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ORIGINAL PAPER

Atopy and new-onset asthma in young Danish farmers and CD14, TLR2, and TLR4 genetic polymorphisms: a nested case–control study

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Summary

Background Evidence exists that exposure to high levels of microbial agents such as endotoxin in the farm environment decreases the risk of atopic sensitization. Genetic variation in innate immunity genes may modulate the response to microbial agents and thus influence susceptibility to asthma and atopy.

Objective To study potential associations between single nucleotide polymorphisms (SNPs) in CD14, Toll-like receptor 2 (TLR2), and TLR4 genes, and atopy and new-onset asthma in young farmers.

Methods A nested case–control study was conducted within a cohort of 1901 young Danish farmers. We genotyped 100 new-onset asthma cases and 88 control subjects for three CD14 SNPs, three TLR2 SNPs, and two TLR4 SNPs. Atopy at baseline (defined as a positive skin prick test to one or more common inhalant allergens) was found in 17 asthma cases (17.0%) and in 17 controls (19.3%).

Results The CD14/−260T allele was significantly associated with less atopy [odds ratio (OR) 0.39; 95% confidence interval (CI) 0.21–0.72, additive genetic model], whereas the CD14/−651T allele was positively associated with atopy (OR 2.53; 95% CI 1.33–4.80). Similar results were obtained by haplotype analysis. Stratified analysis by farm childhood showed stronger effects of both CD14 SNPs on atopy among farmers who were born and raised on a farm, although no significant interaction was found. No associations between CD14, TLR2, or TLR4 genotypes and new-onset asthma were found.

Conclusion The CD14/−260 and CD14/−651 promoter polymorphisms are associated with atopy prevalence among young adults exposed to farm environments.

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Introduction

Evidence exists that exposure to high levels of microbial agents in the farm environment decreases the risk of atopic sensitization in children and adults [1–5]. Although the precise immunological mechanism is yet to be elucidated, it has been hypothesized that increased microbial exposure induces a shift from atopic T-helper type 2 (Th2) responses to Th1-dominated responses through stimulation of the innate immune system. In addition, it has been suggested that regulatory T cells play a role in suppressing allergic and non-allergic immune responses. Toll-like receptors (TLRs) present on the cell surface of innate immune cells recognize microbial motifs called pathogen-associated molecular patterns (PAMPs). In response to interaction of lipopolysaccharide (LPS) as well as other PAMPs with TLRs, IL-12 and IFN-γ are produced, which induce a Th1 response [6, 7]. The capacity of PAMPs to induce inflammation may also result in adverse respiratory health effects including non-atopic asthma, accelerated lung function decline, and organic dust toxic syndrome [7–10].

Variation in innate immunity genes such as TLR2, TLR4, or CD14 may modulate responsiveness to LPS and other PAMPs and thus play a role in the development of atopy and respiratory disease. TLR4 functions predominantly, if not exclusively, as a receptor for LPS whereas TLR2 is involved in the recognition of multiple products of Gram-positive organisms, mycobacteria, and yeast [11]. CD14 is the receptor that binds LPS and transfers it to TLR4, thus forming the CD14–TLR4 complex. Arbour et al. [12] demonstrated an association between the Asp299Gly polymorphism in TLR4 and a blunted response to inhaled endotoxin in humans. However, subsequent studies on TLR4 polymorphisms as a risk factor for atopy or asthma in adults or children have shown conflicting results [13–17]. Carriage of the wild-type TLR2/−16934T allele was found to be associated with less asthma and allergies among German and Austrian farmers' children, whereas such an effect was absent in non-farmers' children [15]. A single nucleotide polymorphism (SNP) in the promoter region of CD14 (C −260T, also reported as C −159T) has been associated with increased levels of soluble CD14 (sCD14) [18, 19], and with lower levels of total serum IgE or atopic sensitization in farming and non-farming populations [17, 18, 20, 21]. Moreover, functional studies revealed increased transcriptional activity of the T allele [22]. On the other hand, other investigators did not find an association between CD14/−260 and atopy or allergic disease, or obtained opposite results [19, 23–26]. Farming is associated with high levels of exposure to a wide range of microrganisms and PAMPs [27]; therefore we hypothesized that the susceptibility to asthma and atopy in farmers might be associated with polymorphisms in innate immunity genes. We carried out a nested case–control study to explore associations between atopy and new-onset asthma, and CD14, TLR2, and TLR4 genetic polymorphisms in young farmers from the Danish SUS cohort [28].

