

Associations of the *IL12B* promoter polymorphism in longitudinal data from asthmatic patients 7 to 42 years of age

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Background: The *IL12B* gene encodes the p40 chain of IL-12, a proinflammatory cytokine that antagonizes T_H2 expression and hence may play a critical role in the pathogenesis of airway inflammation observed in asthma. A promoter polymorphism of the gene was recently shown to be associated with asthma severity in children but only in heterozygotes.

Objective: The aim of the present study was to test the hypothesis that the *IL12B* promoter polymorphism is associated with asthma susceptibility, severity, and related phenotypes in a cohort with longitudinal phenotypic data, from childhood to adulthood.

Methods: Four hundred one 7-year-old children (106 control children, 295 asthmatic children) and 83 10-year-old children with severe asthma were recruited from a 1957 birth cohort. Atopic status and respiratory functions were determined at ages 7, 10, 14, 21, 28, 35, and 42 years. At age 42 years, blood samples were taken from 244 individuals for genotyping and the determination of plasma IgE levels and PHA- and house dust mite-induced IFN- γ responses. Genotyping was done by the PCR restriction fragment length polymorphism method, using *Alu I*, and confirmed in 10% of the samples by direct sequencing.

Results: The *IL12B* genotypes were not associated with asthma susceptibility, severity, or atopy at ages 7 and 42 years. Total serum IgE levels at age 42 of men with at least one CTCTAA allele were higher than those homozygous for the GC allele ($P = .042$), whereas no difference was observed for women. At all ages, female subjects with at least 1 copy of the CTCTAA allele had lower mean percent predicted levels of FEV₁ and FVC compared with those without this allele; these differences were significant at ages 10 and 14 years ($P < .05$) and in the asthmatic subgroup at age 7 years ($P = .001$).

Conclusions: In this long-term study of asthmatic subjects with comprehensive data on asthma severity, we found no evidence to support the presence of a heterozygote effect of the *IL12B* promoter polymorphism on the level of asthma in early childhood or adulthood. The polymorphism was also not associated with asthma susceptibility, but the CTCTAA allele may have been associated with elevated serum IgE levels in male subjects and reduced pulmonary function in female subjects in early childhood. (*J Allergy Clin Immunol* 2004;113:475-81.)

Key words: Asthma, *IL12B*, promoter, polymorphism, longitudinal study, association

Asthma is a common disorder with an increasing incidence in Westernized societies, and in Australia the disease affects up to 1 in 4 children under the age of 14 years.¹ Asthma is a complex disease that is characterized by airway inflammation and bronchial hyperresponsiveness and results from an interaction of inherited and environmental factors. Large-scale genome screens have identified several chromosomal regions, including chromosome 5q31-33, linked to the diagnosis of asthma,² and asthma-related phenotypes such as elevated serum IgE levels^{3,4} and bronchial hyperresponsiveness.⁵ The cytokine gene cluster is located on chromosome 5q31-33, and this region contains several potential candidate genes for asthma, including IL-3, IL-4, IL-5, IL-9, IL-12B, IL-13, CD14, and the β_2 -adrenergic receptor.⁴⁻⁷ Polymorphisms in several of these genes have been associated, in more than 1 population, with asthma phenotypes including several in the IL-13 gene, which have been associated with asthma, atopy, and total serum IgE^{8,9} and the CD14-159C/T promoter polymorphism, which was associated with total serum IgE levels.¹⁰

The *IL12B* gene is a potential candidate for asthma because of its role in regulating T_H1/T_H2 responses by preferentially generating T_H1 cells and IFN- γ ^{11,12} while suppressing the release of T_H2 cytokines such as IL-4.¹¹ IL-12 deficiency thus skews the immune system toward a T_H2-type profile, which is thought to play a crucial role in the pathogenesis of airway inflammation observed in asthma. IL-12 is also important in cell-mediated immunity against many bacterial and parasitic infections, and, if deficient, predisposes the individual to severe mycobacterial and salmonella infections^{13,14} as well as to recurrent episodes of pneumonia.¹⁵ Severe and repeated infections have been associated with lower incidences of asthma and atopy,^{16,17} suggesting that exposure to infectious pathogens may reduce the risk of development of

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Abbreviations used

FVC: Forced vital capacity
HDM: House dust mite

such allergic diseases. Furthermore, IL-12 mRNA expression was significantly lower in bronchial biopsy specimens from allergic asthmatic patients compared with those from normal control subjects,¹⁸ and stimulation of whole blood cells resulted in reduced production of IL-12 in allergic asthmatic subjects.¹⁹ In murine studies, recombinant IL-12 had significant therapeutic effects in inhibiting antigen-induced airway hyperresponsiveness and inflammation.^{20,21}

