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Association of Toll-like receptor 4 alleles with symptoms and sensitization to laboratory animals

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Abstract

Background—Researchers and technicians working with laboratory animals (LAs) are exposed to animal allergen and endotoxin, which can interact to potentiate or inhibit symptoms or allergic responses. We hypothesized that functional genetic variants of Toll-like receptor 4 (TLR4), a key surface receptor for endotoxin, interface between worker and workplace and affect animal sensitization, symptoms, or both.

Objective—We sought to determine whether *TLR4*/8551 variants alter the risk for LA sensitization, symptoms, or both.

Methods—Three hundred thirty-five researchers, 195 of whom worked with animals, completed questions on workplace practices and symptoms and underwent skin prick tests or RASTs to common and animal allergens. Real-time PCR assessed *TLR4*/8551 and *TLR4*/8851 variants. Nominal logistic regression was used to analyze the contribution of demographic, exposure, and genetic variables to outcomes of interest.

Results—Twenty-one percent of workers were LA sensitized, and 29% reported 1 or more symptoms to LAs. The *TLR4*/8551 G variant, which is less responsive to endotoxin, was detected in 9% and in linkage disequilibrium with the *TLR4*/8851 T allele. The G variant significantly associated with atopy and LA sensitization. Workers with the G variant spent significantly longer hours in high endotoxin/animal allergen tasks compared with those with the AA variant, which is perhaps less affected by endotoxin exposures. In multivariate analyses the G variant and longer animal research hours increased the risk of LA sensitization. Job tasks and LA sensitization, but not *TLR4* variants, were predictors of LA-induced symptoms.

Conclusion—Workers with *TLR4* variants that reduce responsiveness to endotoxin have higher risks for LA and other allergen sensitization but spend longer hours in tasks with high endotoxin and animal allergen exposures.

Keywords

Laboratory animal; allergy; asthma; Toll-like receptor 4; endotoxin

The laboratory animal (LA) workplace is a bioaerosol-rich environment characterized by a complex mixture of animal allergens, endotoxin, particulates, volatile organic compounds, ammonia, and other substances encountered both separately and together on the same particles. We and others have detected moderate-to-high levels of airborne animal allergen in this workplace and more modest levels of airborne endotoxin^{1,2} that might play a role in both symptoms and sensitization to laboratory animals. Evidence from this³ and other endotoxin- and allergen-rich work environments, such as poultry farms and hog-confinement barns, shows that endotoxin exposure elicits upper and lower respiratory symptoms, such as rhinorrhea, eye and nose irritation, cough, wheeze, and chest tightness, and is associated with airflow limitation. From 20% to 50% of LA workers report respiratory symptoms, skin symptoms, or both when handling laboratory animals,^{4,5} although fewer, 10% to 20% of employees, are sensitized to laboratory animals based on skin tests or RASTs.^{6–8}

Animal models⁹ have demonstrated that in addition to symptoms, endotoxin also affects the process of allergic sensitization. Coexposure to endotoxin and allergen can either enhance or inhibit the allergic process, depending on the timing and dose of endotoxin. Toll-like receptor 4 (TLR4) is the predominant and best-characterized cell-surface receptor specific for endotoxin that also might modulate the allergic response.^{10–12} Mice deficient in TLR4 and challenged with ovalbumin have increased numbers of lung dendritic cells and higher titers of IgE and airway eosinophils, all markers of atopic asthma,¹³ compared with wild-type mice with normal levels of TLR4. Immune downregulation by TLR4 is mediated in part through regulatory T cells, and LPS treatment increases CD4⁺CD25⁺ cell suppressor efficiency by 10-fold, without requiring antigen-presenting cells.¹⁴ We questioned whether the ability to respond to airborne endotoxin in conjunction with allergen could similarly affect risk for sensitization to LAs in this work environment.

Several functional variants of *TLR4* show reduced responsiveness to endotoxin.^{15,16} Carriers of the *TLR4*/8551 minor G variant (the Asp299Gly protein site) have less bronchospasm to endotoxin inhalation and release less IL-1 β and TNF- α from airway epithelial cells stimulated with endotoxin *in vitro*.¹⁵ They have greater atopy scores based on the number of positive skin prick test responses and specific IgE levels¹⁷ but are less likely to have asthma in high endotoxin environments.¹⁸ Together, these studies suggest that subjects carrying the less responsive *TLR4*/8551 G variant might be less efficient at limiting the allergic response or inducing tolerance.