Methods

Study population and design

In 1992–1994, a cohort of 1901 Danish farming school students (1691 men and 210 women, mean age 19.2 years, range 16–26 years, response rate 81%) was recruited into a longitudinal study to investigate the incidence of asthma in relation to farm exposures. Details of the baseline population have been reported previously [4, 28, 29]. Within this cohort, we conducted a nested case–control study. Each consecutive year, up to 1999, all participants received a questionnaire, and subjects with new onset of respiratory symptoms were contacted by phone for an interview. Those who fulfilled the questionnaire criteria for new-onset asthma were invited to a clinical evaluation together with a randomly selected asthma-free control subject [30, 31]. For each new case, one control subject was selected by incidence density sampling to obtain odds ratios (ORs) that are unbiased estimates of the relative risk. Among the young farmers, 106 cases of new asthma were identified, and 102 controls were included at the same time. DNA samples were available for 100 cases and 88 controls. The study was approved by the ethics committee and all participants gave written consent.

Health outcomes

The clinical evaluation included an interview that consisted of questions about onset of asthma symptoms, smoking habits, and occupational history. Asthma was diagnosed if the subjects answered yes to at least one of the group A questions and two of the group B questions in Table 1 [28]. A skin prick test (SPT) was performed at baseline with a panel of eight common inhalant allergens; house dust mite, cat, dog, pollen from grass (mix of five species), birch, mugwort (*Artemisia*), and moulds (*Alternaria alternata* and *Cladosporium herbarum*) (Soluprick, ALK, Copenhagen, Denmark). The panel was extended with six 'farm-related' allergens from three different storage mites (*Acarus siro*, *Tyrophagus putrescentia*, and *Lepidoglyphus destructor*): cow, pig, and horse [4, 28]. As we wanted to compare our results with other studies, we focused mainly on the common allergens. Atopy was defined as a positive SPT to one or more of the eight common inhalant allergens (weal diameter of at least 3 mm).

Genotyping

We selected two SNPs in the 5′ flanking region of the CD14 gene (C −651T and C −260T), and a SNP in the 3′

Table 1. Questions on asthma

Group A	Group B
Have you been told by a doctor that you have asthma?	Do you ever have chest tightness?
Do you have asthma?	Do you wake in the morning with chest tightness?
Have you ever had asthma?	Do you wake in the night with wheeze?
Do you ever wheeze?	Do you cough when you wake up in the morning?
	Do you wake in the morning with cough?
	Do you wheeze by exposure to cold air?
	Do you wheeze when you exercise?
	Do you wheeze by exposure to pollen?
	Do you wheeze by exposure to animals?
	Do you use asthma drugs?

Table 2. Identification numbers and primer sequences (SSP-PCR) for the identification of biallelic single nucleotide polymorphisms in the CD14, TLR2, and TLR4 genes

Polymorphism	dbSNP*	Specific primer	Consensus primer
CD14/−651 C/T	rs5744455	†	
CD14/−260 C/T	rs2569190	5′CAGAATCCTTCCTGTTACGG C/T	5′CTGAGGTTCGGAGAAGTTGC
CD14/+1342 C/A	rs2563298	†	
TLR4 Asp299Gly A/G	rs4986790	†	
TLR4 Thr399Ile C/T	rs4986791	5′TCTCAAAGTGATTTTGGGACAA C/T	5′GAGAGAGGTCCAGGAAGGTC
TLR2/−16934 A/T	rs4696480	5′ATTGAAGGGCTGCATCTGG A/T	5′GTGTGCCCCAAAGCTCATG
TLR2 Pro631His C/A	rs5743704	5′CTGCTGGGAGCTTTCCTG G/T	5′AGCAAGCACTGGCCAAAGTCT
TLR2 Arg753Gln C/T	rs5743708	5′AGGTCTTGGTGTTCATTATCTTC T 5′GGTCTTGGTGTTCATTATCTTC C	5′ATGATGTGGGCCTGGCTC

