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Published on: 01 Mar 2009 - American Journal of Respiratory and Critical Care Medicine (American Thoracic Society)

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Lidwien Smit, Valérie Siroux, Emmanuelle Bouzigon, Marie-Pierre Oryszczyn, Mark Lathrop, et al..
CD14 and toll-like receptor gene polymorphisms, country living, and asthma in adults.: CD14, TLR2,
rural living and asthma. American Journal of Respiratory and Critical Care Medicine, American
Thoracic Society, 2009, 179 (5), pp.363-8. 10.1164/rccm.200810-1533OC . inserm-00349146

HAL Id: inserm-00349146

<https://www.hal.inserm.fr/inserm-00349146>

Submitted on 21 May 2014

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CD14 and Toll-like receptor gene polymorphisms, country living, and asthma in adults

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Funded by ANR-SEST, AFSSET.

Lidwien Smit is supported by an EAACI-GA²LEN exchange fellowship award.

Running head: *CD14*, *TLR2*, rural living and asthma

Subject category: Asthma: epidemiology (57)

Manuscript: 3304 words

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

AT A GLANCE COMMENTARY**Scientific Knowledge on the Subject**

Several studies have suggested that gene-environment interactions play an important role in the occurrence of asthma and allergy. Genes dependent on the innate immunity pathway are obvious candidates for the understanding of the protective effects of exposure to microbial agents on allergy and asthma.

What This Study Adds to the Field

TLR2 and *CD14* SNPs were associated with asthma and atopic asthma respectively. Variation in *CD14*, *TLR2*, *TLR4*, and *TLR9* genes modified associations between country living during childhood and asthma, in particular among atopic subjects.

ABSTRACT

Rationale: It has been shown that country living protects against asthma, which may be explained by microbial exposures. **Objectives:** To study whether single nucleotide polymorphisms (SNPs) in *CD14* and Toll-like receptor (*TLR*) 2, *TLR4* and *TLR9* genes are associated with asthma in adults, and whether these SNPs modify associations between country living and asthma. **Methods:** Twenty-five SNPs in *CD14*, *TLR2*, *TLR4*, and *TLR9* genes were genotyped in adult subjects from the French Epidemiological study on the Genetics and Environment of Asthma, Bronchial Hyperresponsiveness, and Atopy (EGEEA). We conducted a case-control analysis on unrelated subjects (239 asthmatics and 596 non asthmatics), and a family based association test (FBAT) in 192 families ascertained through asthmatic probands. **Measurements and Main Results:** The *TLR2*/+596 C allele was associated with an increased risk for asthma in both case-control and family-based analyses (under a dominant model, OR [95%CI]=1.91 [1.34-2.72], *P*=.0003; z statistics from FBAT=2.48, *P*=.01). In skin prick test (SPT) positive subjects, the *CD14*/-260 C allele was negatively associated with asthma (additive model, OR[95%CI]=0.66 [0.48-0.91]). Significant gene-environment interactions between variation in *CD14* and *TLR* genes and country living during childhood were found for ten SNPs. In SPT positive subjects carrying *CD14*/-260 CC, country living protected against asthma (OR=0.32 [0.12-0.85]), whereas country living was not associated with asthma in atopics carrying *CD14*/-260 T (OR=1.11 [0.65-1.90]) (gene-environment

interaction $P < .05$). **Conclusions:** *TLR2* and *CD14* SNPs were associated with asthma and atopic asthma respectively. In addition, *CD14*, *TLR2*, *TLR4*, and *TLR9* SNPs modified associations between country living and asthma.

Abstract: 249 words

Keywords: asthma; atopy; epidemiology; gene-environment interaction; hygiene hypothesis

INTRODUCTION

Epidemiological studies have shown a lower prevalence of asthma and allergic sensitization in children and adults who lived on a farm or in a rural area during childhood (1-6). According to the hygiene hypothesis, the development of allergic disease may be influenced by bacterial and viral infections, and environmental exposure to noninfectious microbial agents such as endotoxin – which are abundantly present in the farm environment (7). Both exposure to environmental factors and genetic susceptibility play a role in the occurrence of asthma and allergy. Therefore, genes dependent of the innate immunity pathway are obvious candidates for the understanding of the protective effects of exposure to microbial agents on allergy and asthma. Indeed, several single nucleotide polymorphisms (SNPs) in genes encoding pattern recognition receptors such as CD14 and Toll-like receptors (TLR) have been associated with atopic sensitization and asthma (8, 9). The first report by Baldini et al. (10) on the functional (11) *CD14 C -159 T* SNP (*C -260 T* when counting from the translation start site) has shown increased levels of circulating soluble CD14 (sCD14) in TT homozygotes, and a lower number of positive skin prick tests in atopic children carrying this genotype. Although subsequent studies have often replicated the association between *CD14/-260 T* and increased sCD14, results on atopic sensitization have been conflicting and results for asthma were mainly negative (9, 12-14). Several studies have suggested that gene-environment interactions play an important role, since associations between *CD14/-260* genotype and allergy and asthma-related outcomes have been shown to depend on the level of microbial exposures (15). A few studies have also shown gene-environment interactions in relation to SNPs in *TLR2* and *TLR4* genes, and other SNPs in the *CD14* gene (*-1721* showing a high linkage disequilibrium with *CD14/-260*) (16-18).

In the French Epidemiological study on the Genetics and Environment of Asthma, Bronchial Hyperresponsiveness, and Atopy (EGEA) a lower prevalence of asthma has been shown in adults who lived in the country during childhood (3). The purpose of the present study was to investigate whether SNPs in the candidate genes *CD14*, *TLR2*, *TLR4*, and *TLR9* are associated with asthma in adults from the EGEA study by conducting a case-control analysis on unrelated subjects and a family based association test (19). We further investigated whether these SNPs modify associations between living in the country and asthma. Studies in children and adults have shown that exposure to endotoxin and other microbial agents may protect against asthma or wheeze in atopic subjects, whereas increased exposure may be a risk factor for nonatopic asthma or wheeze (20-22). Therefore, the role of atopy was evaluated by conducting separate analyses for atopic and nonatopic subjects. Some of the results presented here have been previously reported in the form of an abstract (23).

