Extended Haplotype in the Tumor Necrosis Factor Gene Cluster Is Associated with Asthma and Asthma-related Phenotypes

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Rationale: **Tumor necrosis factor is a proinflammatory cytokine found in increased concentrations in asthmatic airways. The TNF-α (TNF) and lymphotoxin-α (LTA) genes belong to the TNF gene superfamily located within the human major histocompatibility complex on chromosome 6p in a region repeatedly linked to asthma. The TNF position –308 and LTA *Nco*I polymorphisms are believed to influence TNF transcription and secretion, respectively.** ***Objectives:*** **This study sought to determine whether polymorphisms in TNF or LTA, or in TNF-LTA haplotypes, are associated with asthma and asthma phenotypes.** ***Methods:*** **We genotyped the TNF –308 and LTA *Nco*I polymorphisms, and two other haplotype-tagging polymorphisms in the TNF and LTA genes, in 708 children with mild to moderate asthma enrolled in the Childhood Asthma Management Program and in their parents. Using an extension of the family-based association tests in the PBAT program, each polymorphism was tested for association with asthma, age at onset of asthma, and time series data on baseline FEV_1 % predicted, post-bronchodilator FEV_1 % predicted, body mass index, and log of PC_{20}.** ***Measurements and Main Results:*** **Although no associations were found for the individual single-nucleotide polymorphisms, the haplotype analysis found the LTA *Nco*I_G/LTA 4371T/TNF –308G/TNF 1078G haplotype to be associated with asthma and with all five phenotype groups.** ***Conclusions:*** **We conclude that it is unlikely that the TNF –308 or LTA *Nco*I polymorphisms influence asthma susceptibility individually, but that this haplotype of variants may be functional or may be in linkage disequilibrium with other functional single-nucleotide polymorphisms.**

Keywords: asthma; haplotypes; lymphotoxin-α polymorphism; tumor necrosis factor

Tumor necrosis factor is a proinflammatory cytokine that is found in increased concentrations in asthmatic airways. The TNF-α (TNF) and lymphotoxin-α (LTA) genes are members of the TNF gene superfamily located within the human major histocompatibility complex on chromosome 6p in a region repeatedly linked to asthma (1, 2). As shown in Table 1, association between a position –308 guanine (G)-to-adenine (A) polymorphism in the TNF promoter and asthma has been tested in 18 published studies that included different age groups of individuals with asthma from a range of ethnic backgrounds, and an association between the A allele and asthma was reported in seven of these studies (3–9). In nine additional studies (10–18) listed in Table 1, however, authors reported no association between asthma and this single-nucleotide polymorphism (SNP), and two other studies showed an association between the wild-type allele and asthma (19, 20). The TNF –308A allele is of interest because it is associated with increased *in vitro* transcription of TNF and with increased TNF levels in stimulated human white blood cells (21, 22). As shown in Figure 1, the LTA gene, previously called the TNF-β gene, is located close upstream to the TNF gene. An *Nco*I polymorphism in the LTA gene has also been associated with asthma in children (19) and adults (5), although each study reported that a different allele was associated and 13 other studies (4, 7, 9, 11, 13, 14, 16, 17, 20, 23–26) showed no association (*see* Table 1).

Conflicting findings in previous studies could be explained by the different populations studied, by population stratification, or by small sample sizes in case-control study designs. The TNF –308 and LTA *Nco*I polymorphisms are in strong linkage disequilibrium (5, 19). Moffatt and colleagues (16) have advocated that haplotypes created by the TNF –308 and LTA *Nco*I polymorphisms and surrounding innate immunity genes in the major histocompatibility complex may explain the conflicting results. A Japanese study showed significant transmission disequilibrium with haplotypes in the TNF genes (26). Using a family-based design to control for population stratification in a large cohort of children with mild to moderate asthma, we tested the TNF –308 and LTA *Nco*I polymorphisms, and extended TNF/LTA haplotypes for association with asthma. Some of the results of these studies have been previously reported in the form of an abstract (27).

