OR (95% CI)</td>
<td>1.00</td>
<td>0.50 (0.19 - 1.30)</td>
</tr>
</tbody>
</table>

# Chi square test across the 3 wheezing categories: p < .005
§ p = .0005
^ Chi square test across the 3 wheezing categories: p < .05
* p = .004
Table III. Adjusted OR (with 95% CI and p values) associated with infrequent and recurrent wheezing found in multinomial logistic regression (n = 238).

<table>
<thead>
<tr>
<th></th>
<th>INFREQUENT WHEEZING</th>
<th>RECURRENT WHEEZING</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Low IFNγ at 3 months</td>
<td>1.44</td>
<td>0.52 - 4.01</td>
</tr>
<tr>
<td>Low sCD14 at birth</td>
<td>0.94</td>
<td>0.40 - 2.24</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>0.70</td>
<td>0.34 - 1.42</td>
</tr>
<tr>
<td>Parental ethnicity: ‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian/Hispanic</td>
<td>1.21</td>
<td>0.45 - 3.23</td>
</tr>
<tr>
<td>Hispanic/Hispanic</td>
<td>0.29</td>
<td>0.09 - 0.98</td>
</tr>
<tr>
<td>Other</td>
<td>0.77</td>
<td>0.27 - 2.22</td>
</tr>
<tr>
<td>Early exposure to other children</td>
<td>5.32</td>
<td>2.12 - 13.32</td>
</tr>
<tr>
<td>Maternal age in years</td>
<td>0.91</td>
<td>0.85 - 0.97</td>
</tr>
<tr>
<td>IgE levels at 3 months ‡</td>
<td>2.46</td>
<td>1.13 - 5.35</td>
</tr>
</tbody>
</table>

‡ Caucasian/Caucasian is the reference group; ¶ Log-transformed IU/ml
FIGURE LEGENDS

Figure 1. Survival curves for wheezing by IFNγ production at 3 months and sCD14 levels at birth. P values represent log-rank tests.

* Numbers are slightly lower than those shown in table II because of cases who failed to report age at first wheezing episode
Figure 1.

IFNγ at 3 months (n = 161) *

\[ p = .0007 \]

sCD14 at birth (n = 205) *

\[ p = .019 \]
APPENDIX A.2

Factors Affecting Remission and Persistence of Childhood Asthma at Puberty: a Population-based Longitudinal Study

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Submitted to the American Journal of Respiratory and Critical Care Medicine
The aim of this study was to determine rates and predictors of remission of childhood asthma after the onset of puberty. We used data collected at ages 6, 8, 11, 13 and 16 from the Tucson Children's Respiratory Study, a population-based birth cohort. Onset of puberty was defined as the time of appearance of the first signs as reported by parents. Information on wheezing both during childhood and after puberty (mean ± SD follow-up from onset of puberty: 3.9 ± 1 yr) was available for 732 children. 158 children had asthma (either frequent wheezing or a physician-confirmed diagnosis plus any wheezing) during childhood. Of these, 60% reported presence of wheezing after onset of puberty (persistent asthma). The proportion of persistent wheezing after puberty was much lower (29%) among the 126 children with only infrequent wheezing during childhood. In addition to frequent wheezing, the continuous recurrence of wheezing in all the surveys during childhood, overweight, an early onset of puberty, bronchial hyperresponsiveness, skin test sensitization to Alternaria, recurrent cough and active sinusitis were other significant predictors of persistent asthma after puberty. Our findings from a population-based longitudinal cohort challenge the idea that asthma remits
during adolescence in most cases and provide a profile of risk factors for persistence of asthma after puberty.

INTRODUCTION

In western societies, asthma prevalence has been dramatically increasing in the last decades and, at the present time, asthma represents the most common chronic disease in childhood. Children with asthma are at increased risk for experiencing asthma-related respiratory symptoms in adult life(1-3) and, possibly, for developing long-term non-reversible airway obstruction(4). In this regard, it has been long known that adults with COPD are more likely to recall respiratory troubles during childhood than those without such illnesses(5).

Prospective studies on pediatric cohorts as they enter adulthood can shed important light on the natural history of childhood asthma and on the risk factors affecting the persistence of the disease into adult life. This is a matter of particular interest, as it is plausible that persistence of asthma symptoms may be more frequently associated with long-term sequelae on lung function than remitting or intermittent asthma.
Adolescence represents a transition phase from childhood to adulthood and is characterized by rapid hormonal, physical and behavioral changes, all of which may affect the natural course of asthma. Indeed, it has been long recognized that many children with asthma may outgrow the disease after the onset of puberty(6). However, this observation has been based on “anecdotal” clinical experience, although previous epidemiological studies have tried to substantiate it with longitudinal data. To date, it remains unknown what proportion of asthmatic children experience a remission of the disease after the onset of puberty and what factors affect the outcome(7). Only longitudinal population-based cohort studies may provide unbiased answers to these questions. Retrospective studies, in fact, are very sensitive to recall bias and cannot resolve the temporal relationship between risk factors and asthma outcome in adolescence. Similarly, longitudinal studies on children selected from “at risk” cohorts or in different clinical settings can provide inflated rates of asthma persistence and distorted profiles of risk factors, reflecting the experience of the selected group of children rather than that of the general population.

To date, a few longitudinal population-based cohort studies have investigated the factors associated with the outcome of
childhood asthma in adult life(1, 2, 8, 9) and adolescence(10-13), but each of them had inherent limitations for identifying specific rates and predictors of asthma remission after the onset of puberty. To study such an outcome, longitudinal cohorts of children need to be followed at least at 2-3 yearly intervals in order to identify reliably the onset of puberty, which can occur within a relatively wide range of ages. The longitudinal birth cohort of the Tucson Children’s Respiratory Study has been followed consistently with frequent follow-up surveys and it is now approaching its third decade. We used data from this cohort up to the Year 16 Survey to study the factors influencing persistence and remission of childhood asthma after the onset of puberty.

METHODS

Study Population and Design
The children included in this study are a subset from the birth cohort enrolled in the Tucson Children’s Respiratory Study between May 1980 and October 1984. Detailed information on the design and enrollment process of this longitudinal study has been published previously(14). The parents of the 1,246 enrolled children completed an initial
questionnaire on parental history of respiratory diseases and smoking habits and, subsequently, a series of questionnaires regarding their child's health status at different ages: YR2 survey (mean age ± SD, 1.62 ± 0.3 years), YR3 (2.93 ± 0.5), YR6 (6.27 ± 0.9), YR8 (8.62 ± 0.7), YR11 (10.90 ± 0.7), YR13 (13.47 ± 0.6), YR16 (16.55 ± 0.5).

Questions on whether and when puberty started were included in the YR13 and YR16 questionnaires. Parents completing these questionnaires were provided with specific examples of signs identifying the onset of puberty: "breast development or menstruation in girls; pubic and/or underarm hair; voice changes in boys". The prepubertal period was defined as that between YR6 survey and the reported onset of puberty.

Outcomes during adolescence were studied in the follow-up period included between the onset of puberty and up to YR16 survey.

Questions on the presence and frequency of wheezing episodes during the previous year were asked in each of the surveys. Children reporting wheezing neither in the prepubertal period nor after the onset of puberty were included in the "No Wheezing" group. Children reporting episodes of wheezing in the prepubertal period were divided in two groups:
"Infrequent Wheezing" and "Asthma". The "Infrequent Wheezing" group included subjects experiencing infrequent wheezing (≤ 3 wheezing episodes during the previous year) in at least one survey and never reporting a physician-confirmed diagnosis of asthma. The "Asthma" group included children reporting the presence of frequent wheezing (> 3 wheezing episodes during the previous year) in at least one survey or a physician-confirmed diagnosis of asthma. Based on the outcome in adolescence, the "Infrequent Wheezing" and "Asthma" groups were then further classified as "Unremitting" if any wheezing episodes were reported in at least one survey after the onset of puberty and "Remitting" if they were not.

Based on the proportion of surveys in the prepubertal period in which the subjects reported wheezing episodes during the previous year, three mutually exclusive categories of "episodic" (< 50% of surveys), "recurrent" (≥ 50% and < 100%) and "continuous" (100%) wheezing were coded.

Table 1 gives information on the definition and time of assessment of some of the risk factors included in our analyses and provides references (15-17) for a more detailed description of them.
**Statistical Analyses**

Statistical comparisons were performed across the 5 groups of "No Wheezing", "Remitting Wheezing", "Unremitting Wheezing", "Remitting Asthma", and "Unremitting Asthma" (one-way analysis of variance for continuous variables and $\chi^2$ tests for proportions assessed at a single survey).