METHODS

Study population and design

The design of EGEA combines a case-control study and a family study of adult and childhood asthma. The design, ascertainment of asthmatic cases, and descriptive characteristics have been described earlier (24-27). Briefly, asthmatic patients (age 7-70 years) were recruited from six chest clinics in five French cities. Family members of asthmatic probands were included, either by including the proband's parents and siblings, or by including the proband's spouse and children. Controls were population-based. Inclusion criteria used to define asthma in probands were based on self-reported answers to the four questions "Have you ever had attacks of breathlessness at rest with wheezing?", "Have you ever had asthma attacks?", "Was this diagnosis confirmed by a physician?", and "Have you had an asthma attack in the last 12 months?", or a positive response to at least two questions and a positive expertise of their medical record (25). Asthma in relatives of probands was defined as a positive answer to the question "Have you ever had attacks of breathlessness at rest with wheezing?" or "Have you ever had asthma attacks?" (25). In the present study, "asthmatics" were defined as probands and relatives with asthma, "nonasthmatics" as population-based controls and relatives without asthma. Atopy was defined as a positive skin prick test (SPT) to one of the 11 common allergens tested (28). Country living during childhood was defined as a positive answer to the question "Have you ever lived in the country for at least one year?", and being 16 years of age or younger when beginning to live in the country (3). Age 16 was chosen as a cut-off because in the initial study (3), living in the country before age 16 was most strongly associated with a reduced prevalence of asthma. Sensitivity analyses were conducted using country living before being 1 year of age, and additional information on the definition of country living is given in the online data supplement.

A case-control analysis was conducted in 825 genetically unrelated adults from the parental generation (aged 27-70 years), i.e. adult probands and their spouses, parents of probands from families where the proband is offspring, and population-based controls from the same generation. In addition, a family-based analysis was

conducted in 708 adult subjects from 192 families, comprising 378 parents who were included in the case-control analysis as well, and 330 adult offspring subjects who were not included in the analysis among unrelated subjects. All participants gave written informed consent. Additional information is given in the online data supplement.

Genotyping

Twenty-five SNPs belonging to the four candidate genes considered were selected using a tagging approach: *CD14* (5 SNPs), *TLR2* (5 SNPs), *TLR4* (10 SNPs) and *TLR9* (5 SNPs). This set of SNPs allowed us to capture the majority of common haplotype variation of these four genes (i.e., haplotype with a frequency > 5%). Details on genotyping are given in the online data supplement.

Statistical analysis

In the 825 genetically unrelated adults, odds ratios (ORs) for associations between genotype and asthma were calculated by generalized estimating equations (GEE model; Proc GENMOD using SAS statistical software) to adjust for dependence between subjects sharing the same household (proband and their spouses, and mothers and fathers of probands). ORs were adjusted for age and sex. The effect of each SNP on disease was tested under a general genetic model (2-degrees-of-freedom-test) and ORs were calculated using homozygotes for the most frequent allele as reference group. In addition, dominant, recessive, and additive genetic models were considered and the best-fitting model was selected (likelihood ratio test). We tested the null hypothesis that effects of genotype and country living on asthma were independent by including an interaction term in the model (gene-environment interaction) and testing the significance (GEE model, Z-score). False Discovery Rate (FDR) adjusted *P*-values were calculated to take multiple comparisons (n=25 SNPs) into account (29).

In order to confirm associations among unrelated subjects, a family-based association test, as implemented in FBAT (30) was performed as a complementary method to test for association between asthma and genetic polymorphisms. Details on the FBAT method are given in the online data supplement.

RESULTS

Identification numbers, position, and minor allele frequencies of studied SNPs are shown in Table E2 in the online data supplement. All SNPs were in Hardy-Weinberg equilibrium ($P > .05$). Tables E3-E6 in the online data supplement show linkage disequilibrium (LD) between polymorphisms for the four genes under study.

Characteristics of nonasthmatic (n=586) and asthmatic (n=239) subjects are shown in Table I. Living in the country during childhood was associated with a lower prevalence of asthma with an OR of 0.67 (0.48-0.92) (before 16y) and 0.72 (0.52-1.01) (before 1y, $P = .06$), which confirmed the earlier report in a different subpopulation of the EGEA study (3). Adjusting for age, sex, and smoking habits did not change the association.

Association between *CD14*, *TLR2*, *TLR4*, and *TLR9* genetic polymorphisms and asthma

The genotypic distribution of *CD14*, *TLR2*, *TLR4*, and *TLR9* SNPs and the associations between these polymorphisms and asthma in the 825 unrelated subjects are shown in Tables E7-E10 in the online data supplement. Carrying the *TLR2*/+596 C allele was associated with an increased risk for asthma (OR (95%CI) = 1.91 (1.34-2.72); $P = .0003$, *P* value adjusted for multiple comparisons = .008). Two other *TLR2* SNPs, *TLR2*/-24438 and rs2289318, and two *TLR9* SNPs, rs353547 and *TLR9*/+2848, were also associated with asthma, but not significantly after adjusting for multiple comparisons. Since low LD was observed among the three *TLR2* SNPs on the one hand (LD coefficient $r^2 \leq 0.34$; Table E4) and between the two *TLR9* SNPs on the other hand (LD coefficient $r^2 = 0.53$; Table E6), we conducted two separate multivariate-SNP analyses by including these polymorphisms in the regression model. *TLR2*/+596 was the only SNP significantly associated with asthma in the model testing for three *TLR2* markers. Estimates for each *TLR9* SNP in the multiple regression model were similar to those in the univariate model. However, none of them was significant after mutual adjustment. Associations between genotype and asthma were not different for men and women (interaction $P > .10$ for all SNPs). Similar results were obtained when either population-based controls (n=117) or non-asthmatic family members (n=469) were excluded from the control group (data not shown). The positive association between the *TLR2*/+596 C allele and asthma under a dominant model was confirmed by a family-based association test in 192 families ($Z = 2.48$; $P = .01$, FBAT). In the family-based analysis, asthma outcome of 330 offspring subjects determined the magnitude of the test statistic, whereas the case-control analyses in the 825 genetically unrelated subjects as described above included only the parental generation. For other SNPs no significant association was found using FBAT ($P > .05$; data not shown).

In the 825 genetically unrelated subjects, the influence of *TLR2*/+596 on associations between other SNPs and asthma (i.e. gene-gene interactions) was assessed by including an interaction term in the model and testing the significance (GEE model, Z-score). For three SNPs (*CD14*/+1188 and two *TLR9* SNPs), a significant interaction with *TLR2*/+596 genotype was found (Table II), showing significant associations between these SNPs and asthma in *TLR2*/+596 TT homozygotes, but ORs around 1 in subjects carrying *TLR2*/+596 C.