METHODS

Population

The Childhood Asthma Management Program (CAMP) was a multicenter, randomized, double-masked, placebo-controlled clinical trial evaluating the long-term effects of inhaled antiinflammatory medications in children with mild to moderate asthma (28). The diagnosis of asthma was based on methacholine hyperreactivity (PC_{20} no greater than 12.5 mg/ml) and the meeting of one or more of the following criteria for at least 6 months in the year before recruitment: (*1*) asthma symptoms two times or more per week; (*2*) at least two uses per week of an inhaled bronchodilator; and (*3*) daily asthma medication. *See* the online supplement for a description of spirometry, including methacholine challenge (29), and other testing methods.

Of the 1,041 children participating in the original CAMP study (29), DNA samples were obtained from 968 participating children and 1,518 of their parents. Complete family trios were available for 652 nuclear families that included 708 children. Some families had multiple children with asthma (49 families had two children and two families had three children). The characteristics of these children are shown in Table 2.

Choice of SNPs and Molecular Methods

Choice of SNPs and SNP locations are described in detail in the online supplement, including representation of the SNPs reported as being

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TABLE 1. STUDIES EVALUATING THE ASSOCIATION OF TNF 308 AND LTA *Nco*I POLYMORPHISMS FOR ASTHMA

				TNF –308*‡		LTA *Nco*I‡	
First Author, Year	Reference	Population	Design	G	A	G	A
Moffatt, 1997	5	White, 413 subjects from 88 families: 318 nonasthmatic and 92 with asthma (32 asthmatic parents and 60 children)	Case-control	–	+	+	–
Albuquerque, 1998*	19	White, 74 children with atopic asthma and 50 unrelated control subjects	Case-control	+	–	–	+
Moffatt, 1999†	16	White, 1,004 subjects in 230 nuclear families; 179 were asthmatic (66 parents and 113 children)	Case-control	NS	NS	NS	NS
Li Kam Wa, 1999*	4	Mixed race, 556 subjects tested for bronchial hyperreactivity (246 hyperreactive); also sample of 60 extended families of an asthmatic proband	Case-control	–	+	NS	NS
Chagani, 1999	3	White, 92 with mild–moderate asthma, 159 with fatal/near-fatal asthma, 43 nonasthmatic, 252 random control subjects	Case-control	–	+		
Trabetti, 1999	17	White, 600 families with 131 children with atopic asthma	Sibling-pairs	NS	NS	NS	NS
Winchester, 2000	8	White, 20 subjects with asthma identified by questionnaire for history of childhood asthma and 416 control subjects	Case-control	–	+		
Louis, 2000	15	White adults, 95 subjects with asthma and 98 control subjects	Case-control	NS	NS		
Zhu, 2000	18	White children, 12 with asthma and 269 nonasthmatic	Family based	NS	NS		
Cardaba, 2001	23	Spanish gypsies, 5 families with 87 people	Case-control			NS	NS
Izakovicova, 2001	25	Czech, 243 white with atopy (167 had asthma) and 184 control subjects	Case-control			NS	NS
Immervol, 2001	24	White, 97 families with 2 or more siblings with asthma	Family based			NS	NS
Lin, 2002	14	Taiwanese, 80 subjects with high-IgE asthma and 69 nonasthmatics	Case-control	NS	NS	NS	NS
Noguchi, 2002	26	Japanese, 144 atopic asthma families; transmission disequilibrium tests done	Family based			NS	NS
Witte, 2002	9	White, 169 asthma cases and 170 control subjects	Case-control	–	+	NS	NS
Buckova, 2002	11	Czech, 151 patients with atopic asthma and 155 control subjects	Case-control	NS	NS	NS	NS
Di Somma, 2003	12	Italian, 70 patients with asthma and 169 without asthma from 51 nuclear families	Case-control	NS	NS		
El Bahlawan, 2003	13	Mixed race, children: 38 with asthma and 231 control subjects	Case-control	NS	NS	NS	NS
Sandford, 2004	6	Chinese, 107 children with wheeze in the last 12 mo and 118 without wheeze	Case-control	–	+		
Shin, 2004	20	Korean, 550 with asthma and 171 control subjects	Case-control	+	–	NS	NS
Beghe, 2004	10	142 toluene diisocyanate–induced asthma cases and 50 asymptomatic exposed control subjects	Case-control	NS	NS		
Wang, 2004	7	Taiwanese children, 128 with atopic asthma, 51 with nonatopic asthma, 55 atopic control subjects, and 78 nonatopic control subjects	Case-control	–	+	NS	NS

