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TNFA-308 in Two International Population-Based Cohorts Shows Increased Risk for Asthma

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At a Glance Commentary:

Inconsistent findings about the role of Tumour Necrosis Factor Alpha polymorphisms were obtained in previous works. This population based study confirms the association of *TNFA -308 G/A* polymorphism with an increased risk of asthma.

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

Abstract

Rationale. Genetic association studies have related the Tumour Necrosis Factor alpha (*TNFA*) -308 G/A polymorphism with increased risk to asthma but results are inconsistent.

Objectives. To test whether two single nucleotide polymorphisms of the *TNFA* and lymphotoxin alpha (*LTA*) genes are associated with asthma, bronchial hyper-responsiveness and atopy in adults, by combining the results from two large population-based multicenter studies and to conduct a meta-analysis of previously published studies.

Methods. The European Community Respiratory Health Survey (ECRHS) and the Swiss Cohort Study on Air Pollution and Lung and Heart Disease in Adults (SAPALDIA) have used comparable protocols including questionnaires for respiratory symptoms and measures of lung function and atopy. DNA samples from 11,136 participants were genotyped for *TNFA-308* and *LTA+252*. Logistic regression with fixed and random effects models and non-parametric techniques were used.

Results. The prevalence of asthma was 6%. The *TNFA-308* polymorphism was associated with increased asthma prevalence (odds ratio for the A allele = 1.3, 95%CI 1.1-1.5) and with bronchial hyper-responsiveness (OR = 1.2, 95%CI 1.0-1.3). No consistent association was found for atopy. The *LTA+252* polymorphism was not associated with any of the outcomes. A meta-analysis of 17 studies showed an increased asthma risk of the *TNFA-308 A* allele (OR = 1.3, 95%CI 1.2-1.5).

Conclusions. The *TNFA-308* polymorphism is associated with a moderately increased risk for asthma and bronchial hyper-responsiveness, but not with atopy. These results are supported by a meta-analysis of previously published studies.

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Key Words: asthma, genetics, TNF, LTA, polymorphism

Introduction

Asthma is a complex disease with both genetic and environmental components. It is characterized by obstruction of the airways of the lung and is related to atopy and bronchial hyper-responsiveness (BHR). Several chromosome regions and candidate genes have been associated to asthma, although the individual genes identified so far have only modest effects and an unknown pattern of inheritance [1,2,3].

Tumour Necrosis Factor (TNF) is a potent proinflammatory cytokine involved in the inflammation of asthmatic airways [4]. TNF is located within the class III region of the major histocompatibility complex (MHC) region on chromosome 6p21.3 [5], which has previously been linked to asthma in different genome screens [1,3,6]. TNFA and lymphotoxin α (*LTA* also named *TNFβ*) genes are members of the TNF super-family. *TNFA* plays an important role in generating and maintaining inflammatory responses and airway hyper-reactivity [7,8]. TNFA protein has been found in increased concentrations in the airways [8] and bronchoalveolar lavage fluid of asthmatic patients [9]. Moreover, the TNFA secretory response to allergens differs between atopic and non-atopic subjects [10]. The LTA gene is closely located upstream to TNFα, and has similar biological activity as TNFA [11].

Polymorphisms in the two genes may affect the levels of TNF in the airways. *TNFA-308G/A* polymorphism, located in the promoter region of TNFA, has been associated with increased secretion and promoter activity [12]. The LTA+252A/G polymorphism, located in the first intron of LTA gene seems to be associated to a high LTA production [13]. The *TNFA-308 A* and *LTA+252 G* alleles have been positively associated with asthma in many [14,15,16,17,18,19,20,21,22] but not all studies [23,24,25,26,27].

We evaluated whether polymorphisms in the *TNFA* and *LTA* genes are associated with asthma, bronchial hyper-responsiveness and atopy in adults in two large population-based European cohorts that had used comparable methods, the Swiss Cohort Study on Air Pollution and Lung Diseases in Adults (SAPALDIA) and the European Community Respiratory Health Survey (ECRHS). We also evaluated the role of TNF in asthma susceptibility across smoking categories. Finally, we evaluated the consistency of our results through a meta-analysis of all published papers on polymorphisms in the two genes.

Material and Methods

Study Population

This study includes 11,136 subjects derived from two different population cohorts. The European Community Respiratory Health Survey (ECRHS) is a population-based multicentric cohort study. In a first phase of the study, taking place in most countries in the early 1990s, a random sample of the population aged 20-44 years living in the study areas was contacted and asked to complete a short questionnaire on respiratory symptoms [28]. In a second phase, an approximately 20% random sub-sample of the study population was contacted together with a complementary "symptom" sub-sample. The symptom sub-sample included all subjects reporting asthma-related respiratory symptoms in the short questionnaire but who had not been selected in the random sample [29]. Subjects in most centres were followed up with a median length of follow-up time of 8.9 years from the first phase (ECRHS) to the second phase (ECRHS-II). The studied population for the present analysis consisted of 5,065 subjects with interview

information, from whom in addition DNA had been extracted in ECRHS-II (19 centres from 10 countries).