Because different genes are functionally important in different environments and the LA workplace is characterized by both endotoxin and allergen exposures, we hypothesized that carriers of the *TLR4*/8551 minor G variant would be more likely to have allergic

sensitization but less likely to have respiratory symptoms in endotoxin-rich environments. This article will focus primarily on the effect of the *TLR4*/8551 polymorphism (the Asp299Gly protein sequence) and secondarily on the *TLR4*/8851 polymorphism (the 399 protein sequence), 2 of the best-characterized and functional endotoxin response elements, on the risk for symptoms and sensitization to LAs.

METHODS

Description of the cohort

We recruited all workers in a major research institution between 18 and 70 years of age who performed research (n = 311) or worked as an animal handler (n = 24). The study was approved by the institutional review board, and all subjects provided informed consent. Subjects completed a previously validated, American Thoracic Society–based questionnaire³ that elicited demographic information, past work history, current job tasks, and allergy and smoking history. Subjects were asked to report nasal, chest, and skin symptoms associated with work with specific animals and with specific tasks. Chest symptoms associated with methacholine reactivity in a prior study^{19,20} were assessed. Skin prick testing was performed on 314 (94%) subjects, and 10 mL of blood or a buccal sample was provided for DNA analysis. Those who could not complete skin testing (n = 21, 6%) provided blood for RASTs.

Symptom characterization

Subjects were considered symptomatic to LAs if they ever reported nasal symptoms (itchy, runny, or stuffy nose or sneezing), chest symptoms (cough, chest tightness, wheeze, or shortness of breath), or skin rash when working with specific LAs. The same symptoms were elicited during specific job tasks: cage changing, cleaning, or dumping; experimental work in the animal facility or in individual research laboratories with LAs; and research without LAs.

Sensitization measures

Skin prick testing was performed on the volar surface of both arms to (1) environmental allergens (mixed grasses, mixed weeds, and dust mites; Greer, Lenoir, NC); (2) pets (cat, dog, and horse; Greer); and (3) the following LAs housed in the research facility: mice (ALK-Abelló, Hørsholm, Denmark; Greer; and National Jewish Medical and Research Center, Denver, Colo), rats (Greer), rabbits (ALK-Abelló and Greer), hamsters (Greer), and guinea pigs (ALK-Abelló and Greer). Because commercial allergens for LAs vary in concentration, we used at least 2 commercial extracts, where available, and also created a mouse allergen extract from male mouse urine collected at our institution. A positive skin test response was defined as a 3-mm or larger wheal that was larger than that elicited by the negative control. RAST (Phadia US, Inc, Chicago, Ill) results were considered positive at 0.35 kUA/L or greater. One or more positive skin test responses or RAST results defined a subject as atopic. One or more positive skin test responses or RAST results to LAs defined a subject as LA sensitized.

TLR4 allele characterization

We used allele-specific PCR amplification to genotype 316 subjects for *TLR4*/8851 and 162 subjects for *TLR4*/8551 (see Table E1 in this article's Online Repository at www.jacionline.org). We changed to a custom-designed real-time PCR *TLR4*/8551 genotyping assay (Applied Biosystems, Foster City, Calif) when it became available because of higher throughput and lower DNA requirements for 173 subjects. Allele determination was performed with DNA Engine Opticon 2 System version 2.02 software (MJ Research, Waltham, Mass). Thirty-eight subjects were genotyped with both methods to ensure comparability of results, and the results were the same regardless of which method was used.

Ten other *TLR4* single nucleotide polymorphisms (SNPs) were genotyped in 200 subjects by using allele-specific PCR, including 2 in the promoter region, 1 in an intron, and 7 nonsynonymous SNPs reported to be present in 5% or more of a cohort of European patients with sarcoidosis (unpublished data). These 10 sites included *TLR4*/–55, *TLR4*/–20, *TLR4*/4308, *TLR4*/8392, *TLR4*/8680, *TLR4*/8810, *TLR4*/8854, *TLR4*/9075, *TLR4*/9185, and *TLR4*/9943 (see Table E1).