Definition of abbreviations: LTA = lymphotoxin-α; NS = not significant; TNF = tumor necrosis factor.

* Positive association found in individuals homozygous for the allele.

† The Moffatt and coworkers (1999) study (16) includes the same population as the Moffatt and Cookson (1997) population (5).

‡ A significant ($p \leq 0.05$) association, with the + allele increased and the – allele decreased in the subjects with asthma; NS, no significant differences found.

significantly overtransmitted to Japanese individuals with atopic asthma (26). Briefly, haplotypes were identified with the PHASE program (30) and this output was analyzed by the BEST program (31) to identify haplotype-tagging SNPs. As shown in Table 3, the four SNPs chosen for genotyping should have been able to distinguish all of the common (more than 7%) haplotypes identified in Europeans. SNP genotyping was performed with the SEQUENOM system (*see* the online supplement for a detailed description).

Statistical Analysis

The PedCheck program was used to assess the genotype data for pedigree errors (32). Hardy-Weinberg equilibrium was tested in the parental data for each locus, using the χ^2 goodness of fit test in SAS (version 8.1; SAS Institute, Cary, NC).

The primary analysis was for association of individual SNPs and haplotypes with asthma, applying the PBAT (33) program to the entire sample for the SNP analysis and to white individuals for the haplotype analysis. In the PBAT program the recessive model was used because that model had optimal power, as has been shown in previous studies (34, 35). The secondary analysis was to test each individual SNP/haplotype for association with a time series of asthma-related quantitative phenotypes, using the PBAT program (36). The time series data were obtained during multiple visits (before, at, and after study entry) to children in the CAMP population (*see* the online supplement for details). We tested the age at onset of asthma and the following four-time series: body mass index (BMI), logarithm of the provocative concentration of methacholine causing a 20% fall in FEV_1 from baseline ($LNPC_{20}$), baseline FEV_1 % predicted (Pre-FEV), and postbronchodilator FEV_1 % predicted (Pos-FEV). The time series data were tested with the FBAT-PC statistic for repeated measurements (36) and the time-to-onset data were tested with FBAT-LOGRANK (37). Both tests are available in PBAT for SNP and haplotype analysis.

Pairwise linkage disequilibrium between each pair of SNP loci was evaluated by a maximum likelihood method to infer phases for dual heterozygotes, expressed as r^2.

RESULTS

Genotyping Results and Haplotype Evaluation

Genotypes for families with pedigree check errors were set to zero. The number of PedCheck errors for each SNP were as follows: LTA *Nco*I (23), LTA 4371 (9), TNF –308 (3), and TNF 1078 (3). Genotyping success rates were 97% or greater for each SNP. Hardy-Weinberg equilibrium was confirmed for all loci in the parents (all p values were greater than 0.1). There was significant linkage disequilibrium between the TNF –308 and LTA *Nco*I SNPs ($r^2 = 0.424$). The LTA *Nco*I SNP and the LTA 4371 SNP had an r^2 value of 0.172, and the TNF 282 SNP and LTA 4371 SNP had an r^2 value of 0.083. The r^2 values for all other SNP pairs were less than 0.04.