Since, this sample of the general population was composed of children with different ethnic background, distribution by ethnicity was compared across the groups. Since there were not significant differences, subsequent analyses were not adjusted for ethnicity. Furthermore, statistical comparisons were specifically performed between Remitting and Unremitting Wheezing and between Remitting and Unremitting Asthma groups in order to identify risk factors associated with the persistence of asthma symptoms after the onset of puberty. Student T test was used for continuous variables and $\chi^2$ test for proportions. The effects of categorical variables on persistence of asthma symptoms after the onset of puberty were also expressed as Odds Ratios (OR) and tested for potential confounding in logistic regression models. In those analyses, risk factors measured at age 11 were considered only if the child had not yet started puberty by that age.

Proportions of positive skin prick tests and means of Body Mass Index (BMI) were compared across the 5 groups at YR6,
YR11 and YR16 Surveys. In order to take into account the serial correlation between observations on the same subject, these statistical comparisons were performed using linear mixed models (18) (Generalized Estimating Equations for skin tests and Random Effects Models for BMI). As compared with traditional parametric tests and logistic regression models, mixed models offer the advantage of not requiring that observations are independent, modeling the serial correlation between observations and being less sensitive to missing observations (under the assumption that these observations are missing at random).

RESULTS

In our study, information on wheezing in the prepubertal period and after the onset of puberty was available for 732 subjects (58.8% of the original 1,246 enrolled children). A mean follow-up period of 3.9 ± 1 years after the onset of puberty was available for this study population. As shown in table 2, 369 (50.4%) children never experienced wheezing attacks (neither in the prepubertal period nor after onset of puberty, "No Wheezing" group) and 79 (10.8%) reported wheezing attacks only after the onset of puberty ("Incident
Wheezing). Among the remaining 284 subjects who experienced wheezing episodes during the prepubertal period, 126 (17.2%) had only infrequent wheezing and 158 (21.6%) met the criteria for asthma definition. The majority of the children with infrequent wheezing experienced remission of wheezing episodes after the onset of puberty and were classified in the "Remitting Wheezing" group (89/126, 70.6%) as contrasted with the "Unremitting Wheezing" group (37/126, 29.4%). In contrast, almost 60% of the children with asthma in the prepubertal period had wheezing episodes after the onset of puberty and were included in the "Unremitting Asthma" group (94/158, 59.5%) as contrasted with the "Remitting Asthma" group (64/158, 40.5%). Table 2 summarizes inclusion criteria and sample sizes for the 6 groups. Because of the specific aims of this study, subjects in the incident wheezing group were excluded from the current analyses.

Demographics, smoking and BMI

As shown in Table 3, the distribution by gender significantly differed across the 5 groups. The proportion of boys was higher in the wheezing and asthma groups than in the "No Wheezing" group. The proportion of girls was higher in the unremitting groups as compared with the correspondent remitting groups, but the association did not reach statistical significance. An early onset of puberty was
associated with the persistence of asthma symptoms in adolescence, as children in the unremitting wheezing and asthma groups had on average the onset of puberty significantly earlier than children in the correspondent remitting groups.

No significant difference in the proportions of exposure to environmental tobacco smoking (i.e., maternal smoking during pregnancy and/or the presence of at least one smoker parent between age 6 and 11) was found among the 5 groups or between the unremitting and remitting groups. The percentages of active smoking during adolescence were also evenly distributed across the groups.

The mean BMI significantly differed across the 5 groups at YR11 and YR16 (figure 1). When comparisons between correspondent pairs were performed, the mean BMI was found significantly higher in the "Unremitting Wheezing" group than in the "Remitting Wheezing" group at each point in time and in the "Unremitting Asthma" group than in the "Remitting Asthma" group at YR11 and YR16. The average slopes of increase of BMI between YR6 and YR11 also differed significantly across the 5 groups (ANOVA, p = .006). In the Post Hoc analysis, the mean slopes of BMI increase for the groups of "Unremitting Wheezing" and "Unremitting Asthma"
were higher than those of the "Remitting Wheezing", "Remitting Asthma" and "No Wheezing" groups (0.600 ± 0.61, 0.435 ± 0.37, 0.547 ± 0.50 and Kg/m² per year, respectively).

**Respiratory Symptoms, Diagnoses and BHR**

As shown in table 4, the recurrence of wheezing episodes during the prepubertal period had a strong predictive value for persistence of asthma symptoms after the onset of puberty following a dose-response relationship. When compared with the reference category of episodic wheezing, children in the "Unremitting Wheezing" group were, in fact, 24% more likely to have experienced recurrent wheezing but almost 5 times more likely to have experienced continuous wheezing than children in the "Remitting Wheezing" group (OR 1.24, 0.50 - 3.12, and 4.70, 1.68 - 13.14, respectively). This trend was even stronger among children with asthma. Comparing the "Unremitting Asthma" to the "Remitting Asthma" group, the ORs associated with recurrent and continuous wheezing in the prepubertal period were 2.47 (CI 0.98 - 6.26) and 6.97 (2.79 - 17.42), respectively.

The proportions of subjects with other respiratory symptoms and diagnoses listed in table 4 differed significantly across the 5 groups. However, only recurrent cough and
physician-confirmed sinusitis had a significant predictive value for persistence of asthma symptoms after the onset of puberty. At the last completed survey before the onset of puberty, in fact, 50% of the children in the "Unremitting Asthma" group versus less than 30% in the "Remitting Asthma" group reported the presence of active recurrent cough (OR 2.37, 1.21 - 4.64). The correspondent proportions for active sinusitis in the two groups were 40.4% and 23.4%, respectively (OR 2.20, 1.04 - 4.65).

Methacholine challenge test at YR11 was considered only for subjects who completed it before the onset of puberty (n = 379). About 20% of the children in the "No Wheezing", "Remitting Wheezing" and "Unremitting Wheezing" groups showed bronchial hyperresponsiveness, as compared with more than 40% and 70% in the "Remitting Asthma" and "Unremitting Asthma" groups (p < .0001). When we compared the last two groups, bronchial hyperresponsiveness was significantly associated with persistence of asthma after the onset of puberty (OR 4.09, 1.16 - 14.43).

**Atopy**

As shown in table 5, the proportion of children with skin tests positive to any of the tested allergens was significantly different across the five groups at YR6, YR11
and YR16, the "No Wheezing" and "Remitting Wheezing" groups showing the lowest percentages of sensitization. Similarly, in each survey we found the proportion of atopic children to be higher in the "Unremitting Wheezing" group than in the "Remitting Wheezing" group and in the "Unremitting Asthma" group than in the "Remitting Asthma" group.

When we analyzed the effect of the sensitization to specific allergens on the persistence of asthma symptoms after the onset of puberty, we found Alternaria to be the only allergen with significantly higher percentages of sensitization among unremitting than remitting cases at YR6 and YR11. Up to 39% of the children in the "Unremitting Wheezing" group were sensitized to Alternaria at YR11 as compared with only 17% in the "Remitting Wheezing" group (p < .001). Similarly, 50% and 46% of the children in the "Unremitting Asthma" group were positive to Alternaria at YR6 and YR11 respectively, as compared with 23% and 30% in the "Remitting Asthma" group (p < .005 and p < .05, respectively). For all the other allergens, the percentage of skin sensitized subjects did not differ significantly between the remitting and unremitting groups at YR6 and YR11. In contrast, at YR16 sensitizations to Alternaria, Bermuda and other pollens were all significantly more common among unremitting than remitting asthma cases.
Multivariate Analysis

In Table 6, the results of the multivariate analysis for predicting persistence of wheezing after onset of puberty are shown separately for subjects who experienced only infrequent wheezing (model 1) and for subjects who had asthma (model 2) in the prepubertal period. Bronchial hyperresponsiveness was the only independent variable that was significant in the bivariate analysis that was not tested in the multivariate analysis because of the limited number of subjects who completed the test. Despite the non-significant ORs, gender was included in the models in order to control for its potential confounding effect. An early onset of puberty, the presence of continuous wheezing during the prepubertal period, being overweight at YR11, and skin tests positive for Alternaria at YR11 were independently associated with persistence of asthma-like symptoms in both the models. The results of the Hosmer and Lemeshow tests showed a satisfactory goodness-of-fit for both the models.