Statistics

Genotypic distributions at each polymorphism were evaluated for departures from Hardy-Weinberg equilibrium by using χ^2 goodness-of-fit tests ($P < .001$). Univariate analyses used Wilcoxon rank sum tests for continuous variables and χ^2 goodness-of-fit tests for categorical variables. Multiple logistic regression models first included all univariate predictors with a P value of less than .15, and then predictors were removed sequentially using a step-down method if the log-likelihood did not significantly change. The outcome variables used in the multiple logistic regression models included skin prick test responses (positive/negative) to LAs and symptoms (no symptoms vs 1 symptom). The analysis software package was JMP 6.0 (SAS Institute Inc, Cary, NC), and an α value of .05 was used as the criterion for retaining variables in the models. All tests were 2-sided, and a P value of less than .05 determined statistical significance.

RESULTS

Description of the cohort

We recruited 335 workers engaged in research or animal work at a major research institution (Table I). A majority were female and white, and most had never smoked. Self-reported rates of physician-diagnosed allergy were high and supported by current skin test results. Fifty-nine percent of the workers had a response to at least 1 allergen, 21% were sensitized to 1 or more LAs, and 13% were specifically sensitized to mice (Table II). Of the 69 workers sensitized to LAs, 84% had positive skin prick test responses to other environmental allergens, suggesting that LA sensitization is not a unique allergy (see Table E2 in this article's Online Repository at www.jacionline.org). There were no significant differences in the frequency of specific sensitization by job category.

Workers self-identified their job categories (Table II). More than half worked with LAs, and research scientists were the largest group exposed to LAs. Cage changing, dumping, and washing were considered tasks with high prolonged exposures to animal allergen and endotoxin based on previous studies.¹ Animal experiments on the bench in the animal facility or individual laboratories were considered tasks with moderate and intermittent exposures to animal allergen and endotoxin.

Almost one third of the cohort reported 1 or more symptoms to LAs, and nasal symptoms were the most frequent. Interestingly, nasal and skin symptoms (ever) to LAs were significantly more often reported by animal handlers than those in other job categories, whereas the distribution of chest symptoms induced by LAs did not differ between jobs. In addition, not all symptomatic workers were sensitized to LA allergens. Nasal symptoms to LAs were more likely to be reported by LA-sensitized workers ($P = .007$), whereas chest symptoms were similarly distributed among workers regardless of sensitization status (not significant), suggesting that exposures other than allergen were the trigger (see Fig E1 in this article's Online Repository at www.jacionline.org). Of the 29 subjects reporting chest symptoms to specific LAs, 59% also endorsed 1 or more chest symptoms associated with methacholine reactivity ($P = .015$).¹⁹

TLR4 genotypes and LA workers

TLR4/8551 and *TLR4*/8851 alleles are distributed similarly among LA job categories (Table II). The 8551 and 8851 *TLR4* alleles were in Hardy-Weinberg equilibrium and similarly distributed across job categories. They were also noted to be in strong linkage disequilibrium. The minor alleles were each present in 9.0% of the total cohort, which is comparable with published results¹⁵ and in nearly complete linkage disequilibrium with only 5 discordant subjects. Based on a lack of heterogeneity in our population, 10 other *TLR4* sites were not analyzed further (data not shown).

The *TLR4*/8551 G allele is a risk factor for sensitization to LA and other allergens (Fig 1). Subjects with 1 or 2 copies of the *TLR4*/8551 G allele were 2.5 times more likely to be sensitized to LA allergens than were subjects homozygous for the major A allele (odds ratio, 2.5; 95% CI, 1.5–5.5; $P = .03$). The minor G allele was associated with the presence of atopy overall ($P = .05$), and a trend was noted for a similar association with sensitization to environmental allergens ($P = .08$) and pets ($P = .09$). Most workers sensitized to LAs were also allergic to environmental and pet allergens (see Table E2). As expected, because of linkage disequilibrium, the *TLR4*/8851 minor allele showed similar associations with sensitization (data not shown).