Separate analyses were conducted for atopic and nonatopic subjects. Associations between genotype and asthma outcomes for 11 SNPs associated with at least one of the asthma outcomes are presented in Table III (unadjusted $P < .05$ for 11 SNPs, FDR-adjusted $P < .05$ for 6 SNPs). Carrying the *TLR2/+596 C* allele was a significant risk factor for asthma in atopic subjects (OR 2.39, $P = .0006$), whereas a positive but not significant association was found in nonatopic subjects (OR 1.55, $P = .13$). The *CD14/-260 C* allele protected against asthma in atopic subjects with an OR of 0.66 (0.48-0.91). Four other *CD14* SNPs were also significantly associated with a lower risk of asthma in atopic subjects, and for these SNPs, associations differed significantly from those observed in non-atopic subjects, who had ORs around 1 (gene-atopy interaction $P < .05$). Atopic subjects carrying a protective allele for each *CD14* SNP had significantly less asthma compared with subjects carrying none of the protective alleles, with an OR of 0.34 (0.18-0.67). Carrying a protective allele for one to four *CD14* SNPs showed an OR of 0.64 (0.37-1.16) compared with subjects carrying none. Multiple regression models including more than one *CD14* SNP showed independent effects that were similar in size for each different SNP, thus the effect was not dominated by one particular SNP.

Role of *CD14*, *TLR2*, *TLR4*, and *TLR9* genetic polymorphisms in the associations between living in the country and asthma

Gene-environment interactions between 10 SNPs and living in the country during childhood were found for at least one of the asthma outcomes (unadjusted P -value for interaction $< .05$ for 10 SNPs, FDR-adjusted $P < .10$ for 6 SNPs; Table IV). In atopic subjects with *CD14/-260 CC* genotype, country living protected against asthma (OR 0.32), whereas country living was not associated with asthma in atopics carrying the *CD14/-260 T* allele (OR 1.11). Conversely, in non-atopic subjects carrying *CD14/-260 CC*, country living was not associated with asthma (OR 1.33), whereas country living was associated with a lower risk of asthma in non-atopic subjects carrying the *CD14/-260 T* allele (OR 0.41). For this SNP, a significant second-order interaction term (gene x environment x atopy) was found ($P = .005$).

In atopics, gene-environment interactions were found as well for three other *CD14* SNPs, one *TLR4* SNP, and one *TLR9* SNP (all FDR-adjusted P -values for interaction were borderline significant). In nonatopic subjects, gene-environment interactions were found for four SNPs, including *TLR2/+596*. In nonatopic carriers of the *TLR2/+596 C* allele, country life protected against asthma. Alternatively, when the results were stratified for living in the country, carrying at least one *TLR2/+596 C* allele (CC/CT vs TT) was a risk factor for asthma in nonatopic subjects who had not grown up in the country with an OR of 2.94 (1.16-7.41), but not in those who had grown up in the country (OR 0.77 (0.35-1.70)).

Sensitivity analyses using 1 year of age as a cut-off for beginning to live in the country showed very similar results, but the P -values for interaction were less often statistically significant, especially for atopic asthma (Table E11 in the online data supplement).

DISCUSSION

In the present study, an association was found between the *TLR2/+596* polymorphism and asthma among French adults from the EGEA study. The well-studied *CD14/-260* SNP was associated with asthma in SPT positive subjects – once again confirming the importance of this SNP in the occurrence of allergy and asthma related phenotypes. Gene-environment interactions were found between ten SNPs in candidate genes *CD14*, *TLR4*, *TLR2*, and *TLR9* and living in the country during childhood, which was presumed to represent higher exposures to various microbial agents. Main effects and gene-environment interactions were stronger in atopic subjects than in non-atopic subjects. Significant interactions between *CD14* SNPs and atopy were found in the association with asthma, moreover for *CD14/-260*, a significant interaction between genotype, atopy, and country living was found.

Eder et al. found that a SNP in *TLR2* (*A -16934 T*) was associated with asthma, atopy, and hay fever in children of farmers (16). On the other hand, two studies investigating genetic variation in *TLR2* reported no association with childhood-onset atopic asthma in a Japanese population (31), and no association with atopy or new-onset asthma in young Danish farmers (32). In the present study, we observed replication for the association between *TLR2/+596* and asthma in case-control and family-based analyses. In the case-control analysis only the parental generation was included, whereas in the family-based analysis, genotype and asthma outcome of offspring determined the magnitude of the test statistic. Thus, results of the two different analyses were based on a different population within the EGEA study, which substantiates findings on *TLR2*. Moreover, although we tested a large number of SNPs in four candidate genes which increases the risk for false positive results, the findings on *TLR2/+596* were still statistically significant after adjusting for multiple comparisons. *TLR2* is involved in the recognition of microbial motifs of a wide range of Gram-positive microorganisms, mycobacteria and yeast, and exposures to these microorganisms are likely to be higher in rural than in urban areas. We can only speculate on a possible mechanism explaining our findings, as the functional significance of *TLR2/+596* is so far unknown. A possible explanation could be a lower expression of *TLR2* on the surface of innate immune cells in carriers of the *TLR2/+596C* allele. As a consequence, carriers of *TLR2/+596C* would

benefit less from the protective effects of environmental exposures to TLR2 ligands on asthma. Further analyses of different asthma outcomes showed that *TLR2/+596C* was in particular associated with asthma in SPT positive subjects, whereas in nonatopic subjects, a significant interaction with childhood environment was found, showing that *TLR2/+596C* was only a risk factor for asthma in nonatopic subjects who had not grown up in the country. Thus, variation in *TLR2* appears to be associated with asthma, but environmental exposure, asthma subphenotype, and also interactions with other genes such as *CD14* and *TLR9* were shown to play a role.

TLR9 is a receptor for bacterial CpG DNA motifs, which have been found in increased levels in farm barn dust and dust from rural homes (33). Only few studies have investigated *TLR9* SNPs in relation to allergy or asthma, showing inconsistent results (31, 34, 35). In the present study, only minor associations with asthma were found for *TLR9* SNPs when other factors were not taken into account. However, significant gene-gene interactions with the *TLR2/+596* SNP were found, showing effects of two *TLR9* SNPs on asthma in *TLR2/+596 TT* subjects. Since TLR9 and TLR2 have different ligands, this finding might reflect an interactive effect of multiple microbial exposures.

In contrast to *TLR2* and *TLR9*, which have not been studied widely in relation to asthma or allergic outcomes, *CD14* is one of the best established susceptibility genes for asthma-related phenotypes such as allergic sensitization and total IgE (36). Studies investigating asthma in relation to *CD14/-260* have often been negative, whereas either the C allele or T allele were alternately found to be a risk factor for allergic sensitization, number of positive SPT, or increased total IgE (9, 15). In the present study, *CD14* polymorphisms protected against asthma, but only in atopic subjects, showing significant gene-atopy interactions.