DISCUSSION

In this study, we estimated remission rates of asthma symptoms after the onset of puberty and to define patterns
of risk factors for persistence of asthma in adolescence. We found that about 30% of children with infrequent wheezing during childhood and up to 60% of children with asthma during childhood keep experiencing wheezing episodes after the onset of puberty. These proportions represent the experience of asthmatic children in the first 4 years after the onset of puberty. Obviously, with a longer follow-up period the rates of asthma persistence would be higher. The findings of this study may be expected to reflect the experience of the general population, although it should be acknowledged that we had missing information on 41.3% of the children originally enrolled in this cohort.

Despite the commonly held view that asthma may frequently remit in adolescence, very few studies have reported estimates of remission rates of asthma symptoms during adolescence from samples of the general population. Nicolai and coworkers(11) found that almost 70% of children with asthma at age 10 reported no acute asthma symptoms during the last 12 months in the follow-up survey completed at age 14, if they had had signs of late puberty (change of voice in boys and menarche in girls). In this study, children with asthma were identified from a large cohort of all fourth-grade schoolchildren in Munich and may be representative of the general population. However, caution should be paid in interpreting these remission rates of asthma during
adolescence since they are based on a single follow-up survey and reflect the experience of a single year (last 12 months before the survey completed in adolescence). Indeed, in a similar cohort from UK(12) only 45% of children with wheeze at age 6-8 yr reported no current wheeze at age 14-16 yr. These two cohort studies also shed some light on the effect of gender on persistence of childhood asthma in adolescence. It is well-known that the male/female ratio among asthma cases is high during childhood but it tends to decrease or even reverse after puberty(19). This can be related to higher rates of incidence or higher rates of persistence of asthma, or both, among females than males after puberty. Consistent with these previous reports(11, 12), we did not find gender to be a significant predictor of persistence of asthma after the onset of puberty, although a trend towards a higher proportion of persistent wheezing among girls than boys was present. Of note, in the UK cohort(12) being female was found to be a significant risk factor for late-onset wheeze (wheeze present at age 14-16 but absent at age 6-8), suggesting that increased incident cases among girls account, at least partly, for the reversed gender ratio of asthma prevalence after puberty.

Several other longitudinal studies with longer follow-up periods have investigated factors influencing the outcome of childhood asthma in adult life, but none of them has
identified rates and predictors of asthma persistence in adolescence. Using a random stratified sample from the 1968 Tasmanian Asthma Survey, Jenkins et al. (2) found that only 26% of the subjects with asthma or wheezy breathing by the age of 7 reported current asthma as an adult (age 29-32). Interestingly, the severity of childhood asthma, as assessed by the presence of more than 10 asthma attacks, was among the strongest predictors of persistent asthma in adulthood. Similarly, in another Australian cohort, the Melbourne Asthma Study (8), a clear trend for persistent asthma at age 35 (3) and age 42 (20) was found across increasing levels of wheezing severity at age 7. Thirty-five percent of children with mild wheezy bronchitis at age 7 reported wheezing in the last 3 years at age 35, but this proportion increased to 37% for children with wheezy bronchitis, 70% for children with asthma and 90% for children with severe asthma. However, it is difficult to generalize the overall rates of persistence of asthma from this study to the population of asthmatic children, since this cohort was enriched with a further sampling of children with severe asthma at age 10. Prognosis of wheezing illness by age 11 and 16 was found to be related to severity at age 7 also in a large national British cohort (10). Consistent with these reports, we found that subjects experiencing only infrequent wheezing during childhood were much more likely to report no wheezing episodes after the onset of puberty than children with
asthma (61% of whom experienced frequent wheezing during childhood). Furthermore, the recurrence of wheezing during childhood, as assessed by the proportion of surveys in the prepubertal period in which the subjects reported wheezing, had a strong predictive value for persistence of asthma after the onset of puberty. The effect of frequency and severity of wheezing in childhood on persistence of asthma after puberty illustrates the importance of using population-based cohorts, rather than "at risk" cohorts or outpatient populations, in order to study the outcome of childhood asthma in adult life.

In addition to wheezing frequency and recurrence, we found bronchial hyperresponsiveness to be a predictor of persistence of asthma after the onset of puberty. Recently, in a longitudinal cohort of third and fourth grade schoolchildren in Australia, Xuan et al(9) found children with a positive histamine challenge test at age 8-12 to be at significantly increased risk for persistence of wheeze up to age 27. In the same cohort, severity of BHR at school age was correlated with persistence of both BHR and respiratory symptoms at age 12-14(13). In our study, because of the limited sample size of children who underwent methacholine challenge test in the asthma group, we could not test BHR in the multivariate analysis and, therefore, we cannot conclude
whether the effect of BHR on persistent asthma is or not independent of wheezing severity.

As contrasted with many of the above-mentioned cohort studies which enrolled schoolchildren, the Tucson Children's Respiratory Study followed a large cohort from birth and, therefore, it can provide information insensitive to recall bias on early wheezing and lower respiratory illness. Although the proportions of children with wheezing in the first 5 years of age or with Respiratory Syncytial Virus (RSV) lower respiratory tract illness in the first 3 years of age significantly differed across the 5 groups, neither of these factors was a significant predictor of persistence of asthma or wheezing after the onset of puberty. This is consistent with previous reports from this cohort showing that RSV lower respiratory tract illnesses in early childhood are an independent risk factor for the subsequent development of wheezing up to age 11 but not later(16).

One of the major findings of this study is the strong and independent effect of elevated BMI and early onset of puberty on the persistence of wheezing in adolescence. After adjusting for other risk factors, being overweight at age 11 was a significant risk factor for both persistence of infrequent wheezing and persistence of asthma after the onset of puberty. Consistently, the "Unremitting Wheezing"
and "Unremitting Asthma" groups had mean BMI significantly higher than those of the correspondent "Remitting" groups. These findings expand our understanding of the relationship between obesity and asthma. In recent years, several studies have shown that obesity is associated cross-sectionally\(^{(21, 22)}\) with the presence and longitudinally\(^{(15, 23, 24)}\) with the incidence of asthma symptoms, particularly among females. In this same cohort, we have already found females who were overweight at age 11 to be at increased risk for incident wheezing and the strongest association between overweight status and asthma risk was observed among girls whose puberty started before the age of 11\(^{(15)}\). Here, by studying a different outcome (persistence of asthma after puberty), we report similar findings. We found both the presence of obesity during the pre-pubertal period and an early onset of puberty to be significant and independent risk factors for persistent asthma. We did not observe an interaction between obesity and gender or between obesity and age at onset of puberty in predicting persistence of asthma (data not shown), but this finding should be interpreted with caution because of the possibly limited statistical power related to the small sample size.

It has been argued that subjects with asthma might be less likely to exercise and, in turn, more likely to gain weight
and that obesity might be an effect rather than a cause of asthma. It should be acknowledged that in a study design like ours is virtually impossible to rule out this possibility and, indeed, we found the "Unremitting Wheezing" and "Unremitting Asthma" groups to show the steeper slope of BMI increase between YR6 and YR11. However, if this were related to an effect of asthma symptoms on weight gain we should expect the increase of BMI to correlate with the severity of asthma symptoms. In contrast, we found children with infrequent, rather than frequent, wheezing to be the group gaining weight most rapidly between YR6 and YR11. The hypothesis that obesity plays a direct role in the persistence of asthma symptoms is also supported by the fact that weight reduction in obese patients with asthma improves lung function and symptoms, at least among adults(25, 26).

It is known that obesity is associated with early puberty(27). Yet, in our study an early onset of puberty was still a significant risk factor for persistent asthma, after adjusting for obesity in the multivariate model. This association was present according to a dose-response rather than threshold relationship. This finding can be interpreted in several ways. First, the association between early onset of puberty and persistent asthma might be an artifact, since children with an early onset of puberty had on average a
longer follow-up and, therefore, an increased opportunity to report wheezing in adolescence. We cannot rule out this possibility. However, findings from the multivariate analysis argue against it, since in the final model age at onset of puberty showed a much stronger effect on persistence of asthma than length of follow-up, which indeed was not even a significant predictor. Second, early onset of puberty and asthma persistence may be affected by common risk factors, such as dietary or psychological factors or the exposure to some endocrine disrupters. In this regard, it is interesting that, at the population level, in the last decades the decrease of mean age at puberty and the increase of asthma prevalence have shown to some extent similar geographical and temporal trends. Alternatively, the relationship between early onset of puberty and persistent asthma may be a real biological phenomenon. It is well-known that asthma course can drastically change during pregnancy or the menstrual cycle, most likely in relation to hormonal fluctuations. Sex hormones are known to alter β2-adrenergic responsiveness and female hormones have been shown to increase the production of Th2-like cytokines from peripheral blood mononuclear cells. In addition, leptin represents another potential candidate molecule to explain the link between early onset of puberty and persistent asthma. It has been, in fact, proposed as one
of the signals controlling sexual maturation(33) and, at the same time, leptin receptors have been shown to be present in airway and lung cells and possibly to be involved in the peripheral regulation of respiratory function(34). Further research will be required to dissect the complex interrelationships between the developmental processes of body growth and sexual maturation and their impact on the natural history of childhood asthma.