IL-12 has 2 disulfide-bonded polypeptide chains, a 35-kd chain encoded by *IL12A* on chromosome 3p12-q13.2 and a 40-kd chain encoded by *IL12B* on chromosome 5q31-33.²² The *IL12A* gene has 1 reported polymorphism,²³ but no associations have been noted for this polymorphism. Several polymorphisms have been identified in the *IL12B* gene,^{23,24} including a single nucleotide polymorphism in the 3' untranslated region, which has been associated with resistance to insulin-dependent diabetes mellitus²⁵ and multiple sclerosis²⁶ but not with asthma and allergic rhinitis in a Japanese population.²⁷ Recently, an association was reported between a promoter polymorphism of the *IL12B* gene and asthma severity in a cohort of Australian children.²⁸ In that study, asthmatic children who were heterozygotes were 4 times more likely to have severe asthma compared with homozygotes for either allele, even though the polymorphism was not associated with asthma per se.

Ideally, to provide the best possible population to critically evaluate the hypothesis that the *IL12B* promoter polymorphism has a heterozygote effect on asthma severity would require a cohort with extensive, accurate, and long-term phenotypic data on asthma. A population that may be best for fitting the attributes is the Melbourne Asthma Study cohort, which has followed children from 7 to 42 years of age.²⁹⁻³³ This population has been the result of intensive study, and numerous important observations relating to asthma have resulted.^{32,34,35} The aim of this study therefore was to test the above hypothesis in the Melbourne population. This population would also provide a unique opportunity to test the hypothesis that the *IL12B* promoter polymorphism affects asthma-related phenotypes ascertained at different ages.

METHODS

Study population

In 1964, a community-based study was initiated in which a cohort of 401 children was selected from 30,000 7-year-old children in Melbourne, Australia.²⁹ The cohort was stratified to provide 3 groups of approximately equal size of children with a history of wheezing of varying frequency and a control group with no history of wheezing. Wheezing and asthmatic status were initially determined by parental response to a questionnaire and were subsequently confirmed by clinical examination. These children were surveyed again at age 10,

at which time an additional 83 children from the same 1957 birth cohort were recruited to enrich the group with severe asthma.³⁶ Hence, these study groups were specifically selected to be representative of children, with and without asthma, in the general population: (1) control group, 106 children who had never wheezed; (2) mild wheezy bronchitis group, 75 children with <5 episodes of wheezing associated with bronchitis or apparent respiratory infection; (3) wheezy bronchitis group, 107 children with ≥5 episodes of wheezing associated with bronchitis or apparent respiratory infection; (4) asthma group, 113 children with wheezing unassociated with apparent respiratory infection on at least 1 occasion; (5) severe asthma group, 83 children with onset of symptoms before 3 years of age and persistence of symptoms at age 10, barrel chest deformity, and/or reduction of FEV₁/forced vital capacity (FVC) ratio to ≤50%.

Phenotypic data

The subjects were followed and reviewed at ages 14, 21, 28, 35, and 42 years.^{30,32} At each review, their atopic status was determined³¹ and lung function parameters were measured. At the 42-year follow-up, blood was taken from each of the available 244 individuals, representative of the 5 subgroups, for genotyping, total IgE measurements, and PHA- and house dust mite (HDM)-induced IFN- γ production.

Informed consent was obtained from all subjects, and the study was approved by the ethics committee of the Royal Children's Hospital, Melbourne.

Atopic status and total serum IgE measurements

At each age of review, subjects were considered atopic if skin test reactivity was present for at least 1 of the allergens tested. The allergens tested were rye grass, cat fur, dog hair, HDM, and egg white, although not all agents were tested at each review. Between the ages of 7 and 14 years, a scratch technique was used; a skin prick test was used for the other follow-ups. Skin test reactivity was considered positive if a wheal diameter of at least 3 mm greater than the wheal diameter of the saline control was obtained. Levels of total IgE, expressed in kilounits per liter, were determined by means of UniCAP total IgE fluoroenzyme immunoassays (Pharmacia and Upjohn Diagnostics).