*The SNP loci were identified using accession numbers according to the SNP database at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp
†SNPs determined using TaqMan® SNP genotyping assay.
SSP, sequence-specific primer; TLR, toll-like receptor; SNP, single nucleotide polymorphism.

untranslated region of the CD14 gene (C +1342A). CD14 polymorphisms C −651T and C −260T are also reported as C −550T and C −159T in the literature. For the TLR4 gene, we genotyped the Asp299Gly and Thr399Ile polymorphisms. In addition, three SNPs in the TLR2 gene were selected: A −16934T, Pro631His, and Arg753Gln. DNA was extracted from whole-blood samples using standard procedures. CD14/−260, TLR4 Thr399Ile, and the three TLR2 SNPs were determined using sequence-specific primer (SSP) and PCR [32]. Primers were obtained from Sigma Aldrich (Zwijndrecht, the Netherlands). The identification numbers of the SNP loci and the sequences of SNP-specific primers with their complementary consensus primers are shown in Table 2. The other SNPs (CD14/−651, CD14/+1342, and TLR4 Asp299Gly A/G) were determined using TaqMan® SNP Genotyping Assays using real-time PCR (7500 FAST; Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands).

Statistical analysis

Haplotypes were inferred using phase, version 2.1 with a 90% probability threshold [33, 34]. Data were analysed using SAS statistical software V.8.2. Differences in genotype frequencies or descriptive characteristics between case and control groups were determined by χ^2 test or by Fisher's exact test when the expected count for any cell was less than five. Allele frequencies were tested for Hardy–Weinberg equilibrium by the χ^2 test. Univariate and multiple logistic regression analyses were used to further study associations between health outcomes and genotypes. Genotypes were coded as dummy variables using wild-type homozygotes as a reference group. In addition, an additive genetic model was considered by categorizing genotypes into a 3-level variable for the number of minor alleles [35]. In the haplotype analysis, calculations were based on the number of copies of the haplotype (0,1,2), assuming an additive model [36]. The most frequent haplotype was used as the reference. When atopy was studied as an outcome variable, data were analysed as cross-sectional data. In order to do so, ORs and 95% confidence intervals (CIs) were adjusted for the original case–control status to prevent confounding by the selection process. Potentially confounding factors such as being born and raised on a farm, and smoking habits were controlled for. A P-value <0.05 was considered statistically significant.

Results

Table 3 summarizes general characteristics and genotype frequencies of CD14, TLR4, and TLR2 among new asthma cases and controls. No differences in genotype frequencies were found between asthma cases and control subjects for any of the CD14, TLR4, and TLR2 SNPs. Atopy as well as a positive SPT to one or more farm-related allergens were found as frequently in new asthma cases as in control subjects. In 18 individuals (11 cases and seven controls) a positive SPT to one or more farm-related allergens did not coincide with common atopy. Stratified analysis for non-atopic subjects also did not show differences in genotype frequencies between asthma cases and controls. Allele frequencies for each SNP were in Hardy–Weinberg equilibrium ($P>0.1$). Pairwise linkage disequilibrium (LD, expressed as r^2) between the SNPs in the CD14 gene was as follows: −651 and −260, 0.24; −260 and +1342, 0.34; −651 and +1342, 0.14. High LD (0.74) was found between the two studied TLR4 SNPs. LD between the SNPs in the TLR2 gene was as follows: −16934 and Pro631His, 0.05; Pro631His and Arg753Gln, 0.00; −16934 and Arg753Gln, 0.03.

When atopy was considered as an outcome variable, the CD14/−260T allele appeared significantly associated with less atopy, whereas the CD14/−651T allele showed a significant positive association with atopy (Table 4). Potential confounders (being born and raised on a farm,

Table 3. General characteristics and CD14, TLR4, and TLR2 genotypes among 188 young Danish farmers by asthma status