Workers with the *TLR4*/8551 G allele work longer hours in high endotoxin and mouse allergen exposure tasks than those with the AA allele (Fig 2). To determine whether these genotypes interacted with exposure variables, we compared the mean and maximum hours per week spent in different animal-related tasks between workers with the AA variant and those with 1 or 2 copies of the G allele (see Table E3 in the Online Repository at www.jacionline.org). As a whole, workers with the G allele were not more likely to perform experimental work with LAs or work in direct animal care. However, among subjects who worked with LAs, those with the G allele spent significantly longer hours per week in high

endotoxin and allergen exposure tasks compared with workers with AA. Conversely, time spent weekly in non-LA research showed no differences between allele groups (data not shown).

The *TLR4*/8551 G allele remains a risk factor for LA sensitization in a multivariate model (Table III). We next considered the contribution of this SNP in the context of other known and probable sensitization risk factors along with exposure variables in multivariate models. The *TLR4*/8551 G allele remained significant in the multivariate model for LA sensitization. As expected, the *TLR4*/8851 minor T allele was similarly predictive, but because of linkage disequilibrium, only 1 allele remained significant in the final model. Different markers of atopy were consistently identified as risk factors for LA sensitization, including positive skin test responses to pets or to environmental allergens or a prior physician's diagnosis of allergy or asthma, although positive skin test responses remained the strongest predictor. Interestingly, the *TLR4* G allele was not only associated with LA sensitization but also with atopy, such that it became a stronger predictor of LA sensitization when all other atopy variables were removed from the model (data not shown).

Avoiding work with animals was also a significant predictor of LA sensitization, probably because some workers with previous LA sensitization have chosen work that avoids symptom-triggering exposures. Of importance, however, most symptomatic or sensitized LA workers reported that they did not avoid work with LAs. Of the 96 workers reporting symptoms to LAs, only 18 (19%) said they avoided work with LAs. Of the 69 subjects with positive skin test responses to LAs, only 7 (10%) avoided work with LAs. Interestingly, all of the 18 workers who avoided LAs had a *TLR4*/8551 AA genotype. Longer hours spent on research tasks with LAs in the animal facility compared with animal care tasks was one of the few current exposure variables significantly associated with LA sensitization in our multivariate model.

Symptoms to work with LAs are predicted by LA tasks and markers of atopy (Table IV). We used a multivariate model to evaluate predictors of symptoms to work with LAs. Several different measures of atopy predicted LA-associated symptoms, including a prior physician's diagnosis of allergy, positive skin test responses to LAs, and prior physician-diagnosed asthma, likely because a large proportion of asthma is associated with atopy. Of interest, the strongest exposure predictor for symptoms was experimental work with LAs in individual laboratories. Although experimental work with LAs in the animal facility was also predictive, it was less strongly associated with symptoms and did not remain in the final model. Average hours per week spent changing cages was the one animal care task associated with symptoms. Women were more likely to report symptoms to LAs than were men, and this association was independent of job status. Women had a higher frequency of symptom reporting in general (data not shown). Neither the *TLR4* AA nor the G genotype was associated with nasal or chest symptoms in the multivariate model.

DISCUSSION

In this study we hypothesized that endotoxin plays a pivotal role in the health effects of the LA workplace in 2 ways: through its modulation of allergic sensitization to LAs^{9,11} and by

triggering respiratory symptoms directly. We studied the effect of functional variants of *TLR4*, an important endotoxin cell-surface receptor, on our primary end points of LA sensitization and LA-related symptoms as a way of assessing the contribution of endotoxin to these outcomes. We found that subjects carrying 1 or 2 copies of the *TLR4*/8551 less-endotoxin-responsive G allele were more likely to be sensitized to LA allergens, as well as to environmental and pet allergens. The *TLR4*/8851 minor variant T, in linkage disequilibrium with 8551, independently showed similar associations. These findings were confirmed in our multivariate models. The positive association of the *TLR4*/8551 G allele with risk for allergic sensitization has also been detected in English asthmatic subjects¹⁷ and in Swedish schoolchildren.²¹ Taken together, results from our and other studies suggest that the ability to respond normally to endotoxin through the dominant *TLR4*/8551 AA genotype might be protective against the development of allergic sensitization.

