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Genetic variants in endotoxin signalling pathway, domestic endotoxin exposure and asthma exacerbations

Blazenka Kljaic-Bukvic MD^{1*}, Mario Blekic MD^{1*}, Neda Aberle MD PhD¹, John A Curtin PhD²,
Jenny Hankinson PhD², Aida Semic-Jusufagic MD PhD², Danielle Belgrave PhD², Angela
Simpson MD PhD^{2§} and Adnan Custovic MD PhD^{2§}

¹General Hospital "Dr Josip Bencevic" Slavonski Brod, University of Osijek, Croatia

²The University of Manchester, Manchester Academic Health Science Centre, University
Hospital of South Manchester NHS Foundation Trust, Manchester, UK

^{*}Equal contribution

[§]These authors contributed equally to the manuscript and are joint senior authors.

Corresponding author: Adnan Custovic MD PhD, University of Manchester, University
Hospital of South Manchester NHS Foundation Trust, Second Floor, Education and
Research Centre, Manchester M23 9LT, UK

adnan.custovic@manchester.ac.uk, phone +441612915869, fax +441612915730

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ABSTRACT

Background: We investigated the interaction between genetic variants in endotoxin
signalling pathway and domestic endotoxin exposure in relation to asthma presence, and
among children with asthma we explored the association of these genetic variants and
endotoxin exposure with hospital admissions due to asthma exacerbations.

Methods: In a case-control study, we analysed data from 824 children (417 asthmatics, 407
controls; age 5-18 years). Amongst asthmatics, we extracted data on hospitalisation for
asthma exacerbation from medical records. Endotoxin exposure was measured in dust
samples collected from homes. We included 26 single nucleotide polymorphisms (SNPs) in
the final analysis (5 *CD14*, 7 *LY96* and 14 *TLR4*).

Results: Two variants remained significantly associated with hospital admissions with
asthma exacerbations after correction for multiple testing: for *CD14* SNP rs5744455, carriers
of T allele had decreased risk of repeated hospital admissions compared to homozygotes for
C allele (OR [95% CI], 0.42 [0.25-0.88], $p=0.01$, FDR $p=0.02$); for *LY96* SNP rs17226566, C
allele carriers were at a lower risk of hospital admissions compared to T allele homozygotes
(0.59 [0.38-0.90], $p=0.01$, FDR $p=0.04$). We observed two interactions between SNPs in
CD14 and *LY96* with environmental endotoxin exposure in relation to hospital admissions
due to asthma exacerbation which remained significant after correction for multiple testing
(*CD14* SNPs rs2915863 and *LY96* SNP rs17226566).

Conclusion: Amongst children with asthma, genetic variants in *CD14* and *LY96* may increase the risk of hospital admissions with acute exacerbations. Polymorphisms in endotoxin pathway interact with domestic endotoxin exposure in further modification of the risk of hospitalisation.

Key words: asthma, children, *CD14*, *TLR4*, *LY96*, endotoxin, gene-environment interactions

Reprint requests to: Adnan Custovic MD PhD, University of Manchester, University Hospital of South Manchester NHS Foundation Trust, Second Floor, Education and Research Centre, Manchester M23 9LT, UK

INTRODUCTION

Expression of allergic diseases is influenced by the interaction between genes and environmental exposures.¹⁻³ Microbial exposure in early life may induce immune system maturation and prevent development of allergies and asthma.⁴ One of the measurable indicators of environmental microbial exposure is endotoxin (lipopolysaccharide-LPS), a structural component of the outer cell membrane of gram-negative bacteria. In contrast to the protective role of endotoxin in the development of allergic phenotypes,^{5, 6} inhalation of endotoxin can induce bronchial obstruction amongst patients with established asthma.⁷

The endotoxin response pathway has CD14/TLR4/ LY96 (MD2) complex of molecules at its core.^{8, 9} In serum, LPS binds to the serum LPS-binding protein which catalyses transfer of LPS to membrane-anchored pattern recognition receptor CD14. At the surface of cell membrane, LPS binds to Toll-like receptor-4/myeloid differentiation-2 (TLR4/MD2) complex, resulting in the expression of inflammatory gene and production of Th1 inflammatory cytokines.¹⁰

Interaction between polymorphisms in *CD14* gene and environmental endotoxin exposure is one of the most replicated gene-environment interactions in the development of atopy.⁹ However, it is of note that published results vary in the specific SNP implicated in the

reported interaction, occasionally differ in the site and method of the endotoxin measurements, and the direction of interaction effects is not always consistent (summarised in Table E1).⁹ The interactions between endotoxin and polymorphisms in other genes involved in LPS recognition, such as *TLR4* and *LY96* (also known as *MD2* and *ESOP-1*) may also play an important role.¹¹⁻¹⁹ For example, two functional polymorphisms in *TLR4* (rs4986790 and rs4986791) were shown to interact with endotoxin exposure in modifying the risk of atopy and asthma in adults,¹⁹ and the association between occupational endotoxin exposure and non-atopic wheeze in agricultural workers is modified by variants in *LY96*.²⁰ We hypothesized that genetic variants in the *CD14*, *TLR4* and *LY96* modify the relationship between endotoxin exposure and childhood asthma, and among asthmatics the risk of severe asthma exacerbations. To address these hypotheses, we examined the interaction between polymorphisms in these genes and endotoxin exposure in relation to asthma in a case-control study of carefully phenotyped children. Among patients with asthma, we then investigated the association between hospital admissions with severe asthma exacerbations, endotoxin exposure and genetic variants in endotoxin pathway.

METHODS

Study design: Case-control study (described in detail elsewhere^{21, 22}). The study was approved by the local ethics committee, and informed consent was obtained for all participants.

Setting: Catchment area of the General Hospital in Slavonski Brod, Brodsko-Posavska County, Croatia (~2000 square km, stable urban-rural population of Caucasian ancestry).

Participants

Cases: Children with asthma aged 5-18 years were recruited from paediatric asthma clinic if all of the following criteria were met: (1) physician-diagnosed asthma; (2) asthma symptoms within the previous 12 months; and (3) current use of anti-asthma medication.