	Control subjects ($n = 88$)	Asthma cases ($n = 100$)	P
Male	79 (89.8)	85 (85.0)	0.3
Current smokers	23 (26.1)	57 (57.0)	<0.001
Born and raised on a farm*	39 (47.6)	24 (29.6)	0.02
⩾1 positive SPT, common allergens (atopy)	17 (19.3)	17 (17.0)	0.7
⩾1 positive SPT, farm-related allergens	16 (18.2)	19 (19.0)	0.9
Genotype†			
CD14/−651 (rs5744455)			0.7
CC	52 (59.1)	53 (53.0)	
CT	33 (37.5)	43 (43.0)	
TT	3 (3.4)	4 (4.0)	
CD14/−260 (rs2569190)			0.7
CC	28 (29.6)	34 (34.0)	
CT	47 (53.4)	47 (47.0)	
TT	15 (17.1)	19 (19.0)	
CD14/+1342 (rs2563298)			0.6
CC	40 (45.5)	52 (52.0)	
CA	38 (43.2)	37 (37.0)	
AA	10 (11.4)	11 (11.0)	
TLR4 Asp299Gly (rs4986790)			0.8
AA	78 (89.7)	91 (91.0)	
AG	9 (10.3)	8 (8.0)	
GG	0 (0.0)	1 (1.0)	
TLR4 Thr399Ile (rs4986791)			0.2
CC	77 (88.5)	93 (93.0)	
CT	10 (11.5)	6 (6.0)	
TT	0 (0.0)	1 (1.0)	
TLR2/−16934 (rs4696480)			0.3
AA	24 (27.6)	22 (22.0)	
AT	41 (47.1)	58 (58.0)	
TT	22 (25.3)	20 (20.0)	
TLR2 Pro631His (rs5743704)			0.3
CC	76 (87.4)	92 (92.0)	
CA	11 (12.6)	8 (8.0)	
TLR2 Arg753Gln (rs5743708)			0.2
CC	80 (92.0)	97 (97.0)	
CT	7 (8.0)	3 (3.0)	

Data are presented as numbers with percentages in parentheses.
*Information on farm childhood is available for 82 control subjects and 81 asthma cases.
†Information on TLR4 and TLR2 genotype is available for 87 control subjects.
SPT, skin prick test; TLR, toll-like receptor.

smoking, and case–control status) had ORs close to one in multiple logistic regression models, and adjusted ORs showed comparable results (Table 4). ORs for both SNPs remained significant in a multiple regression model that included both CD14/−260 and CD14/−651 as independent variables [additive genetic model for the minor T alleles; OR 0.45; 95% CI (0.21–0.97), and OR 2.42; 95% CI (1.09–5.37), respectively]. Similar patterns were observed when one or more positive SPT for farm-related allergens was used as an outcome variable; however, these associations were not significant [additive genetic model for the minor T alleles; CD14/−260, OR 0.59; 95% CI (0.32–1.06), and CD14/−651, OR 1.91; 95% CI (0.95–3.82)].

When the same analyses for CD14/−260 were run separately for farmers with and without farm childhood, we found an OR of 0.21 in farmers who were born and raised on a farm (additive genetic model for the T allele; 95% CI 0.06–0.73) compared with an OR of 0.45 in farmers without farm childhood (95% CI 0.20–1.03). Accordingly, stratified analysis for CD14/−651 showed a stronger positive association in farmers with farm childhood (OR 7.10; 95% CI 1.55–32.59), compared with farmers without farm childhood (OR 2.43; 95% CI 1.01–5.83). Although we did not find a significant interaction between CD14 genotypes and farm childhood, these ORs are suggestive of a stronger effect on atopy of CD14 genotypes in farmers who were born and raised on a farm. Separate analyses for asthma cases and control subjects showed a somewhat stronger effect of the CD14/−260T allele in cases (OR 0.36; 95% CI 0.06–0.73) compared with controls (OR 0.43; 95% CI 0.18–1.02), whereas for CD14/−651, the effect was more pronounced in controls (OR 4.20; 95% CI 1.57–11.21) compared with cases (OR 1.62; 95% CI 0.67–3.91); however, no significant interactions were found between CD14 genotypes and case–control status. TLR4 and TLR2 genotypes were not associated with atopy (data not shown).

Haplotypes were constructed to evaluate the respective contribution of the three CD14 SNPs on atopy. Four haplotypes were obtained for the CD14 gene, of which three were common and one was found in only six subjects (Table 5). The risk of atopy was significantly higher in carriers of haplotype 2 and 3, as compared with haplotype 1, which contained CD14/−260T and CD14/−651C. Haplotype 3, which contained CD14/−651T, was associated with the highest risk of atopy.