There were five four-SNP haplotypes represented in the sample at 5% or greater frequency in any ethnic group. The common haplotypes represented in the sample were the same as those with 5% or greater frequency reported in Table 3 for the Centre d'Etude du Polymorphisme Humain European sample.

Family-based Association Analysis of the TNF SNPs and Haplotypes with Asthma

As shown in Table 4, the A allele for the TNF –308 SNP was not overtransmitted to children with asthma from their parents. In contrast to previous reports of an increased frequency of the

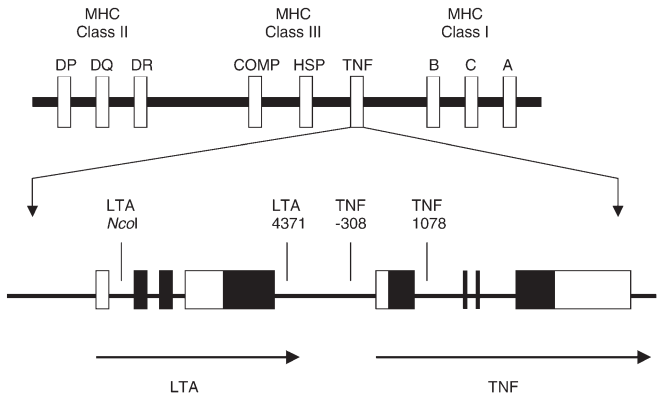

Figure 1. *Top*: Schematic representation of the TNF genes on chromosome 6 in relation to other important innate immunity genes in the area. TNF includes lymphotoxin-α (LTA), tumor necrosis factor-α (TNF), and lymphotoxin-β (LTB). COMP = complement; HSP = heat shock 70-kD protein-1 and 90-kD protein-1; MHC = major histocompatibility complex (human leukocyte antigens DP, DQ, DR); SNP = single-nucleotide polymorphism. *Bottom*: TNF and LTA with SNP locations; the *solid boxes* denote exons and the *open boxes* denote the 5′ and 3′ untranslated regions. Not drawn to scale. (The *top portion* of this diagram was modified from Reference 39.)

A allele in the asthmatic cohorts, we found a trend toward undertransmission of the A allele in the entire cohort. using additive or recessive models. There was also a trend for the G allele of the LTA *Nco*I polymorphism to be overtransmitted, but the p value (less than 0.05) was not corrected for multiple comparisons.

We restricted the extended haplotype analysis to white subjects. As shown in Table 5, there was an increase in transmission of the LTA *Nco*I_G/LTA 4371T/TNF –308G/TNF 1078G (GTGG) haplotype, using the recessive model (GTGG = 167 vs. other haplotypes = 146; relative risk, 2/0 copies GTGG = 3.1 and 1/0 copy GTGG = 2.75, p = 0.05). The p values shown in Table 5 are corrected for multiple comparisons. Table 5 also shows the results of the haplotype analysis for the GTGG haplotype for asthma-related phenotypes. The GTGG haplotype was associated with later age of onset of asthma based on the Kaplan-Meier plot (Figure 2). The GTGG haplotype was also associated with BMI, $LNPC_{20}$, Pre-FEV, and Pos-FEV, using the time-series phenotype data.

TABLE 2. CHARACTERISTICS OF POPULATION

	CAMP Children with Asthma	
	All (*n* = *708*)	White Subjects Only (*n* = *432*)
Sex, % male	59.4	58
Age, yr	8.07 (SD, 2.1)	8.84 (SD, 2.1)
Ethnicity, %		
Non-Hispanic white	73.4	100
African American	9.7	NA
Hispanic	7.5	NA
Other	9.2	NA
Baseline FEV_1, % predicted	95.1 (SD, 13)	96.4 (SD, 14)
Age of asthma onset, yr		
Mean	3.07	3.03
Median, IQR	2.5, 1.0–4.5	2.5, 1.0–4.0
BMI at first visit		
Mean	18.2	17.7
Median, IQR	16.6, 15.6–19.8	17.7, 15.6–19.1
Log of serum IgE, total	2.62	2.56

Definition of abbreviations: BMI = body mass index; CAMP = Childhood Asthma Management Program; IQR = interquartile range; NA = not applicable.