We did not find any association between persistence of asthma after puberty and active smoking at age 16, exposure to environmental tobacco smoking between ages 6 and 11 or maternal smoking during pregnancy. At YR16, the proportions of children who reported smoking cigarettes at least weekly were evenly distributed across the 5 groups (range 13.6% - 19.4%). Information on active smoking was collected using a separate confidential smoking questionnaire to be completed directly by the child and the prevalence rates for tobacco use found in this cohort are very close to those expected based on the 1997 Arizona Youth Tobacco Survey (15% for 14-17 years old children). Active smoking at age 16 was not associated with wheezing prognosis in a large national British cohort(10), but it was associated with persistent wheezing among boys in another cohort of 2,289 children(12). Contrasting findings on the effect of active smoking on the
long-term prognosis of childhood wheezing have been reported also among adults(1, 3). It is likely that the detrimental effects of smoking on asthma symptoms are somewhat masked by the so-called "healthy smoker effect" (subjects with more severe asthma symptoms are less likely to smoke but more likely to experience persistent wheezing). A similar phenomenon could affect the association between passive smoking and persistence of asthma, as parents of children with severe asthma might be more motivated to avoid or quit smoking. Our findings are consistent with previous reports that failed to find an association between parental smoking and persistence of asthma(2, 12).

Atopy is known to be strongly associated with asthma(35). It is also known that an early sensitization to specific allergens, which can be different in different geographic and climatic areas, is associated with asthma in a stronger fashion(36). In the desert environment of Tucson, we have already found Alternaria to be the only allergen independently associated with increased risk of asthma at age 6 and 11(37). In this study, we found the sensitization to Alternaria at YR6 or YR11 among children with asthma to strongly predict the persistence of wheezing after the onset of puberty. No other single allergen showed this association. Although it is unclear why Alternaria is the only asthma-related allergen in this environment, these
findings emphasize the importance of evaluating not only the presence of skin test sensitization but also the specific pattern of sensitization among children with asthma in order to predict their likelihood of outgrowing the disease. It remains to be determined whether the association between early sensitization to Alternaria and persistence of asthma after puberty holds true for other asthma-related allergens, such as mites and cat dander, in different environments.

This study has several limitations. The major concern is related to the fact that the onset of puberty was estimated based on parental report. Although specific examples of signs identifying the onset of puberty were provided in the questionnaires, it is likely that a certain degree of misclassification has occurred. No specific evaluations by study nurses were used to validate parental report of the onset of puberty. Therefore, accuracy of reports remains uncertain. Most likely, this might affect our estimates of rates of asthma remission in adolescence. However, since there is no reason to believe that the degree of misclassification was differential on both exposure and outcome, this should not jeopardize the validity of our findings on risk factors for persistence of asthma in adolescence. Second, in each questionnaire participants were asked about the presence of wheezing episodes in the last 12 months. This approach increases the reliability of self-
reports and minimizes any recall bias. However, since the questionnaires were administered every 2-3 years, this approach disregards information on the presence of wheezing during several "time windows". For example, a subject completing at age 12 and 14 two surveys would have reported on the presence of wheezing episodes between age 13 and 14, but information on the presence of wheezing between age 12 and 13 would have been disregarded. This could have provoked an under-estimation of the rates of period prevalence for wheezing in adolescence. Finally, the small sample size of some of our study groups did not provide sufficient statistical power to test the interaction between gender and other risk factors in predicting persistence of asthma after puberty, although it is biologically plausible that the patterns of risk factors may differ between the two genders.

In conclusion, in a population-based birth cohort we identified during childhood the following risk factors for persistence of asthma after the onset of puberty: presence of frequent and continuous wheezing, obesity, early onset of puberty, bronchial hyperresponsiveness, skin test sensitization to Alternaria, recurrent cough and active sinusitis. Our findings challenge the commonly held view that asthma may remit during adolescence in many cases. Only 40% of children with asthma reported no wheezing in the first 4 years after the onset of puberty, although a
reduction of the frequency and severity of wheezing episodes in adolescence is likely to occur in some of the remaining 60% of the cases. Furthermore, it remains to be determined in what proportion of remitting cases a subclinical airway inflammation persists (38) and to what extent this may increase the risk for relapses of the disease later in life (1).

REFERENCES


limitation and reduced transfer coefficient in patients with asthma after 26 years of follow up. Thorax 58(4):322-7.


31. Mills, P. J., M. G. Ziegler, R. A. Nelesen, and B. P. Kennedy. 1996. The effects of the menstrual cycle, race, and


38. van Den Toorn, L. M., J. B. Prins, S. E. Overbeek, H. C. Hoogsteden, and J. C. de Jongste. 2000. Adolescents in clinical remission of atopic asthma have elevated exhaled
Table 1. Measurement methods, time of assessment and criteria for defining a positive exposure for some of the variables used in this study.

<table>
<thead>
<tr>
<th></th>
<th>Measurement Method</th>
<th>Time of Assessment</th>
<th>Criteria for Positive Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Passive smoking</strong></td>
<td>Questionnaire by parents</td>
<td>YR6, YR8 and YR11</td>
<td>Mother or father current smoker in at least one survey</td>
</tr>
<tr>
<td><strong>Active smoking</strong></td>
<td>Questionnaire by children</td>
<td>YR16</td>
<td>Child smokes cigarettes at least weekly</td>
</tr>
<tr>
<td><strong>Overweight</strong></td>
<td>Direct measurement of height and weight by study nurses</td>
<td>YR6, YR11 and YR16</td>
<td>BMI [weight (Kg) / square of height (m)] ≥ 85th percentile of US age and sex standard values</td>
</tr>
<tr>
<td><strong>RSV lower respiratory illness</strong></td>
<td>Nasopharyngeal swabs and throat specimens</td>
<td>Up to YR3 (if lower respiratory tract illness)</td>
<td>Viral culture positive for RSV</td>
</tr>
<tr>
<td><strong>Active Recurrent cough</strong></td>
<td>Questionnaire</td>
<td>Last completed survey before the onset of puberty</td>
<td>Two or more episodes of cough without a cold in the past year</td>
</tr>
<tr>
<td><strong>Skin Tests</strong></td>
<td>Skin prick tests for allergens common in the Tucson area*</td>
<td>YR6, YR11 and YR16</td>
<td>Wheal at least three mm larger than the control wheal for at least one tested allergen</td>
</tr>
<tr>
<td><strong>Bronchial Hyperresponsiveness</strong></td>
<td>Methacholine Challenge Test</td>
<td>YR11</td>
<td>Methacholine-DRS ≤ -0.403 ml/log dose unit</td>
</tr>
</tbody>
</table>

* For reasons of consistency, only the six common allergens (i.e., Alternaria alternata, Bermuda grass, olive, careless weed, mesquite, and mulberry) tested in each of the skin prick tests (YR6, YR11 and YR16) were considered
Table 2. Criteria used for classifying the 732 subjects into the 6 groups.

<table>
<thead>
<tr>
<th>PREPUBERTAL PERIOD</th>
<th>AFTER ONSET OF PUBERTY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Wheezing</td>
</tr>
<tr>
<td>No Wheezing, No Asthma</td>
<td>NO WHEEZING (n = 369)</td>
</tr>
<tr>
<td>Infrequent Wheezing, No Asthma *</td>
<td>REMITTING WHEEZING (n = 89)</td>
</tr>
<tr>
<td>Asthma **</td>
<td>REMITTING ASTHMA (n = 64)</td>
</tr>
</tbody>
</table>

* During the prepubertal period, these subjects experienced only infrequent wheezing AND did not receive a physician-confirmed diagnosis of asthma.

** During the prepubertal period, these subjects experienced frequent wheezing OR had infrequent wheezing + a physician-confirmed diagnosis of asthma.
Table 3. Demographics and smoking exposure across the groups. Percentages refer to the proportion of subjects in each group positive for the specific factor.