Discussion

In this nested case–control study, we found that the CD14/−260T allele was significantly associated with reduced prevalence of atopy, whereas the CD14/−651T allele was associated with increased atopy. Results of the haplotype analysis confirmed the role of CD14/−260 and CD14/−651 on atopy in our study population. The two SNPs were not in strong linkage disequilibrium, and they were independently associated with atopy in a multiple regression analysis. Unlike CD14/−260, studies taking into account CD14/−651 are scarce and haplotypes including both SNPs have not been reported before as a risk factor for atopy.

Table 4. Associations of the CD14/−260 and −651 promoter polymorphisms and atopy (a positive SPT to one or more common allergens)

	SPT−*n* (%)	SPT+*n* (%)	OR (95% CI)	*P*	Adjusted OR (95% CI)*	*P*
CD14/−260						
CC	43 (27.9)	17 (50.0)	1.0		1.0	
CT	78 (50.7)	16 (47.1)	0.51 (0.24–1.12)	0.09	0.42 (0.17–1.02)	0.05
TT	33 (21.4)	1 (2.9)	0.08 (0.01–0.60)	0.01	0.08 (0.01–0.63)	0.02
T allele†			0.39 (0.21–0.72)	0.003	0.35 (0.17–0.69)	0.003
CD14/−651						
CC	92 (59.7)	13 (38.2)	1.0		1.0	
CT	59 (38.3)	17 (50.0)	2.07 (0.93–4.59)	0.07	3.51 (1.38–8.94)	0.009
TT	3 (2.0)	4 (11.8)	9.63 (1.93–48.21)	0.006	11.13 (1.87–66.19)	0.008
T allele†			2.53 (1.33–4.80)	0.005	3.42 (1.63–7.17)	0.001

*OR adjusted for farm childhood, smoking habits, and case–control status. Information on farm childhood is available for 136/154 non-atopic subjects and 27/34 atopic subjects.

†In the additive genetic model, the genotype was categorized into a three level variable for the number of minor alleles (0,1,2).

CI, confidence interval; OR, odds ratio; SPT, skin prick test.

Table 5. CD14 haplotype frequencies in 34 atopic and 154 non-atopic farmers and association of CD14 haplotypes with atopy

				Haplotype frequency, *n* (%)			
Haplotype	−651	−260	+1342	SPT−	SPT+	OR (95% CI)*	*P*
1	C	T	C	144 (47)	18 (26)	1.0 (reference)	
2	C	C	A	93 (30)	24 (35)	2.24 (1.14–4.41)	0.02
3	T	C	C	65 (21)	25 (37)	4.03 (1.83–8.88)	<0.001
4	C	C	C	6 (2)	1 (1)	1.14 (0.15–9.01)	0.90

*Calculations are based on the number of copies of the haplotype (0,1,2), assuming additivity on the logit scale. ORs estimate the risk of atopy for carriers of one copy of the haplotype as compared with two copies of the reference haplotype [36].

CI, confidence interval; OR, odds ratio; SPT, skin prick test.

We did not find associations between TLR2, TLR4, or CD14 genotypes and new-onset asthma in young Danish farmers. Because of the limited power of our relatively small study, we cannot exclude an effect on asthma for the less-common TLR polymorphisms. However, for the common CD14 polymorphisms and TLR2/−16934, we did not observe any trend suggestive of an association with incident asthma. The majority of new asthma cases had a non-atopic phenotype (83%). Non-atopic asthma is common among farmers, and has been associated with increased exposure to microbial agents [8]. Interestingly, LeVan et al. [37] have shown associations between CD14 promoter polymorphisms and increased wheeze and decreased pulmonary function in a cross-sectional study among non-smoking male farmers, supporting the hypothesis that higher levels of sCD14 may lead to increased susceptibility for endotoxin-induced airway inflammation. The longitudinal design of our study might be one of the reasons as to why we did not find a relationship with the studied SNPs, as new-onset asthma in the cohort of young farmers might include less-severe phenotypes. All subjects were tested for bronchial hyperresponsiveness (BHR) and lung function, and a strong association was found between BHR and case status (OR = 10.8) [31]. The present definition of asthma, however, has been made from several symptoms without reference to lung function data or atopy. Recently it has been discussed in two large population studies that by using asthma definitions that require positive answers to several asthma symptom questions, one increases the precision of the asthma diagnosis [38, 39]. Thus, we believe our symptom diagnosis of asthma was suitable for the analysis.