Controls: Children were selected randomly from patients with non-respiratory conditions attending other hospital departments if all of the following criteria were met: (1) no chronic respiratory symptoms; (2) never diagnosed with asthma; and (3) never received anti-asthma medication (confirmed by interviewer-administered questionnaires). None of the control subjects had a formal diagnosis of other inflammatory conditions which have been reported to be associated with variants in endotoxin signalling pathway.

Cases and controls were not matched by gender and age.

Data sources and definition of variables

Hospital admission with acute asthma: We transcribed all data on acute asthma exacerbations resulting in hospital admissions from hospital records. Children who were admitted to hospital on two or more occasions were considered to have repeated admissions.

Endotoxin exposure: We collected dust samples by vacuuming one-m² area of the child's mattress for two minutes. Endotoxin was measured using a kinetic limulus assay as previously described,²³ and results expressed as endotoxin concentration (EU/mg) and load (EU/m²).

Gene and tagging single nucleotide polymorphisms (SNP) selection and genotyping

Haplotype-tagging SNPs were selected for *CD14*, *TLR4* and *LY96* using GVS (<http://gvs.gs.washington.edu/GVS>), complemented by NCBI's dbSNP (www.ncbi.nlm.nih.gov/projects/SNP) and Haploview, using LD-based tagSNP selection with a pairwise algorithm-LDSelect. SNPs with a minor allele frequency (MAF) $\geq 5\%$ in Caucasian populations were included in the algorithm. We selected LD threshold of $r^2=0.8$. Relevant SNPs reported in the literature were force-included. TagSNPs from all generated bins were selected, and genotyping performed using Sequenom® MassARRAY® iPLEX™. Details of the 30 genotyped SNPs (five in *CD14*, 16 in *TLR4* and nine in *LY96*) are presented in Table E2. Quality control procedures were then applied and redundant SNPs excluded (Online Supplement). Two functional *TLR4* SNPs (rs4986790 and rs498679) were force included.

Statistical Analysis

Statistical analysis was carried out using SVS 7.2.3 (Golden Helix, MT, USA) and SPSS 19. All analyses comparing asthmatics and controls were adjusted for gender and age. For the purposes of multiple comparisons, each gene was analysed separately. In the association analysis between genetic variants and outcomes, for SNPs showing a trend ($p < 0.15$) for association in the additive model, we tested dominant and recessive models. For consistency, all analyses are presented using major allele as reference.

Endotoxin exposure data was log-normally distributed and treated as a continuous variable; the results are expressed as geometric means (GM) and 95% confidence intervals (CIs).

Analysis of gene-environment interactions was carried out using multivariate logistic regression models; we tested dominant and recessive models. In order to display the results graphically, we conducted a stratified analysis, using logistic regression to investigate the effects of endotoxin exposure within each genotype group separately, and produced plots of the predicted probabilities of asthma for different endotoxin exposures.

Correction for multiple comparisons was carried out using Benjamini-Hochberg False Discovery Rate (FDR) method.²⁴ Results are presented as p values (uncorrected and FDR corrected), and odds ratios (OR; unadjusted and adjusted for gender-aOR) and 95% CIs as a measure of effect size.

Statistical power: For gene-environment interactions in the whole population (824 children), we had 88% power to detect a large effect size (OR=2), 42.7% power to detect a medium effect size (OR=1.5), and 6% power to detect a small effect size (OR=1.1). The study was sufficiently powered to detect gene-environment interaction if the effect size exceeded an OR of 1.8 (80% power at the 5% level of significance).

For gene-environment interactions among the 417 children with asthma, the study was sufficiently powered (80%) at the 5% level of significance if the Odds ratio exceeded 2.23.

RESULTS

Participants

We approached 923 children (438 asthmatics, 485 controls), of whom 837 agreed to participate in the study (91%; 423 asthmatics, 414 controls); 824 children provided blood sample for DNA (417 asthmatics and 407 controls). We collected 792 dust samples; endotoxin was measured in 760 (remaining 32 samples had <10 mg dust available).

Demographic characteristics of the study population have been reported previously.^{21, 22}

There was a significant difference in gender between the groups, (the cases were more likely to be boys) and age (controls approximately one year older than cases) with no differences in the area of residence or position in sibship (Table E3). The prevalence of atopy was high among cases (90.5%), with dust mite (78.5%) being the most common sensitising allergen.

Descriptive data

Endotoxin concentration and load correlated significantly ($r=0.7$, $p<0.001$); we observed no material difference in any of the results dependent upon how the endotoxin data were expressed; therefore, we presented environmental endotoxin exposure as concentration (EU/mg). Endotoxin concentration was significantly higher in rural compared to urban/suburban areas (EU/mg; GM [95%CI]:10.83 [9.98-11.75] vs. 8.31 [7.49-9.22], $p=0.04$). The risk of asthma significantly decreased with increasing endotoxin exposure (aOR [95% CI]: 0.75 [0.58–0.98], $p=0.03$).

Genotyping success rates were generally high (>95%); two *LY96* SNPs with a success rate <90% (rs2891354 and rs10504554) and two *TLR4* SNPs (rs10759931 and rs1927906) in high LD with other SNPs ($r^2>90\%$) were excluded (Table E2). We included 26 SNPs (5 in *CD14*, 7 in *LY96* and 14 in *TLR4*) in the final analysis (LD plots are shown in Figure E1).

Association analysis

Asthma presence: We found no associations between five genotyped *CD14* SNPs and asthma (Table E4). One SNPs in *LY96* and one in *TLR4* were associated with asthma, and

for one *LY96* SNP there was a non-significant trend; however, none of these associations remained significant after correction for multiple testing (FDR $p > 0.05$; Table E5).

Hospital admission with acute asthma exacerbation: Among children with asthma, five SNPs (one *CD14*, three *LY96* and one *TLR4*) were significantly associated with hospital admissions due to asthma exacerbations, and for one *CD14* SNP we observed a non-significant trend (Table 1). Two of these associations remained significant after FDR correction (*CD14* SNP rs5744455 and *LY96* SNP rs17226566). For rs5744455, carriers of T allele had decreased risk of repeated hospital admissions compared to homozygotes of C allele (OR [95% CI]: 0.42 [0.25-0.88], $p = 0.01$, FDR $p = 0.02$). Carriers of C allele for rs17226566 were at a lower risk of hospital admissions compared to T allele homozygotes (0.59 [0.38-0.90], $p = 0.01$, FDR $p = 0.04$).