Lung function

Lung function was measured from spirometry performed on a Jaeger MasterLab (Wurzburg, Germany) according to ATS standards.³⁷ Predicted values were calculated by the equations developed by Quanjer et al.³⁸

IL12B genotyping

All samples were genotyped with DNA extracted from peripheral blood.³⁹ Subjects were genotyped for a previously reported *IL12B* CTCTAA/GC promoter polymorphism²⁸ by PCR amplification of the region containing the polymorphism and subsequent restriction endonuclease digestion of the amplicons. PCR amplicons of 196 bp (allele 1: CTCTAA) and/or 192 bp (allele 2: GC) were generated using the primers 5' TACAGCCTGTCTCCGAGAGAA 3' and 5' GAGGAAGTGGTTCTCGTACTTTAGC 3'. As there is no natural restriction site in either of the alleles, the underlined bases were modified to create *Alu* I sites. PCR reactions were carried out in a total volume of 25 μ L, using approximately 100 ng DNA, 2.5 mmol/L MgCl₂, 200 mol/L dNTPs, 12.5 ng of each primer, and 0.5 units of Taq DNA polymerase (Promega, Madison, Wis) in the PCR buffer provided by the manufacturer (10 mmol/L Tris-HCl, pH 9.0, and 50 mmol/L KCl). PCR involved an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds,

TABLE I. *IL12B* promoter genotype frequencies and asthma severity

Population group at age 7 and age 10 y	Control* (n = 49)	Mild wheezy bronchitis* (n = 35)	Wheezy bronchitis* (n = 55)	Asthma* (n = 63)	Severe asthma† (n = 38)	Total (n = 240)
Male subjects (%)	27 (55%)	17 (49%)	35 (64%)	43 (68%)	29 (76%)	151 (63%)
<i>IL12B</i> genotype						
1.1	12 (24%)	8 (23%)	17 (31%)	14 (22%)	6 (16%)	57 (24%)
1.2	22 (45%)	13 (37%)	30 (54%)	31 (49%)	20 (53%)	116 (48%)
2.2	15 (31%)	14 (40%)	8 (15%)	18 (29%)	12 (31%)	67 (28%)
Population group at age 42 y	Control (n = 49)	No recent asthma (n = 73)	Infrequent asthma (n = 33)	Frequent asthma (n = 42)	Persistent asthma (n = 42)	Total (n = 239)
Male subjects (%)	27 (55%)	49 (67%)	21 (64%)	21 (50%)	32 (76%)	150 (63%)
<i>IL12B</i> genotype						
1.1	12 (24%)	20 (27%)	5 (15%)	12 (28%)	8 (19%)	57 (24%)
1.2	22 (45%)	35 (48%)	13 (39%)	23 (55%)	22 (52%)	115 (48%)
2.2	15 (31%)	18 (25%)	15 (46%)	7 (17%)	12 (29%)	67 (28%)

*Recruitment and assessment at 7 years old.

†Recruitment and assessment at 10 years old.

and extension at 72°C for 45 seconds, with a final extension at 72°C for 7 minutes. Amplification was confirmed by electrophoresing 5 μ L of the PCR product on a 2% agarose gel containing ethidium bromide and subsequent visualization under UV illumination. After amplification, 5 μ L of the amplicon was digested in a 10- μ L reaction containing 1.5 units of *Alu* I (Promega) at 37°C for at least 3 hours. The fragments resulting from the digest were separated on 3% agarose gel and visualized as before. Digestion 24 bp from the 3' end served as an internal control to indicate complete digestion. *Alu* I also digested 22 bp from the 5' end of the amplicon in the presence of allele 1 (CTCTAA) to produce a 150-bp fragment. As allele 2 (GC) lacks the second restriction site and is 4 bp shorter than allele 1, digested amplicons with this allele produce a 168-bp fragment. To confirm genotyping results, a 10% random sample representing all 3 genotypes was sequenced by fluorescence-based automated DNA sequencing, using the ABI Prism BigDye Terminator Kit (PE Biosystems, Foster City, Calif).

IFN- γ production assay

At the 42-year follow-up, HDM-induced (*Dermatophagoides pteronyssinus*, CSL, Melbourne, Australia) and PHA-induced (Wellcome Diagnostics, Dartford, United Kingdom) IFN- γ production from cultured PBMCs was assayed through use of a sandwich ELISA.³⁵

Statistical analysis

Hardy-Weinberg equilibrium was assessed with a goodness-of-fit χ^2 test; χ^2 tests were used to analyze the association between the *IL12B* promoter polymorphism and asthma susceptibility, severity, and atopy. One-way ANOVA and *t* tests were used to assess differences among mean values of the quantitative measures of log total IgE, FEV₁% predicted, and FVC% predicted in groups stratified according to sex or genotype. All calculations were performed with the SPSS10.0 statistical package.