Because of the large number of SNPs currently identified and the absence of knowledge about the functional consequences of most of them, our approach was to study supposedly functional SNPs or at least SNPs that have been extensively studied. We covered the most well-known SNPs in the studied genes, but we were not able to detect possible associations between new-onset asthma and other SNPs in the TLR and CD14 genes that were not included in the present study. Moreover, interactions with other, unstudied genes are also possible in complex diseases such as asthma and allergy. Relevance of SNPs – especially from a pathological point of view – is a general challenge in genetic research at this moment – and not easy to resolve.

The inverse association between the CD14/−260T allele and atopy was stronger for common inhalant allergens than for farm-related allergens, perhaps because protection against sensitization through increased susceptibility to endotoxin does not outbalance the burden of farm allergens. Previous studies have reported lower levels of IgE or a lower number of total positive SPT in skin test-positive children and adults carrying the CD14/−260 T allele [17, 18, 20]. Therefore, it has been speculated that the CD14/−260 polymorphism does not modulate the susceptibility to become sensitized, but rather leads to a

more severe atopic phenotype [18]. On the other hand, a recent study among 600 French adults participating in the European Community Respiratory Health Survey-II showed a lower risk for atopic sensitization and nasal allergies in carriers of the CD14/−260T allele. Interestingly, these associations were more pronounced in individuals who lived on a farm during the first year of life compared with individuals who never lived on a farm [21]. Results of our study among farmers also suggest a stronger effect in farmers who were born and raised on a farm. This might indicate an interaction between genetic factors and timing of exposure, although the lack of power prevents us from drawing any conclusions on whether long-term farming exposures modify the effect of the T allele. Gene–environment interactions arguably play an important role in the development of complex diseases such as allergy and asthma. Especially a role for microbial exposure has been described. Most participants in the present study were livestock farmers, who are known to be highly exposed to a wide range of microorganisms and PAMPs, with average endotoxin exposure levels of around 500–2000 EU/m^3 [8, 40, 41]. In a few other cross-sectional studies, endotoxin load (EU/m^2) in mattress dust [42] or living room floor dust [26, 43], or endotoxin level (EU/mg) in living room floor dust [44] were investigated as an interactive factor in the relationship between CD14/−260 genotype and asthma or atopy. In contrast with the results of our study, less atopic sensitization, lower serum IgE levels, or less asthma were observed in subjects with the CC genotype compared with TT homozygotes, but only at elevated endotoxin exposure levels [26, 42–44]. Conversely, at lower endotoxin exposure levels, the T allele was associated with lower serum IgE levels and less asthma [43, 44]. Although these results contradict the findings of our study, it should be noted that seemingly incompatible results in genetic association studies might at least partly be explained by differences in the composition of the study population (age, ethnicity) and timing of exposure. In addition, levels of inhalable endotoxin during agricultural activities arguably exceed those measured in floor or mattress dust.

Thus, the key to the association between innate immunity genes and atopy might lie in complex interactions with environmental exposures. Vercelli [45] proposed the 'endotoxin switch hypothesis' as an explanation for the heterogeneous results among different studies. According to this concept, which is intriguing, but to date now not substantiated by any empirical evidence, each polymorphic allele would have its own multimodal response curve, representing the association between microbial exposure and immune response. At a certain exposure level, a switch from Th2 to Th1 responses might occur, which would occur at lower endotoxin levels for the CD14/−260T allele. At higher endotoxin levels, a second switch would occur, and it can be conceived that our results fit in such a model. Nevertheless, many questions remain unsolved, which illustrates the need for large-scale studies that include detailed microbial exposure data, taking into account both childhood and current exposures.

In conclusion, the CD14/−651 and CD14/−260 promoter polymorphisms are associated with the prevalence of atopy in young adult farmers. Effects appeared to be stronger in farmers who were born and raised on a farm, although interactions between CD14 genotype and long-term farming exposures were not significant. No further associations between CD14, TLR2, or TLR4 genotypes and atopy or new-onset asthma were found.

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