Interaction between genetic variants and endotoxin exposure

Asthma presence: We found statistically significant interactions between two *CD14* SNPs and endotoxin exposure (Table E6). However none of these interactions remained significant after correction for multiple testing (FDR $p > 0.05$). We observed significant interactions between three *LY96* SNPs and endotoxin in relation to asthma (Table E6); none of these interactions remained significant after correction for multiple testing (FDR $p > 0.05$). Of note, for *LY96* SNP rs7822054 there was a strong trend which failed to reach formal statistical significance (FDR $p = 0.06$), suggesting that increasing endotoxin exposure may be protective amongst carriers of A allele, whereas the risk of asthma tended to increase with increasing endotoxin exposure amongst homozygotes for G allele.

Hospital admission with acute asthma exacerbation: Amongst asthmatic children, we observed significant interactions with environmental endotoxin exposure for seven SNPs (three in *CD14*, one in *LY96* and three in *TLR4*) in relation to repeated hospital admissions due to asthma exacerbation (Table 2). Two described interactions remained significant after correction for multiple testing (*CD14* SNPs rs2915863 and *LY96* SNP rs17226566, Table 2). Increasing endotoxin exposure tended to increase the risk of hospitalisation among T allele

carriers of *CD14* SNP rs2915863, but was protective among minor (C) allele homozygotes (Figure 1a). For *LY96* SNP rs17226566, the risk of hospital admissions increased with increasing endotoxin exposure in children who were major (T) allele homozygotes, but decreased with increasing endotoxin exposure among C allele carriers (Figure 1b).

DISCUSSION

Principal Findings

To the best of our knowledge, our study is the first to demonstrate a significant effect of polymorphisms in *CD14* and *LY96* amongst asthmatic children in relation to hospital admissions with acute asthma exacerbations. Children who were carriers of T allele in *CD14* SNP rs5744455 had decreased higher risk of hospital admission compared to homozygotes of C allele; we observed similar decrease in risk among C allele carriers for *LY96* SNP rs17226566. These associations remained significant after correction for multiple testing. Among patients with asthma, we also observed novel significant interactions between variants in endotoxin pathway and endotoxin exposure in relation to repeated hospital admissions. Two such interactions remained significant after correction for multiple testing (*CD14* SNP rs2915863 and *LY96* SNP rs17226566). As an example, endotoxin exposure increased the risk of hospital admissions amongst asthmatic children who were T allele homozygotes of *LY96* SNP rs17226566, but tended to be protective amongst C allele carriers.

Limitations

One of the limitations of the current study is that cases and controls were not matched by gender and age. Therefore, all our case-control analyses were adjusted for gender and age. Even though we have tried to comprehensively cover the endotoxin signalling pathway, we acknowledge that our list of SNPs is not definitive. Our approach is likely to have resulted in missing of rare variants, which may have an important effect. However, considerably larger sample size would be required for a credible investigation of the effect of rare variants.

We measured current endotoxin exposure. We acknowledge that it is possible that this measure is more relevant “index of exposure” in relation to asthma severity, than to asthma development (for this outcome, measurement of early-life exposure may be more appropriate). However, we note that we and others have previously reported a good correlation between endotoxin measured in early-life and in school-age.^{23, 25}

We also acknowledge that we have limited power to detect gene-environment interactions. Another limitation is lack of replication; however, we were unable to identify another comparable population of asthmatic children with measured domestic endotoxin and detailed history of hospitalisations to replicate our findings. Thus, the results need to be interpreted with caution.

Interpretation

We carried out a comprehensive investigation of known common genetic variants within the endotoxin pathway. We used candidate gene approach, with *a priori* biologically plausible hypotheses. For clarity, and in order to make our data easily comparable with previous studies (Table E1), we have summarised our findings in Table E7. In our study, we observed a novel gene-endotoxin exposure interaction in relation to asthma exacerbations for *LY96* SNP rs17226566, which remained significant after correction for multiple comparisons. *LY96* (MD2) functionally modulates TLR4 signalling by inhibiting the formation of an active *LY96*-TLR4 signalling complex, and is an important negative regulatory component of the LPS signalling pathway.²⁶ The essential role of *LY96* in LPS trans-membrane signalling has been confirmed by studies demonstrating LPS hypo-responsiveness in cells that expressed TLR4 alone, or *TLR4* associated with mutant *LY96*; the state of reduced responsiveness could be reverted by transfection with *LY96* cDNA or soluble *LY96* protein.^{27, 28} It has been demonstrated that high level of airborne endotoxin exposure was protective against non-atopic wheeze among agricultural workers with *LY96* rs10808798 CC genotype.²⁰

In our study none of the *CD14* SNPs, including functional polymorphisms rs2569190, were significantly associated with asthma, and there was no significant interaction with endotoxin

exposure in relation to asthma presence. However, among children with asthma we observed statistically significant interaction between variants in *CD14* and current endotoxin exposure in relation to hospital admissions with severe acute asthma exacerbations. Possible explanations include the cross-sectional study design and measurement of endotoxin exposure at school age (as previously noted, for asthma development it is likely that early-life endotoxin exposure is more relevant than exposure in later childhood). In summary, complex interactions between endotoxin exposure and variants in endotoxin signalling pathway genes modify the risk of asthma. We provide evidence that amongst asthmatic children, genetic variants in *CD14* and *LY96* may increase the risk of hospital admissions with acute asthma exacerbations, and that polymorphisms in endotoxin pathway interact with domestic endotoxin exposure in further modifying the risk of hospitalisation. Our findings may facilitate specific targeting of susceptible individuals to reduce the risk of asthma exacerbations through interventions aimed at reducing personal endotoxin exposure (which may also be of relevance in occupational setting, where such exposure is often high).²⁹

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Table 1. The association of *CD14*, *LY96* and *TLR4* SNPs with hospital admissions due to severe asthma exacerbation among children with asthma

rs number	Gene	p value for additive model	Genotype	N	aOR (95%CI)	p value	FDR p value
rs5744455	<i>CD14</i>	0.08	TT+TC vs CC.	196/215	0.42 (0.25-0.88)	0.01	0.02
rs4914	<i>CD14</i>	0.1	CC vs CG+GG	8/407	1.37 (0.92-2.05)	0.12	0.31
rs11786591	<i>LY96</i>	0.02	TT+TC vs.CC	198/201	1.58 (1.10-2.29)	0.01	0.09
rs17226566	<i>LY96</i>	0.01	CC+TC vs.TT	160/245	0.59 (0.38-0.90)	0.01	0.04
rs7822407	<i>LY96</i>	0.12	AA vs.GG+AG	9/398	0.16 (0.02-1.21)	0.02	0.10
rs11536897	<i>TLR4</i>	0.04	AA+AG vs. GG	41/368	0.48 (0.22-1.04)	0.04	0.57

Table 2. Interactions between variants in *CD14*, *LY96* and *TLR4* and endotoxin exposure in relation to repeated hospital admissions (two or more) with acute severe asthma exacerbations among asthmatic children.