RESULTS

Two hundred forty-four individuals from the Melbourne cohort were genotyped for the *IL12B* promoter polymorphism. These individuals, who were representative of the total cohort, were assigned as having genotype

1.1, 1.2, or 2.2 for those homozygous for allele 1, heterozygous, or homozygous for allele 2, respectively. The frequencies of the 1.1, 1.2, and 2.2 genotypes in this cohort were 24.1%, 48.3%, and 27.6%, respectively, which were similar to the frequencies reported for a cohort of Perth children.²⁸ The distribution of these *IL12B* promoter genotypes did not deviate from the expected Hardy-Weinberg equilibrium ($P = .863$).

There was no association between the *IL12B* genotypes and asthma susceptibility per se, nor was the promoter polymorphism associated with the degree of asthma severity at age 7 (Table I). The distribution of the genotypes from the 38 patients with severe asthma recruited at 10 years of age was similar to that observed in the other groups assessed at 7 years of age (Table I). The proportion of individuals with heterozygous genotypes in this severe group was also not significantly different from those found in the groups with milder forms of asthma. The lack of associations between the genotypes and asthma susceptibility and severity were also observed when the subjects were subsequently reviewed at the age of 42 years (Table I).

There was also a lack of association between the *IL12B* promoter polymorphism and atopy at all the ages of review. Analyses involving IgE levels at age 42 were conducted separately for each sex, since the mean total serum IgE levels were significantly higher in male subjects (geometric mean IgE = 112 kU/L) compared with those in female subjects (geometric mean IgE = 64 kU/L) ($P = .003$). No association was identified between *IL12B* genotypes and total IgE levels within the female subpopulation, but male subjects with a 1.1 or 1.2 genotype had higher IgE levels compared with those with a 2.2 genotype (geometric mean IgE = 133 kU/L versus 80 kU/L; $P = .042$).

There was no association found between the genotypes and FEV₁% predicted at age 7 years ($P = .085$), but a significant association was found with FVC% predicted ($P =$

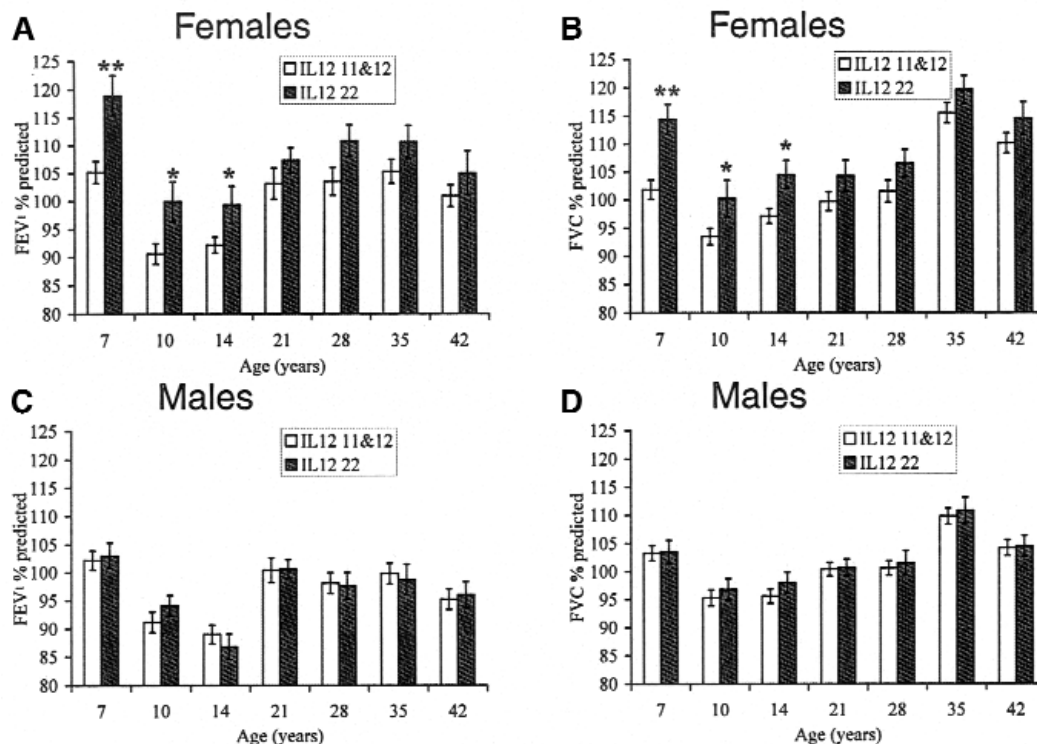


FIG 1. *IL12B* promoter genotype and lung function measurements at different ages of review. **A**, Mean FEV₁% predicted for female subjects; **B**, mean FVC% predicted for female subjects; **C**, mean FEV₁% predicted for male subjects; **D**, mean FVC% predicted for male subjects. ** $P = .001$; * $P < .05$.