DISCUSSION

Our data indicate that the TNF –308 and LTA *Nco*I polymorphisms do not influence asthma susceptibility individually, but that an extended haplotype in the TNF and LTA genes is associated with asthma susceptibility and with multiple common asthma phenotypes. In contrast to seven published case-control studies that showed an increased frequency of the TNF –308A allele in individuals with asthma (3–9), we instead found a strong trend toward an increased frequency of transmission from parents of the G allele to their child with asthma, consistent with other investigators in two case-control studies (19, 20). The TNF and LTA genes are located in the major histocompatibility complex on chromosome 6 between many innate immunity genes (*see* Figure 1). We believe that previous linkage with asthma in this region may be explained by extended haplotypes across the TNF and LTA genes.

Our study has many strengths. We used a family-based genetic study design including a large number of parent–child trios. Our population included more subjects with asthma than have been previously enrolled in other published studies evaluating SNPs in TNF and LTA genes (*see* Table 1). All children had mild to moderate asthma and extensive asthma phenotype data had been collected longitudinally (29). Genotyping success rates were high and we were able to infer haplotypes from family-based data at a high rate.

Our study is limited by the number of SNPs that were genotyped. Because our population was largely white, we chose SNPs to replicate findings in published reports and chose additional SNPs to ensure good representation of common haplotypes. We cannot rule out the possibility that linkage disequilibrium between polymorphisms in the asthmatic population may be different from those in the nonasthmatic population. This is unlikely, however, because our four-SNP haplotype results are consistent with those reported for the nonasthmatic European Centre d'Etude du Polymorphisme Humain sample. With the four-haplotype–tagging SNPs we chose, we were able to test the majority of common haplotypes across the TNF and LTA genes for association with asthma.

Noguchi and colleagues (26) reported an association between a TNF polymorphism and asthma susceptibility in a population of Japanese children with atopic asthma in 144 families. They reported increased transmission of the C allele of the TNF –857C/T SNP to children with asthma in 54 informative families. Further evaluation of haplotypes showed significantly increased transmission of the TNF –1031T/–857C (our SNPs LTA 4371T/TNF 1078G) haplotype in 74 informative families. Although we substituted the TNF 1078 SNP for their SNP labeled –857, these two SNPs are reported to be in 100% linkage disequilibrium in nonasthmatic white individuals. The TNF –308 SNP is not found in the Japanese population (26). Our results do not contradict the finding of Noguchi and colleagues (26), but our haplotypes are more extended. The TNF distal promoter haplotype they reported is broken into subhaplotypes in our mostly white sample, and we had a much larger population of individuals with asthma with many more informative families.

Whether the TNF –308 locus modulates the levels of tumor necrosis factor transcription is controversial. To resolve this controversy, Knight and colleagues (38) used chromatin immunoprecipitation and mass spectrometry (haplo-CHIP analysis) to identify differential protein–DNA binding *in vivo* associated with allelic variants of the TNF and LTA genes. Using Epstein-

TABLE 3. LYMPHOTOXIN-α AND TUMOR NECROSIS FACTOR HAPLOTYPES AND HAPLOTYPE TAGGING SINGLE-NUCLEOTIDE POLYMORPHISMS FOR EUROPEANS IN THE CEPH SAMPLE