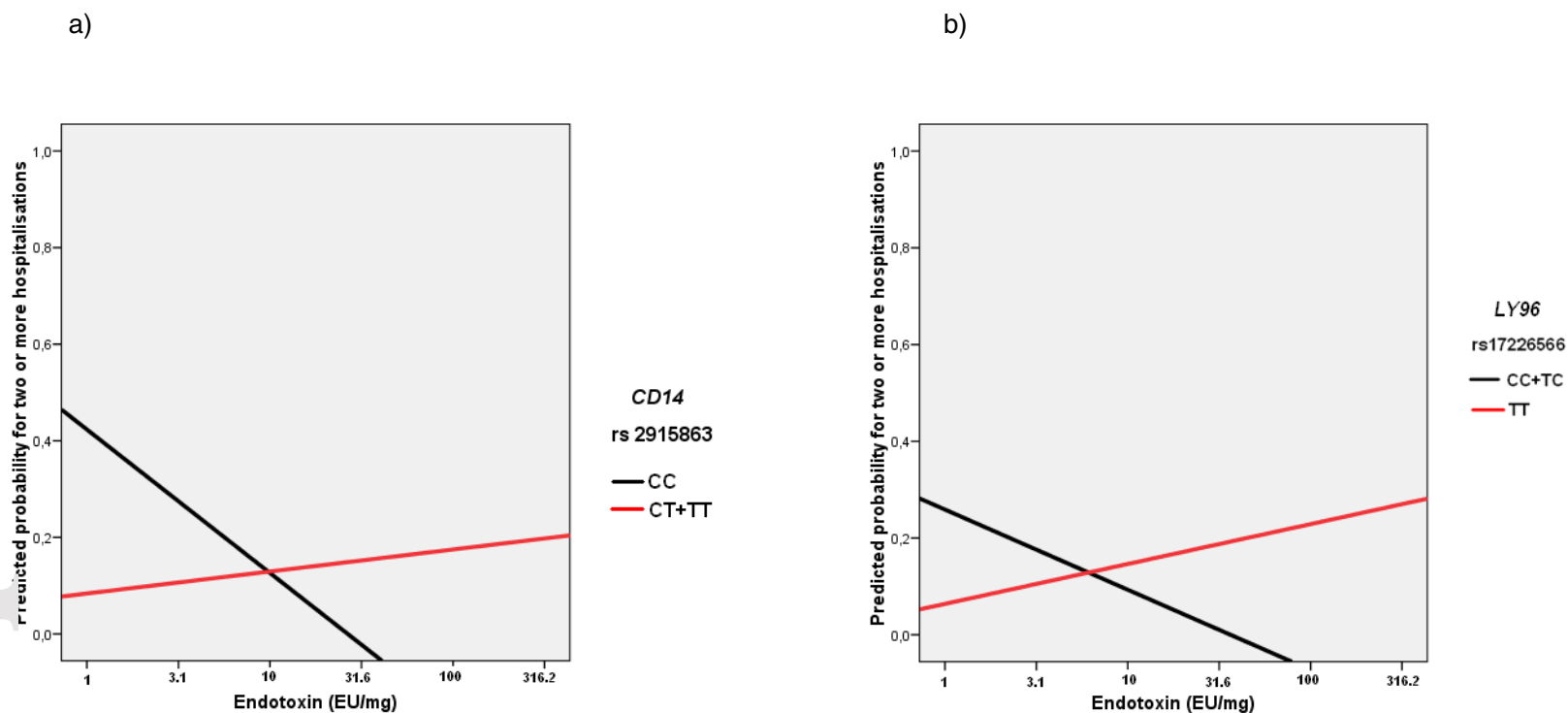
Odds ratios in the stratified analysis are listed for specific genotype groups

rs number	Gene	N	Cases with asthma			Alleles*	Additive				Recessive				Dominant			
			0	1	2		OR	95%CI	p	FDR	OR	95%CI	p	FDR	OR	95%CI	p	FDR
rs2567190	<i>CD14</i>	415	8	101	306	C/G	4.12	0.9-15.3	0.02	0.14	15.2	0.24-1112	0.1	0.14	4.8	1.04-24.9	0.05	0.26
rs2915863	<i>CD14</i>	397	75	201	121	C/T	0.36	0.08-1.31	0.05	0.09	0.15	0.02-1.05	0.04	0.11	0.24	0.06-0.93	0.03	0.08
rs17226566	<i>CD14</i>	406	60	197	149	T/C	0.38	0.00-0.52	0.04	0.09	0.01	0.00-0.62	0.01	0.03	0.36	0.07-1.6	0.2	0.33
rs12377632	<i>LY96</i>	405	25	135	245	T/C	0.12	0.04-0.43	0.02	0.11	3.24	0.01-2049	0.72	0.72	0.06	0.006-0.49	0.01	0.03
rs11536897	<i>TLR4</i>	406	60	201	145	T/C	2.64	1.06-66.1	0.09	0.32	2.56	0.33-22.77	0.39	0.39	8.15	1.16-66.26	0.03	0.46
rs11536857	<i>TLR4</i>	409	1	40	368	G/A	11.29	1.1-123.5	0.03	0.24	ND	ND	ND	ND	11.3	1.11-128.34	0.03	0.24
	<i>TLR4</i>	410	3	53	354	C/T	0.05	0.05-2.02	0.03	0.41	ND	ND	ND	ND	13.89	0.71- 272.6	0.05	0.24

ND -not determined (minor allele frequency too low)

0, 1, 2- represent number of genotypes for minor, heterozygotes and major genotypes respectively *common allele is listed first

Figure 1. Fitted predicted probability curves for repeated hospital admissions with asthma exacerbations in relation to environmental endotoxin exposure in asthmatic children with different genotypes in: (a) *CD14* SNP rs2915863 and (b) *LY96* SNP rs17226566.