In various studies, inverse associations between house dust endotoxin and allergic sensitization, total serum IgE, and asthma have been shown to be strongest in *CD14/-260 CC* homozygotes (15, 37, 38). In the present study, the inverse relationship between a rural childhood and asthma in atopic adults was also strongest in *CD14/-260 CC* subjects, and a significant gene-environment interaction was found. This apparent replication of a gene-environment interaction is remarkable, given the different phenotypes, age of study subjects, timing of exposure, study region, and exposure definitions across these studies. Conversely, in three other studies, the *CD14/-260 TT* genotype protected against atopic dermatitis, atopy, and increased IgE, especially in children with a dog in the home (39, 40), and in adults or young farmers who lived on a farm during childhood (32, 41). Gene-environment interactions are therefore likely to depend on qualitative and quantitative characteristics of environmental exposure, but also on phenotype, interactions with other genes, and on the age of study subjects (9, 36). The importance of atopy as an effect modifier in the present study underlines the complex interrelationships between environmental exposures, genetic variation in *CD14*, and asthma.

The fact that interactions were found between a rural childhood and polymorphisms in genes encoding four different pattern recognition receptors that become activated by a wide array of ligands, suggests that the protective effect of living in the country is – at least partly – a result of increased microbial exposures. In the present study, living in the country was used as a proxy for high exposure to microbial agents during childhood, since measured domestic endotoxin levels or more specific information regarding farm (animal) exposures was not available. Nevertheless, in the EGEA study it has been demonstrated that several lifestyle factors differed substantially between subjects who ever lived in rural communities and subjects who had always lived in urban regions: for instance, rural life was associated with factors such as using wood for heating, household size, and the presence of pets (3). Self-reported country living during childhood was also associated with parental farming and residential history (see Table E1 in the online data supplement). One could argue that concentrations of microbial agents in dust collected in rural homes are lower compared with dust from farm homes (42), and in our study only few subjects had farming parents. However, levels of microbial exposures in rural households have been found to be higher than exposure levels in urban homes (33). Moreover, considering the fact that most of the present study subjects were born in the 1940s/50s, it is likely that other relevant factors such as raw milk consumption, and exposure to stables and livestock were relatively common among those living in the country during their childhood compared with those in urban areas.

For country living during childhood, a 16 year cut-off was used. When we considered subjects with early-life rural exposures (before 1 year), very similar results were obtained, although gene-environment interactions were less often statistically significant. This may partly be due to a decreased power because of the lower number of early-life rural exposed subjects. It is also possible that rural exposures later in childhood may have a protective effect on asthma as well, which is suggested by the observed stronger relationship between country living and asthma when using 16 years as a cut-off.

In conclusion, *TLR2* and *CD14* are important genes for asthma in adults. Variation in *CD14*, *TLR2*, *TLR4*, and *TLR9* genes modified associations between country living during childhood and asthma, in particular for asthma in atopic subjects.

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TABLE I. Characteristics of the study population

	Nonasthmatics	Asthmatics	P
n	586	239	
Age, y, mean \pm sd	47.1 \pm 8.0	46.0 \pm 8.8	.07
Males, n (%)	293 (50.0)	123 (51.5)	.70
Smoking habits			
Never smokers, n (%)	258 (44.3)	104 (43.9)	.01
Exsmokers, n (%)	178 (30.5)	93 (39.2)	
Current smokers, n (%)	147 (25.2)	40 (16.9)	
Living in the country during childhood, n (%) [*]	254 (55.0)	105 (44.9)	.01
Number of years during childhood, y, mean \pm sd [¶]	11.2 \pm 5.2	11.1 \pm 4.9	.97
Living in the country before age 1 year, n (%) [*]	165 (35.7)	67 (28.6)	.06
Atopy, n (%)	182 (31.8)	148 (64.4)	<.0001

^{*} population-based controls (n=117) excluded

[¶] Among the 254 nonasthmatics and 105 asthmatics who lived in the country during childhood.

TABLE II. Associations between *CD14* and *TLR9* SNPs and asthma for SNPs showing significant gene-gene interactions with *TLR2/+596*

SNP	Model	Asthma (OR (95% CI))			<i>P</i> interaction	
		All subjects (n=825) [#]	<i>TLR2/+596 TT</i> (n=259) [#]	<i>TLR2/+596 CT/CC</i> (n=529) [#]	Unadjusted	FDR adjusted
<i>CD14/+1188</i>	CG+GG vs CC (ref)	0.72 (0.49-1.06)	0.30 (0.11-0.81)	0.93 (0.60-1.45)	.03	.16
<i>TLR9</i> rs352143	AG+GG vs AA (ref)	1.15 (0.85-1.55)	2.31 (1.24-4.30)	0.95 (0.66-1.38)	.02	.15
<i>TLR9</i> rs352163	T allele [¶]	0.87 (0.70-1.09)	0.48 (0.30-0.74)	1.04 (0.80-1.35)	.002	.04

[¶] Additive genetic model, the genotype was categorized into a three level variable for the number of minor alleles (0,1,2).

Ref: reference genotype coded as 0.

Bold: p<0.05.

[#] Exact numbers of genotyped subjects for each SNP are given in Table E2. *TLR2/+596* was genotyped in 788 subjects.

TABLE III. Associations between *CD14*, *TLR2*, *TLR4*, and *TLR9* SNPs and asthma for SNPs significantly associated with at least one of the asthma outcomes