Our and other previous studies measured much lower concentrations of endotoxin in the LA research workplace^{1–3} than those reported in other animal worksites, such as poultry and hog-confinement barns.^{22–24} Nonetheless, endotoxin is measurable in the LA workplace, and the modest concentrations detected might be more effective at modulating the allergic process than the extremely high concentrations measured elsewhere. *TLR4* mediates the effects of low-dose endotoxin,¹² receptors that are bypassed by high concentrations of endotoxin.^{25,26} The fact that endotoxin is present in modest quantities in the LA setting might thus be central to its immunomodulatory role,¹⁰ as mediated in part through *TLR4*. An important correlate is the fact that symptoms are frequent in those exposed to high endotoxin concentrations in poultry or hog farms,^{27–30} but allergic sensitization is rare,^{31–34} and rates are much lower than those seen in LA worker cohorts.^{35,36}

Because we and others^{3,37,38} have described nasal and respiratory symptoms in response to endotoxin in both occupational and laboratory challenge settings, we hypothesized that responsiveness to endotoxin through the *TLR4* receptor might also affect symptom reporting in the LA research workplace. Although the *TLR4* G genotype was associated with longer hours of direct work with LAs, we did not find an association with nasal or chest symptom reporting in univariate or multivariate analyses.

Our study characterized a working population of research scientists and LA technicians by genotype and sensitization, although our study could be criticized for relatively small numbers. However, we used objectively characterized outcomes, such as sensitization based on skin tests or RASTs, that might permit detection of significant genetic differences with a smaller number of subjects. Although the prevalence of the *TLR4*/8551 minor allele was only 9%, its associations with sensitization remained robust, even in a multivariate model. Interestingly, we found that longer hours spent on research tasks with LAs in the animal facility compared with animal husbandry tasks was one of the few current exposure variables significantly associated with LA sensitization in our multivariate model. We have noted that LA research work is typically performed by research scientists and technicians, who often have prior exposure to LAs and a different, more intermittent pattern of exposure compared with animal handlers, whose exposure is more consistent from day to day. This lack of association between current exposures and LA sensitization might not be unexpected for a number of reasons.

First of all, we currently have no way of assessing when sensitization occurred. Because the majority of our cohort had worked with LAs previously in college or other laboratories, sensitization might well have occurred in the past.

Second, we have not verified endotoxin and mouse allergen exposures in the current study, although our job and task classifications are based on our previously published measures of airborne mouse allergen and endotoxin concentrations.¹ We also found that workers with the less-endotoxin-responsive *TLR4*/8551 G allele spent longer hours in direct animal exposure tasks, such as experiments with LAs, in the animal facility and in their own laboratories, perhaps because they were less affected by endotoxin exposures. It remains possible that the associations seen with the *TLR4*/8551 and *TLR4*/8851 minor alleles are merely surrogates for another endotoxin receptor or for an exposure other than endotoxin.

Our findings have several important implications. The effect of the *TLR4*/8551 minor allele should be interpreted in the context of the relatively small proportion of our and other study populations that carry the G allele, typically 10% or less. With this low prevalence, the fact that we were able to detect such a substantial increase in the risk for sensitization suggests that endotoxin is an important player in this process and supports those studies that identify the essential role of endotoxin with allergen exposure in other settings. What we are unable to address in the current study is whether skewing of the immune response toward allergy occurs only in childhood or if it is also an ongoing response in adulthood. LA allergy, which typically develops after adult workplace exposures, might reflect an immune system already oriented toward allergy. This interpretation is supported by the fact that atopy (ie, a previous physician's diagnosis of allergic disease, or the presence of environmental or pet allergies) is an important predictor of LA sensitization. However, it is also possible that exposure and response to allergen and endotoxin are ongoing processes that take place throughout life and influence future sensitization, as well as past sensitization.³⁹ Interestingly, we noted that the *TLR4* genotype partially accounted for atopy in our model of LA sensitization because it was a stronger predictor of sensitization when atopy was not included in the multivariate model. This might suggest that genetic factors that help determine allergic responses early in life are also important to allergic responses that occur later in the workplace.