Genetic variants in endotoxin signalling pathway, domestic endotoxin exposure and asthma exacerbations

Blazenka Kljaic-Bukvic MD^{1*}, Mario Blekic MD¹, John A Curtin PhD², Neda Aberle MD PhD¹,
Jenny Hankinson PhD², Aida Semic-Jusufagic MD PhD², Danielle Belgrave PhD², Angela
Simpson MD PhD^{2*} and Adnan Custovic MD PhD^{2*}

¹General Hospital “Dr Josip Bencevic” Slavonski Brod, University of Osijek, Croatia

²The University of Manchester, Manchester Academic Health Science Centre, University
Hospital of South Manchester NHS Foundation Trust, Manchester, UK

*Equal contribution

ONLINE SUPPLEMENT

METHODS

Gene and tagging SNP Selection and LD filtering

In this study, a method of LD-based tagSNP selection was used to choose SNPs for genotyping, as this provides several benefits above typing only a few selected SNPs per gene¹. TagSNPs were selected for each gene using the Genome Variation Server GVS (<http://gvs.gs.washington.edu/GVS/>). This was complemented by data on NCBI's dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/). Haploview was also used to complement tagSNP selection² (www.broadinstitute.org/haploview/haploview).

TagSNP selection was based on samples of populations of European ancestry to match the sample of subjects included in this study. Selection was carried out using a pairwise algorithm developed by Carlson et al. (LDSelect)¹ available in GVS and Haploview² (Tagger)³. A chromosomal region including the gene and 2500 bp upstream and 1500 bp downstream (chosen to try to capture the 5'UTR [promoter] and 3'UTR) was searched and all SNPs identified in populations of European descent with a minor allele frequency (MAF) $\geq 5\%$ were included in the algorithm. This algorithm generates a number of LD bins, each with a corresponding tagSNP (or a choice of more than one where applicable) based on user-defined criteria that include an LD measure threshold $-r^2$ or D' . An LD threshold of $r^2=0.8$ was selected for this study, as this is a generally accepted compromise between optimal variation coverage with a maximally informative set of tagSNPs and reduced genotyping effort. Relevant SNPs reported in the literature were force-included as tagSNPs in the algorithm. TagSNPs from all generated bins (including singleton bins)⁵ were selected for genotyping. Where more than one tagSNP was available for a particular bin, preference was given to exonic, intronic SNPs, missense, frameshift 5'UTR or 3'UTR vs. silent mutations, and SNPs from non-repeat vs. repeat regions. In these cases the algorithm was re-run in Haploview^{2,3}, where the relevant SNPs were force-included as tagSNPs. Additionally, in an attempt to account for genotype failures some redundancy was included and more than one tagSNP was selected from each bin wherever possible⁶. In addition to the tagSNPs

generated by the LDSelect algorithm, all coding missense frameshift5' or 3'UTR SNPs with MAF below the defined cut-off of 5% were appended to the list of SNPs to type as these could potentially be associated with relevant functional changes. Moreover, any additional SNPs previously reported as associated with asthma or related traits not included in the list generated by the method described above (MAF<5%) were also appended to the list of SNPs to genotype. After evaluation of genotyping results all SNPs were subject to quality control measures, excluding all SNPs with a genotype success rate<85% and/or Hardy-Weinberg Equilibrium $p < 0.001$. Genotyping data including all the genes selected in the *CD14*, *LY96* and *TLR4* were uploaded into Haploview and the same methodology was used as for tag SNP selection as described earlier (r^2 cut-off at 0.8).

Measurement of environmental exposure to endotoxin

We collected dust samples from mattress by vacuuming a 1m² area for 2 minutes in a standardised fashion using a dust sampler (airflow 45 l/s). The sampling head was loaded with a stainless steel mesh filter to remove particles >300µm diameter allowing fine dust to be collected onto a 5 µm pore size vinyl filter. Dust sample was transferred into pre-weighed petri dishes and coded. The filled petri dish was weighed to calculate the mass of fine dust collected and stored at 4°C until extraction.

Endotoxin content was measured by a kinetic limulus assay. Briefly, 50 mg of fine dust were extracted by shaking in pyrogen-free tubes with 5-ml endotoxin-free water for 1 hour at room temperature. Following centrifugation (3000 rpm. 30 min) aliquots (100 µl. 1:100-1:100.000) were transferred to a 96 wells microtiter plate and 100 µl of Limulus Amebocyte Lysate were added. Optical densities were read every 30s at 405 nm for 1 hr (BIO-TEK Instruments). Results were calculated from a standard curve run on each plate using Biolise software (Charles River Endosafe). Results were expressed as endotoxin load (EU/m²) and endotoxin concentration (EU/ml).

Table E1. Summary of studies reporting interaction between measured endotoxin exposure and innate immunity genes

Summary of studies reporting interaction between measured endotoxin exposure and innate immunity genes									
Author, year, acronym, country	Population and sample size	Study design	Gene studied	SNPs studied	Definition of exposure; units	Interaction between genotype and endotoxin			
						Allergic sensitisation	Asthma	Eczema	Compared to our study
Werner, ⁴ 2003, ECRH Study, Germany	Caucasian, N=334	Case-control	<i>TLR4</i>	rs4986790 A/G rs4986781C/T	Dust from living room floor Tertiles: first tertile <8.152 EU/m ² second 8.152-32.533 EU/m ² third >32.533 EU/m ²	Not reported	Increased risk for asthma among homozygotes for A allele in the second tertile of endotoxin exposure. and for wheeze in the third tertile.	Not reported	Genotyped but no interaction
Eder, ⁵ 2004, ALEX Study, Germany and Austria	Not reported, N=609	Children living on farms and controls raised in the same area but not on farm, cross-sectional study	<i>TLR2</i> <i>TLR4</i>	-16934 A/T +596 C/T +1349 T/C -6143 A/G -5724 T/C +7263 G/C +8469 T/C +4434 A/G (rs4986790 A/G)	Dust from children mattress, median 27.4 EU/mg (IQR 24.0 EU/mg)	Farmers children who are carriers of T allele -16934 in <i>TLR2</i> gene had less atopy- Homozygotes for A allele +4434 in <i>TLR4</i> exposed to high (>50.ct) endotoxin concentrations, had less atopy.	Farmers children who are carriers of T allele -16934 in <i>TLR2</i> gene had less asthma, compared to non-farmers children.	Not reported	Genotyped functional polymorphisms but no interaction.