<table>
<thead>
<tr>
<th></th>
<th>No Wheezing</th>
<th>Remitting Wheezing</th>
<th>Unremitting Wheezing</th>
<th>Remitting Asthma</th>
<th>Unremitting Asthma</th>
<th>Overall P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>369</td>
<td>89</td>
<td>37</td>
<td>64</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td><strong>Gender: female</strong></td>
<td>57.7%</td>
<td>46.1%</td>
<td>56.8%</td>
<td>34.4%</td>
<td>45.7%</td>
<td>.003</td>
</tr>
<tr>
<td><strong>Ethnic Background: Caucasian</strong></td>
<td>74.7%</td>
<td>64.4%</td>
<td>66.7%</td>
<td>67.8%</td>
<td>65.5%</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Age at onset of puberty in years: mean ±SEM</strong></td>
<td>12.15 ± 0.06</td>
<td>12.33 ± 0.12</td>
<td>11.70 ± 0.24 *</td>
<td>12.73 ± 0.15</td>
<td>11.98 ± 0.12 ¶</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td><strong>Maternal smoking during pregnancy</strong></td>
<td>12.6%</td>
<td>15.1%</td>
<td>16.7%</td>
<td>17.7%</td>
<td>12.1%</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Mother or father smoked at YR6, YR8 or YR11</strong></td>
<td>27.8%</td>
<td>32.9%</td>
<td>43.2%</td>
<td>39.1%</td>
<td>29.8%</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Active smoking at YR16</strong></td>
<td>14.8%</td>
<td>14.0%</td>
<td>14.8%</td>
<td>13.6%</td>
<td>19.4%</td>
<td>NS</td>
</tr>
</tbody>
</table>

* significantly different from 'Remitting Wheezing' (p < .05)
¶ significantly different from 'Remitting Asthma' (p < .0005)
**Table 4.** Respiratory symptoms, diagnoses and bronchial hyperresponsiveness (BHR) in the prepubertal period across the groups. Percentages refer to the proportion of subjects in each group positive for the specific factor.

<table>
<thead>
<tr>
<th></th>
<th>No Wheezing (n = 369)</th>
<th>Remitting Wheezing (n = 89)</th>
<th>Unremitting Wheezing (n = 37)</th>
<th>Remitting Asthma (n = 64)</th>
<th>Unremitting Asthma (n = 94)</th>
<th>Overall P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wheezing in the prepubertal period:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Episodic</td>
<td></td>
<td>52.8%</td>
<td>35.1%</td>
<td>34.4%</td>
<td>10.6%</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Recurrent</td>
<td>N/A</td>
<td>36.0%</td>
<td>29.7%</td>
<td>37.5%</td>
<td>28.7%</td>
<td></td>
</tr>
<tr>
<td>Continuous</td>
<td></td>
<td>11.2%</td>
<td>35.1%</td>
<td>28.1%</td>
<td>60.6%</td>
<td></td>
</tr>
<tr>
<td><strong>Wheezing without cold before age 5</strong></td>
<td></td>
<td>7.7%</td>
<td>13.8%</td>
<td>28.6%</td>
<td>34.4%</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>RSV^ lower respiratory illness in the first 3 years</strong></td>
<td>17.2%</td>
<td>28.9%</td>
<td>35.5%</td>
<td>27.6%</td>
<td>30.3%</td>
<td>.01</td>
</tr>
<tr>
<td><strong>At the last completed survey before the onset of puberty:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active recurrent cough</td>
<td></td>
<td>5.7%</td>
<td>14.6%</td>
<td>24.3%</td>
<td>29.7%</td>
<td>50.0%</td>
</tr>
<tr>
<td>Active rhinitis</td>
<td>21.9%</td>
<td>27.0%</td>
<td>27.0%</td>
<td>50.0%</td>
<td>60.6%</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Active sinusitis</td>
<td>13.3%</td>
<td>18.2%</td>
<td>33.3%</td>
<td>20.6%</td>
<td>36.4%</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>BHR at YR11 (if test completed before the onset of puberty)</strong></td>
<td>18.1%</td>
<td>22.5%</td>
<td>23.1%</td>
<td>40.9%</td>
<td>73.9%</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>
Respiratory Syncytial Virus

* significantly different from 'Remitting Wheezing' (p < .01)
📍 significantly different from 'Remitting Asthma' (p < .0001)
# p value refers to the comparison between 'Remitting Wheezing', 'Unremitting Wheezing', 'Remitting Asthma' and 'Unremitting Asthma'
‡ significantly different from 'Remitting Asthma' (p < .05)
Table 5. Skin tests results at YR6, YR11 and YR16 across the groups. Percentages refer to the proportion of subjects in each group positive for the specific allergen.

<table>
<thead>
<tr>
<th></th>
<th>No Wheezing</th>
<th>Remitting Wheezing</th>
<th>Unremitting Wheezing</th>
<th>Remitting Asthma</th>
<th>Unremitting Asthma</th>
<th>Overall P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>YR6 (n = 514)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any Positive %</td>
<td>26.1%</td>
<td>28.0%</td>
<td>54.2% *</td>
<td>56.7%</td>
<td>73.8% †</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Alternaria Positive %</td>
<td>9.1%</td>
<td>10.7%</td>
<td>20.8%</td>
<td>23.3%</td>
<td>50.0% †</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Bermuda Positive %</td>
<td>17.8%</td>
<td>26.7%</td>
<td>20.8%</td>
<td>43.3%</td>
<td>50.0%</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Other Pollens Positive %</td>
<td>14.9%</td>
<td>17.3%</td>
<td>29.2%</td>
<td>40.0%</td>
<td>46.3%</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td><strong>YR11 (n = 495)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any Positive %</td>
<td>42.6%</td>
<td>49.3%</td>
<td>77.4% ‡</td>
<td>66.1%</td>
<td>85.5% †</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Alternaria Positive %</td>
<td>12.2%</td>
<td>16.9%</td>
<td>38.7% *</td>
<td>30.4%</td>
<td>46.1% †</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Bermuda Positive %</td>
<td>27.5%</td>
<td>29.6%</td>
<td>48.4%</td>
<td>46.4%</td>
<td>56.6%</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Other Pollens Positive %</td>
<td>32.4%</td>
<td>45.1%</td>
<td>54.8%</td>
<td>58.9%</td>
<td>68.4%</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td><strong>YR16 (n = 421)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any Positive %</td>
<td>62.5%</td>
<td>61.0%</td>
<td>85.2% *</td>
<td>80.4%</td>
<td>96.9% †</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Alternaria Positive %</td>
<td>15.6%</td>
<td>25.4%</td>
<td>40.7%</td>
<td>34.8%</td>
<td>60.0% †</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Bermuda Positive %</td>
<td>43.8%</td>
<td>39.0%</td>
<td>63.0%</td>
<td>63.0%</td>
<td>80.0% †</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Other Pollens Positive %</td>
<td>56.3%</td>
<td>54.2%</td>
<td>77.8% *</td>
<td>76.1%</td>
<td>89.2% †</td>
<td>&lt; .0001</td>
</tr>
</tbody>
</table>
Statistical comparisons are performed across the 5 groups (p value reported in the last column), between Remitting and Unremitting Wheezing and between Remitting and Unremitting Asthma.

* significantly different from 'Remitting Wheezing' (p < .05)
§ significantly different from 'Remitting Wheezing' (p < .005)
^ significantly different from 'Remitting Wheezing' (p < .001)
¶ significantly different from 'Remitting Asthma' (p < .05)
† significantly different from 'Remitting Asthma' (p < .01)
f significantly different from 'Remitting Asthma' (p < .001)
Table 6. Logistic regression models for predicting persistence of asthma symptoms after onset of puberty. Subjects from the "Remitting Wheezing" and 'Unremitting Wheezing' groups were included in the first model and subjects from the 'Remitting Asthma' and 'Unremitting Asthma' groups were included in the second model.

<table>
<thead>
<tr>
<th></th>
<th>MODEL 1</th>
<th>MODEL 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>INFREQUENT WHEEZING</td>
<td>ASTHMA</td>
</tr>
<tr>
<td></td>
<td>in the prepubertal period (n = 126)</td>
<td>in the prepubertal period (n = 158)</td>
</tr>
<tr>
<td>OR</td>
<td>95% CI</td>
<td>P value</td>
</tr>
<tr>
<td>Gender: female</td>
<td>1.24</td>
<td>0.49 – 3.11</td>
</tr>
<tr>
<td>Age at onset of puberty in years</td>
<td>0.69</td>
<td>0.46 – 1.04</td>
</tr>
<tr>
<td>Continuous wheezing in prepubertal period</td>
<td>3.23</td>
<td>1.09 – 9.56</td>
</tr>
<tr>
<td>Overweight at YR11</td>
<td>3.61</td>
<td>1.07 – 12.2</td>
</tr>
<tr>
<td>Skin tests at YR11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive for other allergens</td>
<td>1.88</td>
<td>0.55 – 6.45</td>
</tr>
<tr>
<td>Hosmer and Lemeshow Test</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


FIGURE LEGENDS

Figure 1. Mean Body Mass Index among the groups at surveys YR6, YR11 and YR16.