In addition, our findings reinforce the importance of addressing gene effects that affect disease outcomes in the context of environmental interactions. Although many genetic studies in allergy and asthma risk have focused their attention on functional gene polymorphisms important in respiratory disease pathogenesis, results have been disappointing. However, allergic disease and asthma likely have different pathogenetic pathways in different environments. Because different environments will invoke different sets of genes that increase the risk for disease, "allergy genes" might differ in importance depending on the environment and manner of allergen presentation. Although much work remains to characterize the interaction of endotoxin and allergen in the LA research workplace, early steps to identify and quantify these exposures have set the stage for further investigation of the functional importance of *TLR4* and other endotoxin-associated genes in affecting the risk for occupational allergic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical implications

TLR4 functional variants affect the risk for LA sensitization and establish the importance of a gene-environment interaction in the LA research workplace.

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FIG 1.
Distribution of sensitization by *TLR4* alleles. *PST*, Skin prick test.

FIG 2.
Distribution of hours spent per week in LA tasks by *TLR4* alleles.

TABLE I

Demographic findings, smoking status, and physician-diagnosed asthma/allergy (total n = 335)

Age (y), mean (range)	32.7 (18–64)
Sex, no. (%)	
Female	195 (58)
Male	140 (42)
Race/ethnicity, no. (%)	
White	256 (76)
Asian	55 (16)
Hispanic	16 (5)
African	8 (2)
Smoking status, no. (%)	
Current	24 (7)
Never	243 (73)
Past	68 (20)
Allergy and asthma history, no. (%)	
Physician-diagnosed asthma	50 (15)
Physician-diagnosed allergy	139 (41)

TABLE II
Distribution of jobs tasks, symptoms to LAs, sensitization, and *TLR* alleles by job title

Work with LAs	Job titles, no. (%)			
	All	Animal handler, 24 (7%)	Research scientist, 191 (57%)	Research technician, 101 (30%) Research coordinator, 19 (6%)
Current direct work with LAs, no. (%)	172 (51)	75%	56%	48%
Hours/week work with LAs, mean (SD)	3.4 (7.8)	21.6 (16.3)	1.9 (3.1)	2.6 (6.3)
Distribution of tasks				
	No. (%)	% in job group		
Change cage/feed/water	53 (16)	75	10	16
Cage dump/clean	36 (11)	38	8	11
Experiments with LAs in animal facility	138 (41)	21	47	43
Experiments with LAs in research laboratories	141 (42)	13	50	43
Experiments without LAs in research laboratories	258 (77)	8	87	88
Any LA symptoms ever				
	N (%)	% in job group		
Any nasal, chest, or skin symptom induced by LAs	96 (29)	58	29	26
Nasal symptoms to LAs (ever)	86 (26)	50	25	25
Chest symptoms to LAs (ever)	29 (9)	13	9	8
Skin symptoms to LAs (ever)*	23 (7)	21	6	6
Sensitization				
	N (%)	% in job group		
1 positive skin test response	199 (59)	54	60	58
Environmental allergens	182 (54)	50	55	54
Pets	98 (29)	13	29	34
LAs	69 (21)	21	17	26
Mouse	42 (13)	17	11	14
Distribution of <i>TLR4</i> alleles				
	n (%) in job group			
<i>TLR4</i> /8551 AA	305 (91)	21 (88)	177 (93)	91 (90)
AG/GG	30 (9)	3 (13)	14 (7)	10 (10)
<i>TLR4</i> /8851 CC	286 (91)	16 (84)	170 (91)	85 (90)
CT	30 (9)	3 (16)	16 (9)	9 (10)

TABLE III

Model predicting sensitization to LAs

	OR	95% CI	P value
Atopy to environmental or pet allergens	6.1	2.9–12.7	<.0001
Avoid work with LAs	5.1	1.7–14.6	.003
<i>TLR4</i> /8551 G allele	2.4	1.02–5.6	.044
Average hours/week spent in the animal facility	1.1	1.001–1.2	.048

OR, Odds ratio.

TABLE IV

Model predicting symptoms on work with LAs

	OR	95% CI	P value
Sex (female)	3.9	2.1–7.2	<.0001
Experimental work with LAs in individual laboratories	3.4	1.9–5.9	<.0001
Physician-diagnosed asthma	2.5	1.2–5.3	.016
Positive skin test responses to LAs	2.2	1.2–4.2	.013
Physician-diagnosed allergies	1.9	1.1–3.4	.025
Average hours/week spent changing cages	1.06	1.03–1.1	.0002

OR, Odds ratio.