Zambelli-Weiner, ⁶ 2005, BAGS, Barbados	Of African descent/European admixture, N=443	Asthmatic probands and their families (adults and children)	CD14	rs2569190 C/T	Living room composite (75 th percentile) GM 23 144 EU/m ²)	Not reported	More common among TT homozygotes with high exposures	Not reported	Genotyped but no interaction
Eder, ⁷ 2005, ALEX, Germany and Austria	Not reported, N=624	Children living on farms and controls raised in the same area but not on farm, cross-sectional	CD14	rs2569190 C/T	Dust from children mattress, GM in tertiles (2 nd tertile 12 495-30 046 EU/m ²)	Highest endotoxin tertile associated with decreased risk among carriers of C allele	Not reported	Not reported	Genotyped but no interaction
Simpson, ⁸ 2006, MAAS, UK	Mixed European ancestry only, N=442	Population based birth cohort study, age 5 years	CD14	rs2569190 C/T	Living room floor GM 2856 EU/m ² (16.1 EU/mg)	Higher endotoxin associated with decreased risk among CC homozygotes	Higher endotoxin associated with increased risk of non-atopic wheeze among CC homozygotes	Higher endotoxin associated with decreased risk among CC homozygotes	Genotyped but no interaction
Williams, ⁹ 2006, WHEALS mothers, USA	All races, N=517	Mother of population based birth cohort	CD14	rs2569190 C/T	Dust from the home at child's age 1 month GM 16.2 EU/mg	Higher endotoxin associated with lower total IgE, among CC homozygotes	Not reported	Not reported	Genotyped but no interaction
Williams, ¹⁰ 2008, WHEALS, USA	All races, N=90	Population based birth cohort study, age 1 year	CD14	rs2569190 C/T	Dust from the home at age 6 months, GM 18.2 EU/mg	Higher endotoxin associated with lower IgE among CC homozygotes	Not reported	Not reported	Genotyped but no interaction

		rs4986790 A/G rs4986791 C/T rs11536889 G/C LY96 rs10808798 T/C rs11786591 C/T rs16938758 A/T rs17226566 T/C rs7838114 G/A MYD88 rs7744 A/G rs6853 A/G	rs10808798: Carriers of T allele at increased risk of non-atopic wheeze with increasing endotoxin exposure.	LY96 rs10808798 not genotyped in our study, but this SNP is in the same bin as SNP rs7822054 (non- significnat trend) Not genotyped
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Table E2. SNPs selected for genotyping on *CD14*, *LY96* and *TLR4* genes

*common allele is listed first

Marker	Chr	Gene	Position	SNP type/ Function	Alleles*	MAF	Published MAF	Call rate	HWE p controls
rs2563298	5	<i>CD14</i>	139991498	near-gene-3	C/A	0.28	0.24	0.95	0.46
rs4914	5	<i>CD14</i>	139991651	coding-synon	C/G	0.14	0.1	0.98	0.36
rs2569190	5	<i>CD14</i>	139993099	intron.5 UTR	C/T	0.46	0.48	0.92	0.24
rs5744455	5	<i>CD14</i>	139993490	near-gene-5	C/T	0.26	0.01	0.96	0.38
rs2915863	5	<i>CD14</i>	139994560	near-gene-5	T/C	0.41	0.5	0.95	0.55
rs7822054	8	<i>LY96</i>	75070343	intron 1.	A/G	0.44	0.44	0.96	0.10
rs7822407	8	<i>LY96</i>	75070366	intron 1.	G/A	0.17	0.11	0.96	0.04
rs11786591	8	<i>LY96</i>	75078897	intron 1.	C/T	0.27	0.36	0.94	0.19
rs1991262	8	<i>LY96</i>	75081882	intron 1.	G/C	0.25	0.24	0.98	0.01
rs10504554	8	<i>LY96</i>	75082266	intron 1.	T/C	0.20	0.14	0.78	0.01
rs17226566	8	<i>LY96</i>	75082465	intron 1.	T/C	0.23	0.27	0.96	0.41
rs17324476	8	<i>LY96</i>	75088835	intron 2.	C/T	0.01	0.05	0.97	0.92
rs7838017	8	<i>LY96</i>	75098252	intron 2.	C/T	0.18	0.13	0.94	0.11
rs2891354	8	<i>LY96</i>	75103559	intron 3.			0.10	0.00	
rs11536857	9	<i>TLR4</i>	119503956	Intergenic	C/T	0.08	0.06	0.95	0.29
rs10759931	9	<i>TLR4</i>	119503967	near-gene-5	G/A	0.42	0.38	0.95	0.75
rs11536869	9	<i>TLR4</i>	119509652	intron 1.	A/G	0.03	0.16	0.96	0.70
rs1927911	9	<i>TLR4</i>	119509874	intron 1.	C/T	0.23	0.29	0.96	0.76
rs11536878	9	<i>TLR4</i>	119511373	intron 1.	C/A	0.12	0.13	0.96	0.20
rs12377632	9	<i>TLR4</i>	119512550	intron 2.	T/C	0.41	0.36	0.95	0.37
rs1927907	9	<i>TLR4</i>	119512584	intron 2.	G/A	0.09	0.14	0.95	0.86
rs2770146	9	<i>TLR4</i>	119513158	intron 2.	A/G	0.28	0.34	0.94	0.88
rs2149356	9	<i>TLR4</i>	119514019	intron 2.	C/A	0.31	0.29	0.96	0.16
rs4986790	9	<i>TLR4</i>	119515122	Missense	A/G	0.07	0.4	0.96	0.42
rs4986791	9	<i>TLR4</i>	119515422	Missense	C/T	0.08	0.4	0.96	0.10
rs11536889	9	<i>TLR4</i>	119517951	untranslated-3	G/C	0.13	0.12	0.96	0.32
rs7873784	9	<i>TLR4</i>	119518756	untranslated-3	G/C	0.15	0.16	0.94	0.72
rs11536897	9	<i>TLR4</i>	119519830	near-gene-3	G/A	0.06	0.07	0.95	0.73
rs1927906	9	<i>TLR4</i>	119519935	near-gene-3	A/G	0.09	0.05	0.98	0.82
rs1554973	9	<i>TLR4</i>	119520632	Intergenic	T/C	0.24	0.20	0.98	0.50

rs10504554 and rs2891354 were excluded from analysis due to low call rate; rs10759931 and rs1927906 were excluded from analysis because they were in high LD with other SNPs.