Statistical Comparisons of BMI values (after adjusting for gender):

* YR6: Across the groups: $p = \text{NS}$;
  Remitting versus Unremitting Wheezing: $p = .04$;
  Remitting versus Unremitting Asthma: NS.

** YR11: Across the groups: $p = .0001$;
  Remitting versus Unremitting Wheezing: $p = .0005$;
  Remitting versus Unremitting Asthma: $p = .01$.

*** YR16: Across the groups: $p < .0001$;
  Remitting versus Unremitting Wheezing: $p < .0001$;
  Remitting versus Unremitting Asthma: $p = .02$. 
Figure 1.
APPENDIX A.3

The Relation of Beta-2 Adrenoceptor Polymorphisms at Codon 16 and 27 to Persistence of Asthma at Puberty

Authors’ list:
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Penelope E. Graves, Sc.D.
Catherine J. Holberg, PhD
Anne L. Wright, PhD
Fernando D. Martinez, MD

Arizona Respiratory Center, University of Arizona, College of Medicine, Tucson, AZ
SUMMARY

**Background.** It has been long recognized that in many cases children with asthma may outgrow the disease after the onset of puberty, but little is known about the factors affecting the outcome. The aim of the present study was to determine whether the polymorphisms at codon 16 and 27 of the β2 adrenoceptor are significant predictors of persistence of asthma at puberty.

**Methods.** We used data from the birth cohort of the Tucson Children's Respiratory Study. 189 children who were genotyped for the polymorphisms at codon 16 and 27 and experienced wheezing episodes between age 6 and the reported onset of puberty were assessed for persistence or remission of wheezing after the onset of puberty up to age 16.

**Findings.** Boys homozygous for Gly at codon 16 were twice as likely to experience persistent wheezing at puberty than carriers of the other genotypes (RR 2.01, 1.32-3.06, p = .0008). Among boys, the risk associated with the Gly16/Gly16 genotype linearly increased according to the frequency of wheezing episodes after the onset of puberty. These findings held true after adjusting for ethnicity and after selecting only Caucasian children. No association was found among
girls. The polymorphism at codon 27 did not affect the risk for persistent wheezing.

**Interpretation.** These findings provide the first evidence for a strong effect of the Gly16 polymorphism on persistence of asthma at puberty among boys, but not girls. A differential functional regulation of adrenergic receptors by male and female sex hormones might be involved in explaining this interaction by gender.

**INTRODUCTION**

Asthma is a common disease among school-age children. According to surveillance data from the Centers for Disease Control and Prevention, about 60 per 1,000 children aged 5-14 reported episodes of asthma attacks in the preceding 12 months in the United States in 1999. Population-based longitudinal studies have reported even higher rates of cumulative incidence. In a large British cohort, the cumulative incidence of asthma or wheezy bronchitis was found to be 18.2% and 21.8% by the ages of 7 and 11 years. It has been long recognized that many children with asthma may outgrow the disease after the onset of puberty.
Adolescence represents a transition phase from childhood to adulthood and is characterized by rapid hormonal, physical and behavioral changes, all of which may influence the natural course of asthma. However, little is known about the factors affecting the remission or persistence of asthma symptoms at puberty.

Population-based longitudinal cohort studies have shown that severity and frequency of asthma symptoms are among the strongest predictors for persistence of asthma from childhood to adulthood. Using a birth cohort from Tasmania, Australia, Jenkins and coworkers found that having had more than 10 asthma attacks by age 7 almost doubled the likelihood of having current asthma at age 30. In the Melbourne Asthma Study, Oswald et al found a similar trend for asthma prevalence at age 35 across increasing levels of severity of asthma at age 7. Sixty-three per cent of the children with severe asthma at school age reported persistent asthma at age 35, as contrasted with only 8% of the children with mild wheezy bronchitis.

Severity of asthma has been shown to be affected by the genetic variation of the β, adrenoceptor (β,AR). Although the polymorphisms at codons 16 (Arg16→Gly16) and 27 (Gln27→Glu27) of the β,AR have not been associated with the
development of the disease per se, several studies have reported an association between the Gly16 polymorphism and severe asthma. In the original report describing these polymorphisms, Reihsaus et al. found a significantly higher proportion of subjects homozygous for Gly16 among patients with asthma requiring oral steroids than among those with a milder form of the disease. Similarly, Holloway et al. found that patients with at least one admission to hospital with asthma were almost twice as likely to be homozygous for Gly16 than controls. No such association was present among patients with mild asthma. In another study, Weir et al. reported the Gly16-Gln27 haplotype to be more prevalent in moderate asthmatics (taking > 400 µg of inhaled beclomethasone or equivalent per day and/or having an FEV1 < 75% of predicted) than in mild asthmatics.

Based on this evidence, we hypothesized that the polymorphisms at codons 16 and 27 of the β2 adrenoceptor may be associated with persistence of asthma after the onset of puberty. Our findings from a population-based longitudinal cohort provide evidence for a strong effect of the homozygous status for Gly16 on persistent asthma at puberty among boys but not girls.
METHODS

The children included in this study are a subset from the large birth cohort of the Tucson Children's Respiratory Study. Detailed information on the design and the enrollment process of this study has been provided elsewhere. Briefly, between May 1980 and October 1984 parents planning to use the pediatricians of a health maintenance organization in Tucson, AZ, were contacted shortly after their child was born and a total 1246 healthy infants were enrolled in the study. Parents completed questionnaires on their child's health status at the time of the enrollment and at different ages: YR2 survey (mean age ± SD, 1.62 ± 0.3 years), YR3 (2.93 ± 0.5), YR6 (6.27 ± 0.9), YR8 (8.62 ± 0.7), YR11 (10.90 ± 0.7), YR13 (13.47 ± 0.6), and YR16 (16.55 ± 0.5).

In the questionnaires of surveys YR13 and YR16, questions on whether and when puberty started were included. In these questionnaires, specific examples of signs identifying the onset of puberty ("pubic and/or underarm hair, breast development or menstruation in girls, voice changes in boys") were provided. We defined the prepubertal period as that between YR6 survey and the reported onset of puberty. Remission and persistence of wheezing and asthma were
studied in the follow-up period included between the onset of puberty and up to YR16 survey (mean follow-up ± SD: 4.0 ± 1 years).

Questions on the presence and frequency of wheezing episodes during the previous year were asked in each of the surveys. Wheezing in the prepubertal period was defined as the report of any wheezing episode in any of the completed surveys between YR6 and the onset of puberty. Asthma in the prepubertal period was defined as the report of more than 3 wheezing episodes during the previous year in at least one survey or a physician-confirmed diagnosis of asthma. Outcomes in adolescence were then assessed. Wheezing and asthma groups were classified as "Persistent" if any wheezing episodes were reported in at least one survey after the onset of puberty and "Remitting" if they were not. The frequency of wheezing after the onset of puberty was also assessed. Wheezing was defined as "Frequent" if the child reported > 3 wheezing episodes and "Infrequent" if the child reported between 1 and 3 wheezing episodes during the previous year in at least one survey after the onset of puberty.

Genomic DNA was prepared from peripheral blood obtained at about 11 yr of age using standard techniques. $\beta_2$AR
genotypes were determined in 479 subjects by a combination of primer-induced restriction site assay and restriction fragment assay as described previously and they were verified by direct dideoxy sequencing in 8 subjects. Results obtained by sequencing confirmed those obtained with the primer-induced restriction site assay for $\beta_2$AR-16 and with the restriction fragment assay for $\beta_2$AR-27.

Informed consent was obtained from the parents of participating children and the study was approved by the Human Subjects Committee of the University of Arizona.

**Statistical Analyses.**

Population genetics analyses were performed using the software Arlequin ver. 2.000. Because of the nature of our data (genotypic data with unknown gametic phase), maximum-likelihood haplotype frequencies were imputed using an Expectation-Maximization (EM) algorithm. Through this method, it is possible to estimate the most likely distribution by haplotype in the total study population as well as in each ethnic group. Differences in the haplotype distributions among the ethnic groups were tested using the exact test of population differentiation. For subjects heterozygous for both $\beta_2$AR-16 and $\beta_2$AR-27, haplotypes were
inferred using the PHASE package with the threshold probability set at 95%.