.028). When the subjects were stratified according to sex, female subjects were found to have higher mean FEV₁% predicted compared with male subjects (108% versus 102%, $P = .009$) but no significant difference in mean FVC% predicted (105% versus 103%, $P = .46$). With female subjects, significant differences in mean FEV₁% and FVC% predicted ($P = .001$ and $P < .001$, respectively) were detected among the three genotypic groups (Table II). Female subjects with either a 1.1 or 1.2 genotype had lower mean FEV₁% predicted and FVC% predicted at age 7 years compared with those with a 2.2 genotype ($P = .001$ and $.001$, respectively) (Fig 1, A and B). These differences in lung function were also observed at the other ages of review, although significant differences were only detected at ages 10 and 14 (FEV₁% predicted: $P = .025$ and $.029$; FVC% predicted: $P = .036$ and $.007$, respectively). No differences were found in either of the lung function measurements in male subjects with or without allele 1 (Fig 1, C and D).

Further assessment of FEV₁% predicted and FVC% predicted in the children at age 7 showed that the differences between female individuals with either a 1.1 or 1.2 genotype and those with a 2.2 genotype were only significant with the asthmatic subjects, who were defined as belonging to either of the "mild wheezy bronchitis," "wheezy bronchitis," or "asthma" groups ($P = .001$ and $.001$ for FEV₁% predicted and FVC% predicted, respectively) (Table III).

No relationship was found between IFN- γ production, induced by either HDM or PHA, and the *IL12B* promoter genotype.

DISCUSSION

There is no previous report of a study that has investigated the effects of a genetic polymorphism in a longitudinal cohort that has been followed from childhood through to adolescence and middle adulthood. Asthma, its severity, and related phenotypes such as atopy and respiratory function were assessed in relation to their associations with the *IL12B* promoter polymorphism. No association was found between the polymorphism and asthma or its severity. There was also no evidence to support the finding by Morahan et al²⁸ that asthmatic children who were heterozygous for the polymorphism have the greatest risk of progressing to severe asthma. A possible explanation for this discrepancy is population stratification. The patients with severe asthma from the Melbourne population may represent a group that did not have access to the medications that are currently available to asthmatic patients. By today's standards, these children may therefore be deemed as grossly undertreated. If given today's level of medical care, some of these children might have progressed to a milder category. Furthermore, it could be argued that comparisons should not be made between children assessed at age 7 years (the

TABLE II. Mean FEV₁% predicted and FVC% predicted by *IL12B* genotypes and sex among children at age 7 years

<i>IL12B</i> promoter polymorphism	Male subjects			Female subjects		
	n	FEV ₁ % predicted	FVC% predicted	n	FEV ₁ % predicted	FVC% predicted
1.1	31	102 (± 2.6)	104 (± 1.9)	18	111 (± 2.6)	106 (± 2.7)
1.2	50	102 (± 2.3)	103 (± 1.8)	41	103 (± 2.5)	100 (± 2.1)
2.2	35	103 (± 2.3)	104 (± 2.1)	18	119 (± 3.6)	114 (± 2.7)
P value		.96	.91		.001	<.001

Values are expressed as mean ± SEM.

TABLE III. Mean FEV₁% predicted and FVC% predicted by *IL12B* genotypes, stratified by sex and asthmatic status, among children at age 7 years

	<i>IL12B</i> promoter polymorphism	Male subjects			Female subjects		
		n	FEV ₁ % predicted	FVC% predicted	n	FEV ₁ % predicted	FVC% predicted
Nonasthmatic subjects	1.1 or 1.2	12	105 (± 4.3)	103 (± 4.2)	18	113 (± 2.1)	106 (± 2.4)
	2.2	12	107 (± 3.2)	105 (± 2.7)	2	120 (± 7.2)	118 (± 7.5)
P value			.74	.68		.26	.13
Asthmatic subjects	1.1 or 1.2	69	102 (± 1.9)	103 (± 1.4)	41	102 (± 2.6)	100 (± 2.2)
	2.2	23	101 (± 3.1)	103 (± 2.8)	16	119 (± 4.0)	114 (± 3.0)
P value			.87	.82		.001	.001

Values are expressed as mean ± SEM.

control and milder groups) and those assessed at age 10 (the severe group), as asthma severity does change during those years. However, it is highly likely that these children with severe asthma would still represent the most severe cases of the population.