RESULTS

Table E3. Demographic characteristics of the study population

		CASES (N=423)	CONTROLS (N=414)	p value
AGE (years): mean (95%CI)		11.1 (10.8-11.4)	12.2 (11.9-12.6)	<0.0001
GENDER	Male	248 (58.6%)	178 (43.0%)	<0.0001
ATOPY	Atopic	383 (90.5%)	73 (17.7%)	<0.0001
	SPT mite	332 (78.5%)	43 (10.4%)	<0.0001
FAMILYHISTORY				
Asthma	Maternal	37 (8.7%)	4 (1.0%)	<0.001
	Paternal	27 (6.4%)	9 (2.2%)	0.003
SIBLINGS				
	Older	239 (56.5%)	242 (58.9%)	0.49
	Younger	253 (59.8%)	238 (57.9%)	0.58
AREA OF RESIDENCE				
Current	urban	195 (46.1%)	186 (45.3%)	0.81
	rural	228 (53.9%)	225 (54.3%)	
At birth	urban	196 (46.3%)	182 (44.3%)	0.55
	rural	227 (53.7%)	229 (55.3%)	
ENDOTOXIN, GM (95%CI)				
	load (EU/m ²)	5 317.24 (5 723.4-7 430.6)	6 521.4 (4 670.3-6 053.7)	0.03
	concentration(EU/mg)	9.32 (8.49-10.22)	9.92 (8.49-10.22)	0.35

The following SNPs were excluded from the analysis:

- Two with a success rate<90%: rs2891354 and rs10504554 on LY96

- Two because of high LD with other SNPs (SNP selection included built-in redundancy in anticipation of some genotyping failure): rs10759931 and rs1927906 on *TLR4*
- Two SNPs which are known to be functional were forced included despite LD>0.9 with other SNPs: 2 SNPs on *TLR4* (rs4986790 and rs498679)

Table E4. The association of *CD14* SNPs and asthma (additive model); cases vs. controls, adjusted for age and gender. As there were no associations and no trends seen in additive models, no other models were explored.

*Reference category—homozygotes for major allele

rs number	Genotype	N	p value for additive model
rs2563298	CC	57	0.93
	CA	328	
	AA*	413	
rs4914	CC	13	0.88
	CG	204	
	GG*	602	
rs2569190	CC	153	0.25
	TC	398	
	TT*	218	
rs5744455	CC	49	0.52
	CT	317	
	TT*	435	
rs2915863	TT	129	0.38
	TC	393	
	CC*	274	

Table E5. The association of *LY96* and *TLR4* SNPs with asthma (cases vs. controls)

rs number	Gene	p value for additive model	Genotype	N	aOR (95%CI)	p value	FDR p value
rs11786591	<i>LY96</i>	0.12	TT+TC vs. CC	362/422	1.32 (0.99-1.77)	0.06	0.40
rs1991262	<i>LY96</i>	0.14	CC vs. CG+GG	63/756	0.55 (0.32-0.95)	0.03	0.09
rs1927907	<i>TLR4</i>	0.017	AA+AG vs. GG	138/660	1.50 (1.02-2.19)	0.04	0.53

Table E6 Interactions between variants in *LY96* and endotoxin exposure in relation to asthma presence (cases vs. controls)

*Odds ratios adjusted for gender and age in the stratified analysis for specific genotype groups

rs number	Gene	p value for additive model	Genotype	N	aOR (95%CI)	p value	FDR p value
rs7822054	<i>LY96</i>	0.02	GGvs AA+AG	715	3.54 (1.31-9.56)	0.01	0.06
rs7822407	<i>LY96</i>	0.02	GG+AG vs AA	720	1.92 (1.07-3.43)	0.03	0.20
rs7838017	<i>LY96</i>	0.04	CC+CT vs TT	698	1.65 (0.94-2.89)	0.08	0.29
rs2563298	<i>CD14</i>	0.08	CC vs AC+AA	665	1.79 (1.01-3.16)	0.04	0.22
Rs4914	<i>CD14</i>	0.05	CC vs CG+GG	665	1.80 (0.99-3.29)	0.05	0.13

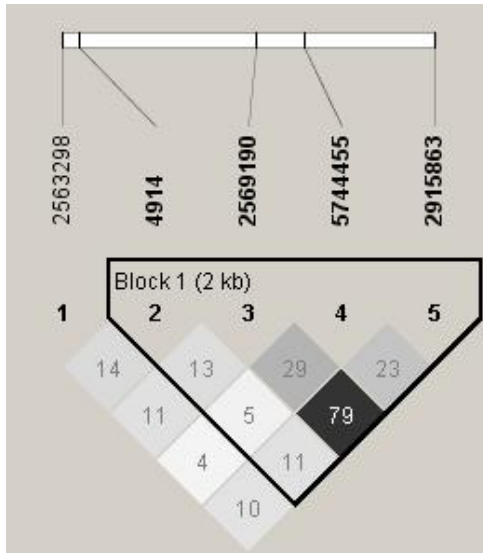
Table E7. Summary of findings in the current study (Croatian Asthma and Genetic Study): statistically significant interactions between SNPs in CD14, LY96 and TLR4 and measured endotoxin in mattress dust.