Proportions of persistent wheezing/asthma were compared using χ² tests across the β₂AR-16 and β₂AR-27 genotypes (analysis by subjects) and the corresponding haplotypes (analysis by chromosomes). The 95% Confidence Intervals (95% CI) of proportions were computed using the binomial distribution. Relative Risks (RR) for persistence of wheezing/asthma associated with the homozygous status for Gly16 were computed and tested for significance through their 95% CI. Relative Risks were adjusted for ethnic background using stratification according to the Mantel-Haenszel method. Interaction by gender was tested through the homogeneity test, testing the null hypothesis that the RRs for the association between the genotype Gly16/Gly16 and persistent wheezing/asthma were equal between males and females. An α=0.05 level of significance was chosen for all the statistical tests performed.

RESULTS
479 children were genotyped for polymorphisms at both codon 16 and codon 27. Genotyped children did not differ significantly from non-genotyped children (n = 767) in terms of gender distribution and average years of maternal and paternal education. In contrast, the 2 groups differed by ethnic distribution. 29.5% of the children genotyped versus 20.1% of the children who were not genotyped had at least one Hispanic parent (p = .003).

The two polymorphisms in codon 16 and 27 were found in strong linkage disequilibrium to the point that the haplotype Arg16-Glu27 was estimated to be absent in the study population (Table I). We found the estimated haplotype frequencies to be significantly different across the ethnic groups (test for population differentiation: p = .0004). No significant differences were found in the distribution of the allele Arg16 (and correspondent haplotype Arg16-Gln27) across the ethnic groups, but the allele Glu27 (and the correspondent haplotype Gly16-Glu27) was significantly more common among children with both parents Caucasian (.418) than children with both parents Hispanic (.261). Children with mixed ethnicity showed an intermediate frequency (.346). Because of these differences in $\beta$AR-27 allele / haplotype frequencies, in this study all the analyses performed were both controlled for and stratified by
ethnicity. Findings stratified by ethnicity will be presented only for children with both parents Caucasian, since this was the only ethnic group with sufficient sample size.

Information on wheezing in the pre-pubertal period and after the onset of puberty was available for 390 genotyped children. There were no differences in the genotype distribution for both $\beta_2$AR-16 and $\beta_2$AR-27 between children with (n = 189) and without (n = 201) wheezing in the pre-pubertal period (data not shown). Consistently, the haplotype frequencies were homogeneous between the two groups of children with and without wheezing in the pre-pubertal period (Arg16-Gln27 34.4% vs 38.1%, Gly16-Gln27 25.7% vs 25.1%, and Gly16-Glu27 39.9% vs 36.8%, respectively; $p = .537$).

Among the 189 children with wheezing in the pre-pubertal period, 95 (50.3%) experienced wheezing episodes after the onset of puberty ("Persistent Wheezing" group) and the remaining 94 (49.7%) did not ("Remitting Wheezing"). In Figure 1, the proportions of children with persistent wheezing at puberty are compared across the $\beta_2$AR-16 and $\beta_2$AR-27 genotypes. About 60% of the children homozygous for Gly16 experienced persistent wheezing versus only 43.5% of the
children carrying a different genotype (RR 1.36, 1.03 - 1.80; p = .032). In contrast, rates of persistence and remission of wheezing after the onset of puberty were homogeneous across the genotypes for β2AR-27. The increased risk for persistent wheezing associated with the Gly16/Gly16 genotype did not change after adjusting for ethnicity (adjusted RR 1.37, 1.03 - 1.82; p = .044) and was stronger within the group of children with Caucasian parents (RR 1.57, 1.08 - 2.26; p = .015).

We found a statistically significant interaction by gender in the association between Gly16/Gly16 genotype and persistent wheezing (Table II). Boys homozygous for Gly16 were twice as likely to experience persistent wheezing after the onset of puberty than those carrying a different β2AR-16 genotype (RR 2.01, 1.32 - 3.06; p = .0008). In contrast, no association between β2AR-16 genotypes and persistent wheezing at puberty was found among girls (RR 0.88, 0.59 - 1.31). The test for homogeneity testing the null hypothesis that the OR for the association between Gly16/Gly16 and persistent wheezing were identical among the groups of males and females was highly significant (p = .008). Therefore, in these data, gender was found to be a significant effect modifier of this relationship. These findings were confirmed among the 118 children who met the criteria for presence of
asthma during the pre-pubertal period (Table II). Furthermore, we could confirm the increased risk for persistent wheezing associated with the Gly16/Gly16 genotype among males also after adjusting for ethnicity (adjusted RR 2.08, 1.35 - 3.23, p = .001) and when analyses were restricted to children with Caucasian parents (RR 2.44, 1.36 -4.36; p = .001).

Figure 2 shows that the risk associated with the Gly16/Gly16 genotype linearly increased among males according to the frequency of wheezing episodes after the onset of puberty. Boys homozygous for Gly16 were, in fact, twice as likely to experience infrequent wheezing (p = .009), but up to 3.4 times more likely to experience frequent wheezing (p = .003) at puberty, as compared with carriers of the other genotypes. The correspondent RRs among Caucasian boys were 2.36 (p = .030) for infrequent wheezing and 5.06 (p = .001) for frequent wheezing. No trend was evident among females, neither in the whole population nor in the Caucasian group.

Results of the haplotype analysis are presented in Figure 3. Because of the significantly different haplotype distributions across the ethnic groups, only children with both parents being Caucasian were selected for this analysis. Persistent wheezing appeared somewhat more common among females than males, but this association reached only
borderline significance. Haplotypes were associated with very similar percentages of persistent wheezing (between 63% and 67%) among females. In contrast, among males the haplotypes carrying the Gly16 allele (Gly16-Gln27 and Gly16-Glu27) showed percentages of persistent wheezing very similar to each other, but significantly higher than those associated with the Arg16 allele (and the correspondent Arg16-Gln27 haplotype) \( p = .009 \).

**DISCUSSION**

Our findings provide the first evidence for a strong effect of the \( \beta_2 \)AR Gly16 polymorphism on persistence of asthma after the onset of puberty among boys, but not girls. Male adolescents homozygous for Gly16 were twice as likely as carriers of the other genotypes to experience persistent wheezing and persistent asthma. In addition, the risk associated with the genotype Gly16/Gly16 linearly increased according to the frequency of wheezing episodes after the onset of puberty.

From *in vitro* studies\(^{16,17}\), it has been long known that, although neither the \( \beta_2 \)AR-16 nor the \( \beta_2 \)AR-27 polymorphism
alters the affinity of the receptor for the agonists, the Gly16 receptor undergoes an enhanced down-regulation as compared with the Arg16 receptor. Interestingly, this effect of the Gly16 polymorphism has been shown directly in primary cell lines of airway smooth muscle, in which the βAR acts to relax and dilate the airway. This is the major mechanism by which β agonists exert their therapeutic effects in asthma treatment and, therefore, it is not surprising that the Gly16 polymorphism has been already shown to reduce the response to β agonists among asthmatic children and adults and to be associated with asthma severity.

The enhanced down-regulation associated with the Gly16 receptor most likely plays a major role also in the increased risk for persistent asthma at puberty shown in our study by males homozygous for Gly16. However, it does not explain why we found no association between βAR polymorphisms and persistent asthma among females. In this regard, it is known that steroid hormones can affect functionally the βAR. An interaction between glucocorticoids and β, agonists is recognized clinically and experimentally and adrenergic receptors have been shown to be regulated by sex hormones, at least in animal models. It is plausible to hypothesize that a differential
functional regulation of adrenergic receptors by male and female sex hormones might be involved in explaining the interaction by gender in the association between Gly16 polymorphism and persistence of asthma at puberty. This would be consistent also with the greater β2AR sensitivity reported in women as compared with men\textsuperscript{23,24}. Experimental studies will be required to determine whether this hypothesis holds true, whether gender-related differences in β2AR regulation affect the receptor per se or the post-receptor activity\textsuperscript{24}, and whether they present any tissue or cell type specificity.