The second cohort study population is the Swiss Study on Air Pollution and Lung and Heart Disease in Adults (SAPALDIA) [30,31]. SAPALDIA subjects were recruited in 1991 as a random sample of adults aged from 18-60 years from eight Swiss communities representing different language and climatic regions and varying degrees of urbanization. The median follow-up time for SAPALDIA was 10.9 years. We included participants with complete interview data and available DNA samples for genotyping (n = 6,071).

Subjects included in our analysis could be considered as mainly of European-Caucasian origin. Some subjects from Basel, Switzerland (n = 400), were in both datasets and were only included in the analysis of SAPALDIA. Ethical approval was obtained for each centre from the appropriate institutional ethics committee and written consent was obtained from each participant.

Asthma, bronchial hyper-responsiveness (BHR) and atopy

ECHRS and SAPALDIA used identical questionnaires for the assessment of respiratory symptoms and asthma. We evaluated asthma at baseline (phase I of both studies) on the basis of reported asthma symptoms and reported physician diagnosed asthma. The presence of asthma symptoms was based on a positive response to either of two questions: attack of asthma during the 12 months preceding the interview or current use of asthma medication. Among subjects reporting an asthma attack, 67% also reported use of asthma medication. We also examined alternative definitions of asthma that have been employed in previous studies. “Wheeze without a cold” was defined as a positive

response to two consecutive questions: “Have you had wheezing or whistling in your chest at any time in the last 12 months?” and if yes “Have you had this wheezing or whistling when you did not have a cold?” Physician diagnosed asthma was defined as a positive response to the question “Have you ever had asthma and was this confirmed by a doctor?”

Data on bronchial hyper-responsiveness was available for a total of 8,043 subjects across the two studies. SAPALDIA and ECRHS used identical spirometry protocols [28,30] and consenting participants underwent a bronchial challenge with methacholine chloride administered by MEFAR® aerosol dosimeters [32]. BHR was defined as a 20% fall in FEV1 from the highest FEV1 post-diluent during methacholine challenge with an accumulated dose of 1 mg for both ECRHS and SAPALDIA [33]. BHR associated with higher accumulated doses delivered in SAPALDIA, were not taken into account. Family history of asthma was defined as a report of asthma of either of the parents.

*S*kin prick test was performed in ECRHS and SAPALDIA (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). Atopic subjects at baseline in both studies were defined as having positive test to at least one common inhalant allergen (house dust mites -*Dermatophagoides Pteronyssinus*-, timothy grass, cat and *Cladosporium Herbarum).*

Candidate SNP Selection and Polymorphism Genotyping

We selected two SNPs, *TNFA-308* (rs1800629) and *LTA+252* (rs909253) on the basis of previous evidence of their correlation with TNFA and LTA serum levels [12,13] and their association with asthma [14,15,16,17,18,19,20,21,22]. In SAPALDIA, the *TNFA-308G/A* and the *LTA+252A/G* polymorphisms were genotyped using liquid–handling

assisted setup and a fluorescent 5'-nuclease real-time PCR (TaqMan, Applera Europe, Rotkreuz, Switzerland) assay on an ABI Prism 7900 sequence detection system (ABI, Rotkreuz, Switzerland). SNP-specific primers were designed for the PCR by Applied Biosystems (Applera Europe, Rotkreuz, Switzerland). SNP-specific minor groove binder (MGB) probes and primers used were as follows: TNF -308A/G: 5'-CCCGTCC[C/T]CATGCC-3', forward 5'-CCAAAAGAAATGGAGGCAATAGGTT-3', reverse 5'- GGACCCTGGAGGCTGAAC-3'; LTA -252 A/G: 5'-CTGCCATG[A/G]TTCCT-3', forward 5'-CAGTCTCATTGTCTCTGTCACACAT-3', reverse 5'-AGAGAGAGACAGGAAGGGAACAG-3'. A random sample of 10% of all DNA samples was regenotyped and all genotypes were confirmed. The genotype call rate was >99%.

In ECRHS, genotyping was performed at the Center for Genomic Regulation (CRG) in Barcelona from the "Centro Nacional de Genotipado" (CeGen) in Spain (http://www.cegen.org). SNPs were genotyped using the SNPlex™ platform (Applied Biosystems, Foster City, CA) according to manufacturer instructions and analyzed on an Applied Biosystems 3730/3730xl DNA Analyzer. Allele-calling was done by clustering analysis using Genemapper software (Genemapper v.4.0). The genotype call rate was >98%. Genotyping quality was controlled in two ways. First, internal positive and negative controls provided by ABI's manufacturer were included in the reaction plates. Secondly, six duplicated samples of two HapMap reference trios were incorporated in the genotyping process. Both genotype concordance and correct Mendelian inheritance were verified. Genotype concordance was tested using SNPator, a web-based tool for genotyping management and SNP analysis developed by CEGEN (http://www.CEGEN.org).