Gene	SNP	Model	OR (95% CI)		
			Asthma	Asthma in atopic subjects	Asthma in nonatopic subjects
<i>CD14</i>	-260	C allele [¶]	0.81 (0.65-1.01)	0.66 (0.48-0.91)**	0.93 (0.65-1.33)
	+1188	CG+GG vs CC (ref)	0.72 (0.49-1.06)	0.44 (0.25-0.78)**	1.00 (0.56-1.82)
	+1341	AC+AA vs CC (ref)	0.77 (0.56-1.06)	0.50 (0.31-0.81)**	1.02 (0.62-1.69)
	rs778584	T allele [¶]	0.86 (0.69-1.06)	0.62 (0.46-0.85)**	1.09 (0.77-1.52)
	rs778583	CT+TT vs CC (ref)	0.80 (0.59-1.09)	0.47 (0.29-0.75)**	1.11 (0.69-1.80)
<i>TLR2</i>	-24438	GG vs AA+AG (ref)	0.57 (0.36-0.90)	0.47 (0.25-0.89)*	0.66 (0.32-1.36)
	+596	CT+CC vs TT (ref)	1.91 (1.34-2.72)**	2.39 (1.45-3.94)**	1.55 (0.88-2.73)
	+1349	C allele [¶]	1.30 (0.90-1.88)	1.04 (0.55-1.98)	1.92 (1.17-3.15)
	rs2289318	CG+GG vs CC (ref)	1.40 (1.01-1.94)	1.61 (1.01-2.56)	0.83 (0.48-1.43)
<i>TLR9</i>	rs353547	AG+AA vs GG (ref)	1.43 (1.03-1.99)	1.27 (0.79-2.06)	1.43 (0.86-2.38)
	+2848	AA vs GG+AG (ref)	0.65 (0.43-0.99)	0.62 (0.34-1.13)	0.77 (0.40-1.46)

Bold: p<0.05, not adjusted for multiple comparisons; * p<0.10, FDR adjusted p value; ** p<0.05, FDR adjusted p value

[¶] Additive genetic model, the genotype was categorized into a three level variable for the number of minor alleles (0,1,2).

Ref: reference genotype coded as 0.

TABLE IV. Gene-environment interactions between *CD14*, *TLR2*, *TLR4*, and *TLR9* SNPs and country living during childhood in asthma outcomes, shown as associations between country living and asthma, stratified by genotype

Gene	SNP	Genotype	Living in the country during childhood, OR (95% CI)		
			Asthma	Asthma in atopic subjects	Asthma in nonatopic subjects
<i>CD14</i>	-260	TT+CT	0.66 (0.45-0.97)	1.11 (0.65-1.90)*	0.41 (0.22-0.79)
		CC	0.63 (0.32-1.24)	0.32 (0.12-0.85)	1.33 (0.46-3.84)
	+1341	CC	0.92 (0.59-1.44)	1.59 (0.81-3.13)*	0.75 (0.36-1.55)
		AC+AA	0.44 (0.26-0.73)	0.48 (0.23-0.99)	0.42 (0.18-1.00)
	rs778584	CC+CT	0.64 (0.44-0.94)	1.07 (0.63-1.82)*	0.42 (0.22-0.78)
		TT	0.72 (0.38-1.40)	0.32 (0.12-0.84)	1.41 (0.51-3.87)
	rs778583	CC	0.89 (0.58-1.37)	1.50 (0.77-2.95)*	0.76 (0.37-1.55)
		CT+TT	0.47 (0.28-0.76)	0.47 (0.23-0.94)	0.48 (0.22-1.05)
<i>TLR2</i>	-24438	AA+AG	0.63 (0.45-0.90)	0.87 (0.54-1.42)	0.47 (0.26-0.85)
		GG	0.94 (0.38-2.33)	0.44 (0.12-1.62)	3.57 (0.58-21.8)
	+596	TT	0.66 (0.35-1.27)	0.53 (0.20-1.35)	1.36 (0.48-3.88)
		CT+CC	0.65 (0.43-0.97)	0.91 (0.52-1.59)	0.40 (0.20-0.78)
	+1349	TT	0.68 (0.47-0.96)	0.78 (0.48-1.27)	0.71 (0.39-1.30)
		CT+CC	0.57 (0.26-1.27)	0.91 (0.26-3.22)	0.23 (0.07-0.76)
<i>TLR4</i>	rs11536889	GG	0.79 (0.54-1.15)	1.17 (0.67-2.05)*	0.70 (0.37-1.32)
		CG+CC	0.43 (0.23-0.80)	0.36 (0.14-0.91)	0.36 (0.13-0.99)
	rs7045953	AA	0.53 (0.37-0.78)	0.66 (0.38-1.13)	0.46 (0.25-0.87)
		AG+GG	1.14 (0.62-2.11)	1.34 (0.54-3.33)	1.16 (0.43-3.10)
<i>TLR9</i>	rs353547	GG+AG	0.72 (0.50-1.03)	1.11 (0.67-1.86)*	0.53 (0.29-0.95)
		AA	0.41 (0.18-0.96)	0.18 (0.05-0.63)	1.66 (0.40-6.92)

Bold: interaction $p < 0.05$, not adjusted for multiple comparisons; * interaction $p < 0.10$, FDR adjusted p value

ONLINE DATA SUPPLEMENT

***CD14* and Toll-like receptor gene polymorphisms, country living, and asthma in adults**

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Study population and design

Subjects were enrolled in six chest clinics from five French cities (Grenoble, Lyon, Marseille, Montpellier, Paris) between 1991 and 1995. Inclusion criteria used to define asthma in probands were based on self-reported answers to the four questions “Have you ever had attacks of breathlessness at rest with wheezing?”, “Have you ever had asthma attacks?”, “Was this diagnosis confirmed by a physician?”, and “Have you had an asthma attack in the last 12 months?”, or a positive response to at least two questions and a positive expertise of their medical record (E1). All probands and their two parents were Caucasian and were born in France. All family members answered a detailed questionnaire based on British Medical Research Council (BMRC)/European Coal and Steel Community (ECSC), American Thoracic Society (ATS), and European Community Respiratory Health Survey (ECRHS) questionnaires. Based on the BMRC/ECSC questionnaire, asthma in relatives of probands was defined as a positive answer to the question “Have you ever had attacks of breathlessness at rest with wheezing?” or “Have you ever had asthma attacks?” (E1). Population-based controls were selected from electoral rolls, a surgery department, and a social security check-up center. In the present study, “asthmatics” were defined as probands and relatives with asthma, “nonasthmatics” as population-based controls and relatives without asthma. Population-based controls with asthma (n=15) were excluded from data analysis.

Figure E1 represents a diagram describing the population used in the present analysis. As shown in Figure E1, a case-control analysis was conducted in genetically unrelated adults from the parental generation. Population-based controls were assigned to the parental generation (controls aged 40-64 yrs) or offspring generation (controls aged 18-47 yrs) on the basis of age in order to obtain an optimal age distribution between cases and controls. The study protocol was approved by the institutional ethics committee and all participants gave written informed consent.

Country living

The present study was restricted to adult subjects of the EGEA study because information on country living was only available for adults. Country living was not taken into account during recruitment, as this was not considered of importance when the study was designed in 1990/1991. As described earlier, the area of residence for asthmatic probands and their relatives was broader than for most population-based controls, reflecting the area of attraction of the chest clinics, and significantly more asthmatic probands were living in the country than population-based controls (E2). Analyses that included country living were therefore conducted in asthmatic probands and relatives only, excluding controls (n=117).