Haplotype No.	LTA 559	LTA 2202	**LTA *Nco*I**	LTA 2820	LTA 3842	**LTA 4371**	LTA 4539	**TNF –308**	TNF 352	**TNF 1078**	Haplotype Frequency
1	T	A	**A**	A	A	**T**	C	**G**	G	G	11 (0.24)
2*	A	C	A	C	A	C	A	G	G	G	7 (0.15)
3	A	C	**G**	A	A	T	C	**G**	G	G	7 (0.15)
4	T	C	G	A	A	T	C	**A**	G	G	5 (0.11)
5	T	A	A	A	A	T	C	G	G	**A**	4 (0.09)
6*	T	C	A	A	A	C	C	G	A	G	3 (0.07)
											37 (0.80)
		Alternative SNPs†									
		LTA 1829 LTA 2490	LTA 2132 LTA 2847		LTA 1071 TNF 2642 TNF 4013	LTA 2619	LTA 306 LTA 1828	TNF 4101	TNF 1009 TNF 1440 TNF 1893 TNF 4040 TNF 4366	LTA 4545* TNF 4765	

Definition of abbreviations: CEPH = Centre d'Etude du Polymorphisme Humain; LTA = lymphotoxin-α; SNP = single-nucleotide polymorphism; TNF = tumor necrosis factor.

Included are all haplotypes at 5% or greater frequency in the European population. SNPs in boldface were genotyped. SNP numbering is as reported by SeattleSNPs, NHLBI Program for Genomic Applications, University of Washington–Fred Hutchinson Cancer Research Center (Seattle, WA), except for LTA 2374 = LTA *Nco*I and TNF 282 = TNF –308 (URL: http://pga.mbt.washington.edu; accessed July 2005).

* Haplotypes 2 and 6 cannot be distinguished from each other. Haplotypes 1, 3, 4, and 5 can be identified, using the SNPs highlighted in boldface.

† Alternative SNPs were in 100% linkage disequilibrium for all haplotypes reported, accounting for any haplotype that occurred in at least two individuals.

Barr virus–transformed B-cell lines, they evaluated RNA polymerase II loading and tumor necrosis factor mRNA expression. Their haplo-CHIP analysis of the –308 TNF polymorphism did not find differential expression of tumor necrosis factor by the two alleles of the SNP. They then extended their haplotypes to include SNPs across the LTA gene. They identified only three major haplotypes across these two genes. Two of the haplotypes had increased transmission of tumor necrosis factor (TNF –1031T_TNF–308A_LTA *Nco*IG and TNF –1031C_TNF –308G _LTA *Nco*IA) in comparison with the third haplotype (TNF –1031T_TNF –308G_LTA *Nco*IA). These haplotypes represent the following respective haplotypes in Table 3: haplotype 4, haplotype 2 or 6, and haplotype 1 or 5. Haplotype 3 (Table 3), which we found to be positively associated with asthma, was not represented in their analysis. Extrapolation from the study by Knight and colleagues (38) is limited because three common haplotypes found in asthmatic and nonasthmatic subjects are missing from their analysis. However, it is clear from their study that the TNF –308 SNP does not upregulate tumor necrosis factor levels and is not likely to be the important SNP as previously hypothesized. We were not able to test whether haplotype 3 (Table 3) had a regulatory effect on tumor necrosis factor levels.

In a large population of children with mild to moderate asthma, we have shown that the TNF –308 and LTA *Nco*I loci are not associated with asthma susceptibility or asthma phenotypes. Our results are consistent with the majority of other case-control and family-based studies shown in Table 1 that failed to find an association between asthma and these two loci. One haplotype across the TNF and LTA genes was found to be associated with asthma susceptibility and with asthma phenotypes in our cohort. Future studies of the association of polymorphisms in the TNF and LTA genes should evaluate extended haplotypes across the TNF and LTA genes and across other nearby genes in the major histocompatibility complex (5).