Consistent with previous reports\textsuperscript{8,25,26}, we found the frequencies of the β2AR haplotypes to be significantly different across the different ethnic groups. Therefore, all the analyses were controlled for and stratified by ethnicity. We could confirm the increased risk for persistent wheezing/asthma associated with the polymorphism Gly16 among boys after adjusting for ethnicity as well as after selecting only subjects with both parents Caucasian. Indeed, the association between the homozygous status for Gly16 and persistent asthma appeared to be even stronger among Caucasians than in the total population. Such an association could not be tested in any other ethnic group because of the reduced sample size.
The polymorphism Gly16 appeared to affect persistence of asthma at puberty according to a recessive model. Subjects with the β,AR-16 genotypes Arg/Arg and Arg/Gly, in fact, showed proportions of persistent asthma very similar to each other (45% and 43%, respectively) and significantly lower than that of carriers of the Gly/Gly genotype (59%). These findings suggest that, in heterozygotes, the expression of the receptor Arg16 might have a protective effect against the consequences of the enhanced down-regulation associated with the receptor Gly16, which is probably clinically relevant only in the absence of any Arg16 receptor. However, caution should be used in interpreting this recessive model because of the reduced number of subjects homozygous for Arg16 (n = 22).

The polymorphism β,AR-27 had no effect on persistence of asthma at puberty. This was evident both from the homogeneous percentages of persistent wheezing associated with the different β,AR-27 genotypes (Figure 1) and from the haplotype analysis (Figure 3). Among males, in fact, the haplotypes carrying the Gly16 allele (Gly16-Gln27 and Gly16-Glu27) showed percentages of persistent wheezing significantly higher than those associated with the Arg16-Gln27 haplotype, but very similar to each other regardless
of the allele present at codon 27. These data are consistent with the \textit{in vitro} finding that the haplotypes Gly16-Gln27 and Gly16-Glu27 show a similar down-regulation phenotype\textsuperscript{15}, suggesting that the Gly16 allele is the major determinant in the process.

In conclusion, our findings from a population-based longitudinal birth cohort suggest that children with asthma homozygous for Gly16 are more likely to experience persistence of asthma at puberty than carriers of the other genotypes. Most importantly, this association is present among boys, but not girls. These findings hold true also after adjusting for ethnicity and after restricting the analyses only to children from the largest ethnic group (Caucasian).

**REFERENCES**


9. Taussig LM, Wright AL, Morgan WJ, Harrison HR, Ray CG. The Tucson Children's Respiratory Study. I. Design and implementation of a prospective study of acute and


Table I. Estimated haplotype frequencies (± SD) across the groups by parental ethnic background.

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Caucasian</th>
<th>Caucasian</th>
<th>Hispanic</th>
<th>Hispanic</th>
<th>Other Ethnicity</th>
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</thead>
<tbody>
<tr>
<td>Arg16-Gln27</td>
<td>.356 (.011)</td>
<td>.385 (.041)</td>
<td>.381 (.027)</td>
<td>.412 (.080)</td>
<td></td>
</tr>
<tr>
<td>Arg16-Glu27</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gly16-Gln27</td>
<td>.225 (.014)</td>
<td>.269 (.049)</td>
<td>.358 (.032)</td>
<td>.324 (.051)</td>
<td></td>
</tr>
<tr>
<td>Gly16-Glu27</td>
<td>.418 (.017)</td>
<td>.346 (.048)</td>
<td>.261 (.040)</td>
<td>.265 (.069)</td>
<td></td>
</tr>
<tr>
<td>N of subjects</td>
<td>282</td>
<td>65</td>
<td>67</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

Test for population differentiation: p = .0004

* Information on ethnic background was missing for 31 children
Table II. Proportion of persistent wheezing and persistent asthma among subjects homozygous for Gly16 and among carriers of other β_{AR}-16 genotypes. Results are stratified by gender.

<table>
<thead>
<tr>
<th>ANY WHEEZING BEFORE PUBERTY (n = 189)</th>
<th>ASTHMA BEFORE PUBERTY (n = 118)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N persistent wheezing / N total (%)</td>
<td>N persistent asthma / N total (%)</td>
</tr>
<tr>
<td>Gly16 / Gly16</td>
<td>30 / 47 (63.8%)</td>
</tr>
<tr>
<td></td>
<td>2.01* (1.32 - 3.06)</td>
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<tr>
<td>Other genotypes</td>
<td>20 / 63 (31.7%)</td>
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<tr>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>FEMALES</td>
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</tr>
<tr>
<td>Gly16 / Gly16</td>
<td>18 / 34 (52.9%)</td>
</tr>
<tr>
<td></td>
<td>0.88 (0.59 - 1.31)</td>
</tr>
<tr>
<td>Other genotypes</td>
<td>27 / 45 (60.0%)</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
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<tr>
<td>Test of homogeneity§</td>
<td>p = .008</td>
</tr>
</tbody>
</table>

* p = .0008; ** p = .001;
§ The test for homogeneity tests the null hypothesis that the RRs for persistent wheezing/asthma associated with the Gly16/Gly16 genotype are equal among males and females.
FIGURE LEGENDS

Figure 1. Proportions (and correspondent binomial 95% Confidence Intervals) of children with persistent wheezing after the onset of puberty across the genotypes of β₂AR-16 and β₂AR-27.

Figure 2. Relative Risks (and correspondent Standard Errors) for infrequent and frequent wheezing at puberty associated with the Gly16/Gly16 genotype. Results are presented stratified by gender within the total population and within the group of children with both parents Caucasian.

Figure 3. Proportions (and correspondent binomial 95% Confidence Intervals) of persistent wheezing after the onset of puberty associated with the β₂AR haplotypes among children with both parents Caucasian. Results are presented stratified by gender. Numbers refer to chromosomes.
Figure 1.

![Figure 1](image_url)
Figure 2.
Figure 3.
APPENDIX B:

HUMAN SUBJECTS APPROVAL LETTERS
Dear Dr. Wright,

We received your 17 April & 24 April 2003 letters and accompanying revised Consent Form and Addendum and updated Verification of Training Form (VOTF) for the above referenced project. The protocol has been modified to change the study title as cited above, to add nasal aspirate collection at time of acute lower respiratory tract illness and to administer previously approved questionnaire at acute visit and at convalescence (2-5 weeks later), and to collect nasal aspirate & administer submitted questionnaire [Nasal Aspirate Specimen Collection Data Sheet] at time of first upper respiratory tract illness in concert with physician consultation; also, Melisa Celaya has been added as research technician and Sally Stamper, Marilyn Lindell, and Lydia de la Ossa have been added as research nurses [a revised Consent Form and newly developed Addendum for previously consented subjects] and data collection sheet and updated VOTF have been provided for review. Approval for these changes is granted effective 1 May 2003.

The Institutional Review Board (IRB) of the University of Arizona has a current Federalwide Assurance of compliance, PWA00004218, which is on file with the Department of Health and Human Services and covers this activity.

Approval is granted with the understanding that no further changes or additions will be made either to the procedures followed or to the consent form(s) used (copies of which we have on file) without the knowledge and approval of the Human Subjects Committee and your College or Departmental Review Committee. Any research related physical or psychological harm to any subject must also be reported to each committee.

A university policy requires that all signed subject consent forms be kept in a permanent file in an area designated for that purpose by the Department Head or comparable authority. This will assure their accessibility in the event that university officials require the information and the principal investigator is unavailable for some reason.

Sincerely yours,

David G. Johnston, M.D.
Chairman, Biomedical Committee
UA Institutional Review Board (IRB)

DGJ
Dear Dr. Martinez:

We received your 25 November 2002 letter and accompanying previously utilized-unsubmitted Year 20 Questionnaire and 3 subject's letters distributed with questionnaires and telephone exclusion form and appointment letter/instructions/map for the above referenced project. Subjects not scheduled for Year 20 testing will receive the submitted subject's letters and health & respiratory history/symptoms questionnaire; also subjects scheduled for testing will be pre-screened using the submitted Telephone Exclusion Form and eligible subjects will receive the questionnaire and the submitted appointment letter/instructions/map. Approval for these changes is granted effective 26 November 2002. Note: All previously utilized questionnaires and subject contact letters must be submitted for IRB review.

The Human Subjects Committee (Institutional Review Board) of the University of Arizona has a current assurance of compliance, number M-1233, which is on file with the Department of Health and Human Services and covers this activity.

Approval is granted with the understanding that no further changes or additions will be made either to the procedures followed or to the consent form(s) used (copies of which we have on file) without the knowledge and approval of the Human Subjects Committee and your College or Departmental Review Committee. Any research related physical or psychological harm to any subject must also be reported to each committee.

A university policy requires that all signed subject consent forms be kept in a permanent file in an area designated for that purpose by the Department Head or comparable authority. This will assure their accessibility in the event that university officials require the information and the principal investigator is unavailable for some reason.

Sincerely yours,

[Signature]

David G. Johnson, M.D.
Chairman
Human Subjects Committee

DGJ:jpm
cc: Departmental/College Review Committee