Country living during childhood was defined as living in a rural area for at least one year in a row, and being 16 years of age or younger when beginning to live in the country. In EGEA2, the 12 year follow-up of the first EGEA survey which has been conducted recently (between 2003 and 2006) (E3), more questions on residential history and farming were asked. In EGEA2 the complete residential history (at the French ‘commune’ level) was obtained and these data were combined with demographic statistics indicating the number of inhabitants of the ‘commune’. Table E1 shows that subjects with and without a rural childhood are significantly different with regard to farming parents and residential history.

Genotyping

Genotyping was performed using either Taqman Probes (Applied Biosystems, Foster City, CA) on an ABI7900HT Sequence Detection System or 1536-plex Illumina Golden Gate assay (Illumina, San Diego, CA) at the Centre National de Génotypage (CNG, Evry, France).

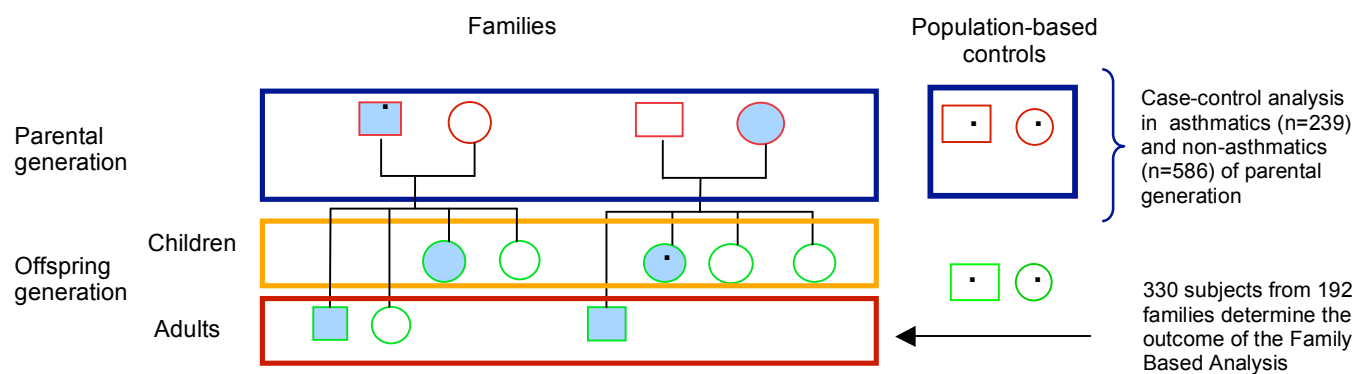
FBAT

A family-based association test, as implemented in FBAT (E4) was performed as a complementary method to test for association between asthma and genetic polymorphisms in the adult offspring sample. This approach tests for the association between the phenotype and excess transmission of a specific allele from parent to offspring. The advantage of this approach is that population stratification is avoided. We used the `-o` option in FBAT program allowing to take into account affected and unaffected offspring in the analysis. Association was initially tested under a general model and subsequently under additive, dominant and recessive models.

TABLE E1. Factors related to living in the country during childhood

	N complete data	Country living during childhood	
		No	Yes
Born in a community <5000 inhabitants, n (%)	500	17 (7.0)	126 (49.0)*
Living in a community <5000 inhabitants during childhood, n (%)	500	27 (11.1)	170 (66.2)*
Farming as parental occupation, n (%)	538	0 (0)	48 (17.2)*

P<.0001

**Figure E1.** Diagram describing the study population used in the case-control and family-based analysis. Asthmatic subjects are represented by shaded shapes. Asthmatic probands and population-based controls are represented by a dot.**TABLE E2.** SNP identification numbers, position, and minor allele frequencies

dbSNP ID	Gene	Genomic location	Position from translation start site	Alleles	Minor allele frequency	N
rs2569190	<i>CD14</i>	5 :139993100	-260	T/C	0.50	777
rs4914	<i>CD14</i>	5 :139991652	+1188	C/G	0.12	783
rs2563298	<i>CD14</i>	5 :139991499	+1341	C/A	0.27	781
rs778584	<i>CD14</i>	5 :139985396		C/T	0.49	819
rs778583	<i>CD14</i>	5 :139985294		C/T	0.28	822
rs13150331	<i>TLR2</i>	4 :154819072	-24438	A/G	0.40	817
rs3804099	<i>TLR2</i>	4 :154844106	+596	T/C	0.43	788
rs3804100	<i>TLR2</i>	4 :154844859	+1349	T/C	0.08	822
rs5743700	<i>TLR2</i>	4 :154845132	+1622	C/T	0.04	786
rs2289318	<i>TLR2, RNF175</i>	4 :154853184		C/G	0.17	822
rs10759930	<i>TLR4</i>	9 :119501442		C/T	0.41	822
rs2737191	<i>TLR4</i>	9 :119502536		A/G	0.27	821
rs4986791	<i>TLR4</i>	9 :119515423	+1196, Thr399Ile	C/T	0.06	792
rs7869402	<i>TLR4</i>	9 :119517853		C/T	0.02	790
rs11536889	<i>TLR4</i>	9 :119517952		G/C	0.17	820
rs1927906	<i>TLR4</i>	9 :119519936		A/G	0.09	821
rs1554973	<i>TLR4</i>	9 :119520633		T/C	0.24	817
rs913930	<i>TLR4</i>	9 :119523830		T/C	0.34	820

rs1927905	<i>TLR4</i>	9 :119525129	A/G	0.06	822
rs7045953	<i>TLR4</i>	9 :119525616	A/G	0.15	821
rs353547	<i>TLR9, PTK9L</i>	3 :52243906	G/A	0.39	821
rs11717574	<i>TLR9, PTK9L</i>	3 :52243286	T/C	0.15	815
rs352143	<i>TLR9, PTK9L</i>	3 :52239947	A/G	0.21	822
rs352140	<i>TLR9</i>	3 :52231737	G/A	0.47	778
rs352163	<i>TLR9, ALAS1</i>	3 :52222150	C/T	0.47	817

TABLE E3. Linkage disequilibrium (r^2) between *CD14* SNPs

	-260	+1188	+1341	rs778584	rs778583
-260		0.14	0.37	0.95	0.37
+1188			0.37	0.14	0.35
+1341				0.38	0.98
rs778584					0.39
rs778583					