Morahan et al²⁸ also studied gene expression and cytokine production in relation to this *IL12B* promoter polymorphism and found that the heterozygous state to be associated in vitro with reduced gene transcription and decreased functional cytokine secretion. This reported heterozygote effect is an unusual finding that cannot be adequately explained from current understanding, especially since the polymorphism is located in the promoter region of the gene.⁴⁰ Associations for heterozygous individuals are more commonly confined to genotypes that are lethal for those homozygous for one of the alleles.^{41,42} Furthermore, the in vitro conditions might not reflect the in vivo environment. In another study by Morahan et al,⁴³ it was reported that homozygosity for the CTCTAA allele was associated with decreased production of nitric oxide in Tanzanian children and increased mortality rates in those children having cerebral malaria. For the same promoter polymorphism to have a heterozygote effect in one disease state but not another would appear to be highly unlikely.

Female subjects in this study with either a 1.1 or 1.2 genotype had significantly lower lung function measurements compared with those with a 2.2 genotype. When assessed in 7-year-old girls, this difference was only found among the asthmatic subjects. The reduction in lung function in female subjects was of a comparable level in FEV₁ and FVC, suggesting that the effect was on

lung development rather than airway tone. Together, these results suggest that allele 1 has more of an effect on lung function in female asthmatic subjects in early childhood than in other subjects. This difference in lung function in female subjects tracked with age to middle adulthood, although significance was lost after 14 years of age. These findings suggest that the *IL12B* promoter polymorphism may play an important role in determining female prepubertal lung development.

If indeed the CTCTAA allele does reduce the level of functional IL-12 protein production, carriers of this allele could have reduced resistance to microbial and viral infections. Neutralization of the cytokine through use of anti-IL-12 antibodies in respiratory syncytial virus-infected mice resulted in an increase in airway hyperreactivity, mucus production, and airway inflammation.⁴⁴ If a similar situation exists in human beings, reduced IL-12 levels in female asthmatic subjects during infection could impair lung development. Hence, until IL-12 levels measured during an exacerbation or bacterial/viral infection are analyzed in relation to the polymorphism, the functional consequences of the polymorphism cannot be fully explored.

Respiratory function in male subjects was not related to genotype at any of the ages reviewed. This finding is consistent with other studies showing sex differences in genetic susceptibility to diseases. A polymorphism in the β₂-adrenergic receptor gene was associated with the propensity to gain weight from childhood to adulthood only in male subjects,⁴⁵ whereas in a study involving 6- to 7-year-old Spanish children, an apoE polymorphism affected total cholesterol, LDL-C, and apoB levels more in girls than in boys.⁴⁶

The IL-12 promoter polymorphism may have a moderate effect on total IgE levels. Total IgE levels at 42 years were higher in men than in women, consistent with previously reported studies involving both unchallenged⁴⁷ and allergen-challenged⁴⁸ subjects. Male subjects with either a 1.1 or 1.2 genotype had higher levels of total IgE than did those with a 2.2 genotype, although no differences were detected in female subjects.

In a previous study, the patients in this cohort who had persistent and severe asthma that continued into adult life had reduced HDM-induced IFN- γ production,³⁵ which is reflective of impaired T_H1 response. As the *IL12B* promoter is associated with maintenance of IFN- γ transcriptional machinery within the T_H1 effector cells, the relationship between this *IL12B* promoter polymorphism and HDM- and PHA-induced IFN- γ production was examined in the current study. There was, however, no relationship between the level of IFN- γ production, induced by either stimulant, and the *IL12B* promoter genotype.

In conclusion, in a population of asthmatic subjects with extensive longitudinal data, no evidence was found to suggest that those who were heterozygous for the *IL12B* promoter polymorphism had increased asthma severity. The polymorphism was also not associated with asthma prevalence, but an association was noted between the CTCTAA allele and elevated serum IgE levels in male subjects and reduced pulmonary function in female subjects in early childhood.

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