Results are presented for asthma presence (N=837 Caucasian children age 5-18 years, case-control study) and asthma hospitalisations (, n= 417 Caucasian children age 5-18 years, asthma cases only)

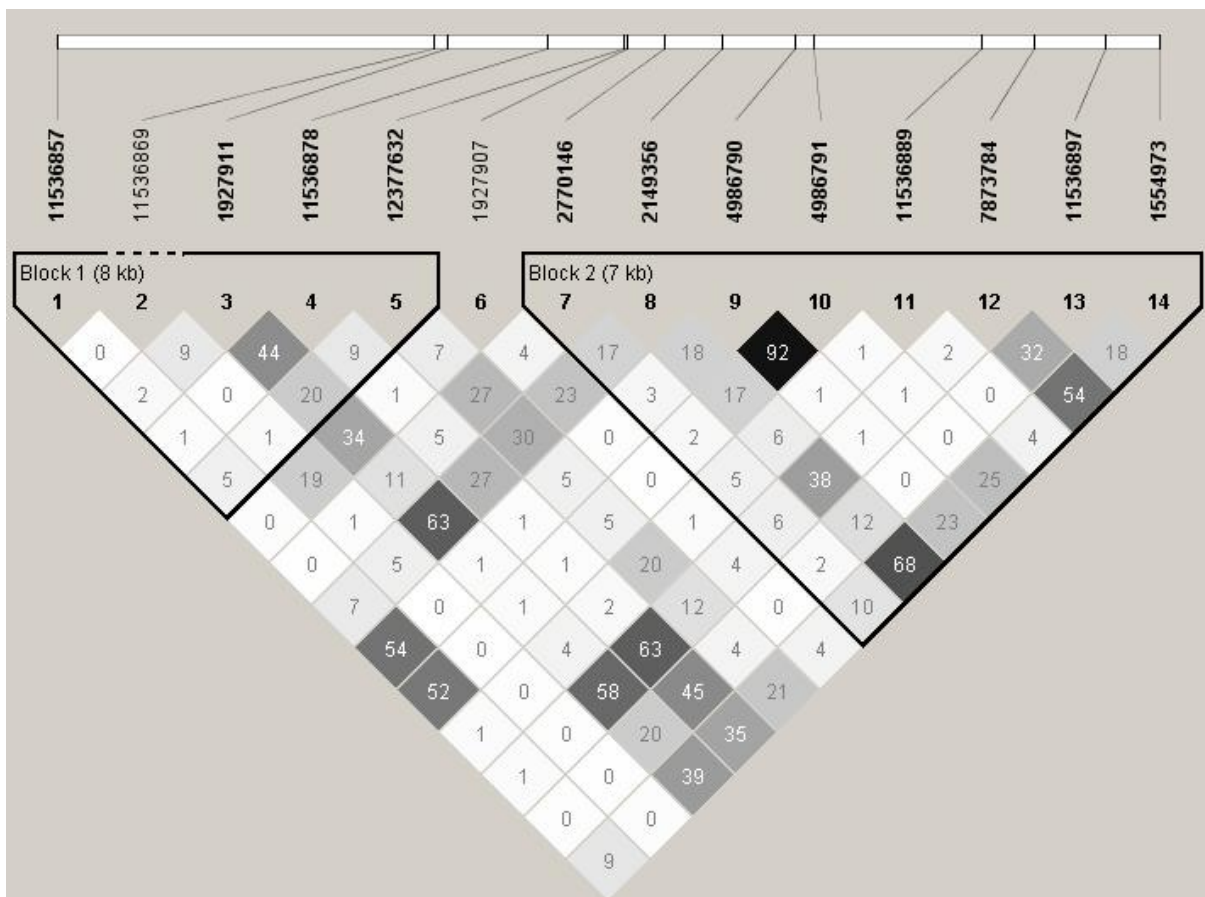
Gene and studied SNPs studied	Asthma presence (case-control study)	Asthma hospitalisations (among asthmatic children)
CD14 rs2563298 C/A rs4914 C/G rs2569190 C/T rs5744455 C/T rs2915863 T/C LY96 rs7822054 A/G, rs7822407 G/A rs11786591 C/T, rs1991262 G/C rs10504554 T/C, rs17226566 T/C rs17324476 C/T, rs7838017 C/T rs2891354 TLR4 rs11536857 C/T, rs10759931 G/A rs11536869 A/G, rs1927911 C/T rs11536878 C/A, rs12377632T/C rs1927907 G/A, rs2770146 A/G rs2149356 C/A, rs4986790A/G rs4986791 C/T, rs11536889 G/C rs7873784 G/C, rs11536897 G/A rs1927906 A/G, rs1554973 T/C	CD14 No statistically significant interactions LY96 No statistically significant interactions TLR4 No statistically significant interactions	CD14 rs2915863: Endotoxin exposure increases the risk among T allele carriers; endotoxin exposure protective among minor (C) allele homozygotes LY96 rs17226566: Endotoxin exposure increases the risk among T allele homozygotes; endotoxin exposure protective among C allele carriers TLR4 No statistically significant interactions

Figure E1. Linkage disequilibrium plot for analysed SNPs in (a) *CD14*, (b) *TLR4* and (c) *LY96* showing pairwise r^2 values

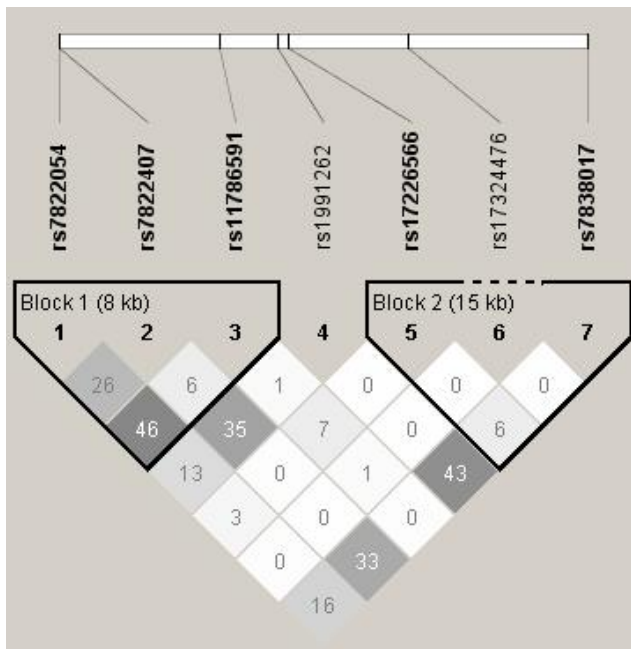
a)



b)



c)



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