TABLE E4. Linkage disequilibrium (r^2) between *TLR2* SNPs

	-24438	+596	+1349	+1622	rs2289318
-24438		0.34	0.05	0.03	0.05
+596			0.11	0.06	0.26
+1349				0	0.02
+1622					0.23
rs2289318					

TABLE E5. Linkage disequilibrium (r^2) between *TLR4* SNPs

	rs10759930	rs2737191	Thr399Ile	rs7869402	rs11536889	rs1927906	rs1554973	rs913930	rs1927905	rs7045953
rs10759930		0.26	0.05	0.02	0.26	0.07	0.21	0.34	0.04	0.12
rs2737191			0.03	0.01	0.06	0.04	0.11	0.65	0.02	0.06
Thr399Ile				0	0.01	0.70	0.22	0.03	0	0.01
rs7869402					0.01	0.25	0.08	0.01	0	0
rs11536889						0.02	0.07	0.11	0.01	0.04
rs1927906							0.31	0.05	0	0.01
rs1554973								0.16	0.21	0.57
rs913930									0.03	0.09
rs1927905										0.37
rs7045953										

TABLE E6. Linkage disequilibrium (r^2) between *TLR9* SNPs

	rs353547	rs11717574	rs352143	+2848	rs352163
rs353547		0.11	0.01	0.53	0.45
rs11717574			0.65	0.13	0.13
rs352143				0.21	0.21
+2848					0.89
rs352163					

TABLE E7. Association between *CD14* genotype and asthma

SNP	Nonasthmatics, n (%)	Asthmatics, n (%)	OR (95%CI)
<i>CD14</i> -260			
TT	133 (24.0)	67 (30.0)	1.00
CT	276 (49.8)	107 (48.0)	0.75 (0.51-1.10)
CC	145 (26.2)	49 (22.0)	0.66 (0.43-1.02)

C allele [¶]			0.81 (0.65-1.01)
<i>CD14/+1188</i>			
CC	428 (76.3)	181 (81.5)	1.00
CG	122 (21.8)	37 (16.7)	0.71 (0.48-1.06)
GG	11 (2.0)	4 (1.8)	0.85 (0.27-2.69)
CG+GG vs CC (ref)			0.72 (0.49-1.06)
<i>CD14/+1341</i>			
CC	292 (52.4)	131 (58.5)	1.00
AC	214 (38.4)	76 (33.9)	0.78 (0.56-1.09)
AA	51 (9.2)	17 (7.6)	0.74 (0.42-1.30)
AC+AA vs CC (ref)			0.77 (0.56-1.06)
rs778584			
CC	146 (25.1)	69 (29.0)	1.00
CT	286 (49.2)	117 (49.2)	0.85 (0.59-1.23)
TT	149 (25.7)	52 (21.9)	0.73 (0.48-1.12)
T allele [¶]			0.86 (0.69-1.06)
rs778583			
CC	303 (52.0)	137 (57.3)	1.00
CT	226 (38.8)	85 (35.6)	0.82 (0.60-1.14)
TT	54 (9.3)	17 (7.1)	0.70 (0.39-1.22)
CT+TT vs CC (ref)			0.80 (0.59-1.09)

[¶]Additive genetic model, the genotype was categorized into a three level variable for the number of minor alleles (0,1,2).

Ref: reference genotype coded as 0.

TABLE E8. Association between *TLR2* genotype and asthma

SNP	Nonasthmatics, n (%)	Asthmatics, n (%)	OR (95%CI)
<i>TLR2/-24438</i>			
AA	192 (33.2)	94 (39.3)	1.00
AG	286 (49.5)	120 (50.2)	0.86 (0.62-1.21)
GG	100 (17.3)	25 (10.5)	0.52 (0.31-0.86)
GG vs AA+AG (ref)			0.57 (0.36-0.90)
<i>TLR2/+596</i>			
TT	207 (36.6)	52 (23.3)	1.00
CT	259 (45.8)	122 (54.7)	1.88 (1.30-2.71)
CC	99 (17.5)	49 (22.0)	1.99 (1.24-3.19)
CT+CC vs TT (ref)			1.91 (1.34-2.72)
<i>TLR2/+1349</i>			
TT	502 (86.1)	199 (83.3)	1.00
CT	77 (13.2)	36 (15.1)	1.20 (0.79-1.85)
CC	4 (0.7)	4 (1.7)	2.58 (0.64-10.39)
C allele [¶]			1.30 (0.90-1.88)
<i>TLR2/+1622</i>			
CC	515 (91.3)	201 (90.5)	1.00
CT	49 (8.7)	21 (9.5)	1.10 (0.64-1.92)
rs2289318			
CC	419 (71.9)	154 (64.4)	1.00
CG	145 (24.9)	81 (33.9)	1.51 (1.08-2.10)
GG	19 (3.3)	4 (1.7)	0.57 (0.19-1.72)
CG+GG vs CC (ref)			1.40 (1.01-1.94)

[¶]Additive genetic model, the genotype was categorized into a three level variable for the number of minor alleles (0,1,2).

Ref: reference genotype coded as 0.