TABLE 4. FAMILY-BASED ASSOCIATION TEST OF TUMOR NECROSIS FACTOR SINGLE-NUCLEOTIDE POLYMORPHISMS FROM PARENTS TO CHILDREN WITH ASTHMA IN THE CAMP COHORT: RECESSIVE MODEL

Marker	Allele	Allele Frequency	Informative Families*	p Value
CAMP cohort				
LTA *Nco*I	A	0.657	337	0.912
	G	0.343	184	0.048
LTA 4371	C	0.208	83	0.257
	T	0.792	303	0.585
TNF –308	A	0.141	48	0.744
	G	0.859	246	0.068
TNF 1078	A	0.094	23	0.251
	G	0.906	163	0.125
White subjects only				
LTA *Nco*I	A	0.646	260	0.775
	G	0.354	134	0.101
LTA 4371	C	0.206	62	0.676
	T	0.794	195	0.382
TNF –308	A	0.128	38	0.855
	G	0.872	195	0.252
TNF 1078	A	0.085	17	0.261
	G	0.915	121	0.686

Definition of abbreviations: CAMP = Childhood Asthma Management Program; TNF = tumor necrosis factor.

* Informative families = at least one parent heterozygous for the allele.

TABLE 5. HAPLOTYPE ANALYSIS RESULTS* FOR ASTHMA AND ASTHMA-RELATED PHENOTYPES†

Phenotype	Test Statistic	p Value‡	h
Asthma	HBAT	0.05	—
Later age of onset	HBAT-LOGRANK	0.007	—
BMI	HBAT-PC	0.025	0.039
$LNPC_{20}$	HBAT-PC	0.014	0.037
Pre-FEV	HBAT-PC	0.019	0.062
Pos-FEV	HBAT-PC	0.044	0.054

Definition of abbreviations: h = percentage of phenotypic variance explained; BMI = body mass index; $LNPC_{20}$ = logarithm of the provocative concentration of methacholine causing a 20% fall in FEV_1 from baseline; Pre-FEV = baseline FEV_1 % predicted; Pos-FEV = postbronchodilator FEV_1 % predicted.

* GTGG = LTA *Nco*I_G/LTA 4371_T/TNF –308_G/TNF 1078_G haplotype.

† Restricted to white individuals in the Childhood Asthma Management Program.

‡ p values are corrected for multiple comparisons.

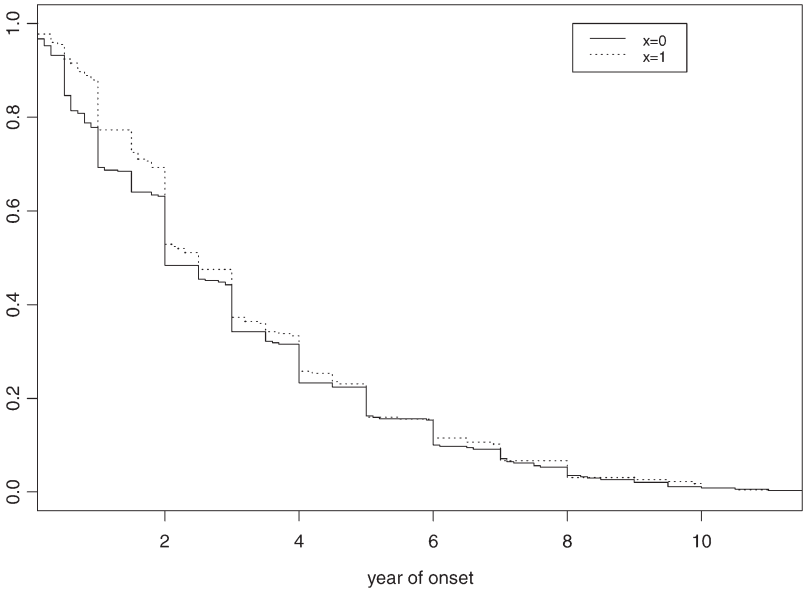

Figure 2. Kaplan-Meier curve of reported asthma age of onset by the presence (x = 1) or the absence (x = 0) of the LTA *Nco*I_G/LTA 4371T/TNF –308G/TNF 1078G haplotype. A statistically significant association for later age of asthma onset in carriers of the haplotype was found (p = 0.007).

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