TABLE E9. Association between *TLR4* genotype and asthma

SNP	Nonasthmatics, n (%)	Asthmatics, n (%)	OR (95%CI)
rs10759930			
CC	192 (32.9)	88 (36.8)	1.00
CT	305 (52.3)	107 (44.8)	0.76 (0.55-1.06)
TT	86 (14.8)	44 (18.4)	1.14 (0.73-1.78)
TT vs CC+CT (ref)			1.34 (0.88-2.03)
rs2737191			
AA	299 (51.4)	123 (51.5)	1.00
AG	248 (42.6)	101 (42.3)	0.99 (0.73-1.34)
GG	35 (6.0)	15 (6.3)	1.00 (0.52-1.89)
AG+GG vs AA (ref)			0.99 (0.74-1.32)
<i>TLR4 Thr399Ile</i>			
CC	492 (86.6)	198 (88.4)	1.00
CT	76 (13.4)	26 (11.6)	0.85 (0.52-1.39)
rs7869402			
CC	544 (96.3)	211 (93.8)	1.00
CT	21 (3.7)	13 (5.8)	1.56 (0.78-3.12)
TT	0 (0.0)	1 (0.4)	n.e.
rs11536889			
GG	385 (66.2)	174 (73.1)	1.00
CG	183 (31.4)	55 (23.1)	0.69 (0.48-0.99)
CC	14 (2.4)	9 (3.8)	1.63 (0.64-4.11)
CG+CC vs GG (ref)			0.72 (0.51-1.00)
rs1927906			
AA	482 (82.7)	196 (82.4)	1.00
AG	99 (17.0)	39 (16.4)	0.97 (0.65-1.47)
GG	2 (0.3)	3 (1.3)	3.33 (0.58-19.08)
AG+GG vs AA (ref)			1.03 (0.69-1.53)
rs1554973			
TT	332 (57.4)	140 (58.6)	1.00
CT	219 (37.9)	83 (34.7)	0.92 (0.67-1.26)
CC	27 (4.7)	16 (6.7)	1.39 (0.75-2.59)
CC vs TT+CT (ref)			1.44 (0.79-2.64)
rs913930			
TT	252 (43.3)	99 (41.6)	1.00
CT	270 (46.4)	113 (47.5)	1.06 (0.78-1.45)
CC	60 (10.3)	26 (10.9)	1.07 (0.64-1.78)
CT+CC vs TT (ref)			1.06 (0.79-1.43)
rs1927905			
AA	513 (88.0)	208 (87.0)	1.00
AG	69 (11.8)	29 (12.1)	1.06 (0.67-1.66)
GG	1 (0.2)	2 (0.8)	4.62 (0.44-49.07)
AG+GG vs AA (ref)			1.11 (0.72-1.72)
rs7045953			
AA	415 (71.3)	174 (72.8)	1.00
AG	157 (27.0)	60 (25.1)	0.92 (0.66-1.29)
GG	10 (1.7)	5 (2.1)	1.21 (0.45-3.21)
GG vs AA+AG (ref)			1.23 (0.47-3.26)

Ref: reference genotype coded as 0

n.e. not estimable

TABLE E10. Association between *TLR9* genotype and asthma

SNP	Nonasthmatics, n (%)	Asthmatics, n (%)	OR (95%CI)
rs353547			
GG	233 (40.0)	76 (31.9)	1.00
AG	263 (45.1)	124 (52.1)	1.46 (1.04-2.07)
AA	87 (14.9)	38 (16.0)	1.35 (0.85-2.15)
AG+AA vs GG (ref)			1.43 (1.03-1.99)
rs11717574			
TT	419 (72.5)	171 (72.2)	1.00
CT	140 (24.2)	62 (26.2)	1.08 (0.77-1.53)
CC	19 (3.3)	4 (1.7)	0.52 (0.17-1.55)
CC vs TT+CT (ref)			0.51 (0.17-1.51)
rs352143			
AA	370 (63.5)	144 (60.3)	1.00
AG	180 (30.9)	83 (34.7)	1.19 (0.87-1.64)
GG	33 (5.7)	12 (5.0)	0.92 (0.47-1.83)
AG+GG vs AA (ref)			1.15 (0.85-1.55)
<i>TLR9</i> /+2848			
GG	154 (27.6)	65 (29.6)	1.00
AG	275 (49.3)	118 (53.6)	1.00 (0.70-1.44)
AA	129 (23.1)	37 (16.8)	0.65 (0.40-1.07)
AA vs GG+AG (ref)			0.65 (0.43-0.99)
rs352163			
CC	159 (27.4)	67 (28.3)	1.00
CT	286 (49.3)	128 (54.0)	1.06 (0.75-1.50)
TT	135 (23.3)	42 (17.7)	0.73 (0.46-1.16)
T allele [¶]			0.87 (0.70-1.09)

[¶] Additive genetic model, the genotype was categorized into a three level variable for the number of minor alleles (0,1,2).

Ref: reference genotype coded as 0

TABLE E11. Gene-environment interactions between *CD14*, *TLR2*, *TLR4*, and *TLR9* SNPs and country living before 1 year in asthma outcomes, shown as associations between country living before 1 year and asthma, stratified by genotype

Gene	SNP	Genotype	Living in the country before 1 year, OR (95% CI)		
			Asthma	Asthma in atopic subjects	Asthma in nonatopic subjects
<i>CD14</i>	-260	TT+CT	0.70 (0.48-1.03)	0.92 (0.53-1.60)	0.54 (0.28-1.06)
		CC	0.80 (0.38-1.70)	0.31 (0.09-1.07)	2.30 (0.80-6.66)
	+1341	CC	0.90 (0.57-1.41)	1.13 (0.57-2.27)	0.76 (0.36-1.61)
		AC+AA	0.53 (0.31-0.92)	0.48 (0.21-1.08)	0.83 (0.36-1.92)
	rs778584	CC+CT	0.69 (0.47-1.01)	0.88 (0.51-1.52)	0.58 (0.30-1.09)
		TT	0.90 (0.43-1.86)	0.32 (0.09-1.11)	2.10 (0.75-5.84)
	rs778583	CC	0.83 (0.53-1.30)	1.12 (0.56-2.23)	0.69 (0.33-1.43)
		CT+TT	0.60 (0.36-1.01)	0.44 (0.20-0.97)	1.00 (0.46-2.20)
<i>TLR2</i>	-24438	AA+AG	0.66 (0.46-0.96)	0.85 (0.49-1.46)	0.57 (0.31-1.04)*
		GG	1.41 (0.58-3.46)	0.42 (0.12-1.46)	7.07 (1.42-35.3)
	+596	TT	0.88 (0.45-1.74)	0.42 (0.14-1.22)	2.49 (0.93-6.66)*
		CT+CC	0.69 (0.46-1.05)	1.00 (0.56-1.81)	0.48 (0.24-0.96)
	+1349	TT	0.71 (0.49-1.03)	0.69 (0.41-1.18)	0.95 (0.52-1.75)
		CT+CC	0.79 (0.34-1.86)	1.17 (0.30-4.58)	0.34 (0.10-1.13)
<i>TLR4</i>	rs11536889	GG	0.77 (0.52-1.15)	0.91 (0.50-1.65)	0.83 (0.45-1.53)

		CG+CC	0.60 (0.30-1.18)	0.52 (0.20-1.39)	0.66 (0.22-2.01)
	rs7045953	AA	0.56 (0.38-0.85)	0.64 (0.36-1.15)	0.56 (0.29-1.09)
		AG+GG	1.34 (0.71-2.55)	1.00 (0.37-2.66)	1.73 (0.66-4.57)
<i>TLR9</i>	rs353547	GG+AG	0.84 (0.59-1.21)	1.09 (0.62-1.92)*	0.82 (0.47-1.45)
		AA	0.28 (0.11-0.73)	0.12 (0.03-0.48)	0.80 (0.19-3.30)

Bold: interaction $p < 0.05$, not adjusted for multiple comparisons; * interaction $p < 0.10$, FDR adjusted p value

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