We compared genotyping across the two laboratories that analysed samples of the two cohorts using subjects from the centre in Basel, Switzerland (n= 400) who were included in both ECRHS and SAPALDIA. The agreement in genotyping was 99.8%. In addition the Basel ECRHS samples had been previously genotyped for *TNFA-308* marker with both RFLP and allele specific PCR methods [34]. Only very small differences in genotype distribution were observed between those results and results reported in this study (X^2= 0.02, df= 2, p = 0.99).

Meta-analysis

Previous articles on *TNFA-308G/A* or *LTA+252A/G* polymorphisms associations with asthma were searched on PubMed (http://www.ncbi.nlm.nih.gov/entrez/), and backward searches of articles cited in earlier literature reviews or original papers were conducted. The keywords used in the PubMed search were “asthma AND gene AND (tumor necrosis factor OR TNF)” for *TNFA-308* and “asthma AND gene AND (lymphotoxin OR LTA OR TNFB)” for *LTA+252*.

In the meta-analysis, we included all studies that met the following criteria: (i) design: either population-based cohort, case-control or cross-sectional studies; (ii) outcome: asthma defined as physician diagnosed [18,20,23,26,27,35,36,37,38] or self reported [16,17,19,25,29,39,40], regardless of age of onset; (iii) ethnicity: information available or if not available in the published report available through the contact with the authors [39]; (iv) method of genotyping reported; (v) complete genotype information available for subjects; (vi) genotypes in Hardy Weingberg equilibrium. Results from SAPALDIA and ECRHS were included in the meta-analysis using asthma symptoms and doctor asthma as the main outcomes definition.

Statistical Analysis

The statistical analysis was performed using R genetic package (version 1.2.1) from R statistical software, version 2.4.0 (http://www.r-project.org/). Exact tests were used to test for Hardy-Weinberg equilibrium in complete control subjects [41]. D' and χ^2 p-values for marker independence were estimated to determine linkage disequilibrium between both genetic markers.

Logistic regression analysis was used to determine adjusted associations between genotypes and disease under co-dominant and additive models. We computed the odds ratio (OR), and p-values corresponding to 95% confidence limits (95%CI) using the glm procedures from R statistical package (http://www.r-project.org/). P-values of <0.05 were considered as statistically significant. Logistic regression models were adjusted for country (ECRHS) or study area (SAPALDIA), sex, age, body mass index (BMI) and smoking status. We also evaluated haplotype-specific adjusted associations. Haplotypes were reconstructed and analysed using the haplo.glm function of the R library HaploStats.

We assessed the impact of population stratification in our data by analyzing 23 unlinked SNPs (supplementary material) using Genomic Control (GC) approach [42]. These SNPs were genotyped in the ECRHS study and in a subsample of SAPALDIA. The significance of the additive model was corrected by the inflation factor (λ) derived from GC for each of the 3 main outcomes in both, the ECRHS and the pooled analysis.

We used multifactor dimensionality reduction (MDR) on genetic and non-genetic potential determinants jointly to find genotype combinations within which the dichotomous outcome variability is much lower than between combinations [43,44,45]. It

is an extension of the combinatorial partitioning method and can be seen as a data reduction technique in that it reduces the dimensionality of multilocus information to a single dimension. The method is non-parametric, assumes no particular genetic model and generates low false positive rates [44].

In the meta-analysis the exact test of Hardy-Weinberg was used to test deviations from Hardy-Weinberg equilibrium only in control groups. Odds ratios were estimated for each study using Fisher's exact test of independence for 2 x 2 (using Fisher's Exact Test for Count Data) tables under the prior hypothesis that the rare allele confers susceptibility to asthma. Meta-analysis was performed using Mantel-Haenszel method under fixed effects and random effects model with R library rmeta version 2.14. Publication bias was evaluated by measuring the asymmetry of funnel plot measuring the intercept from regression of standard normal deviates against precision [46].

Results

Characteristics of participants in ECRHS and SAPALDIA are presented in Table 1. Both populations were comparable with regard to gender, BMI and pulmonary function (FEV1 and FVC), but not with regard to mean age due to differences in the inclusion criteria for age. Smoking status is also slightly different between studies, but the smoking prevalence in SAPALDIA is within the range of prevalences observed between different centres in the ECRHS.

The prevalence of atopy and asthma and the minor allele frequencies (MAF) for *TNFA-308* and *LTA+252* are shown by study and country in Table 2. Asthmatics (and as a consequence also atopics), have been over-sampled in the ECRHS since the sub-

cohort with respiratory symptoms was included. Among the random samples of ECRHS and SAPALDIA, the prevalence of atopy ranged from 15% in Germany (ECRHS) up to 42% in Australia (ECRHS). The prevalence of asthma symptoms ranged from 2% in Germany, Estonia, Spain and Belgium up to 7% in the UK and Australia. Even higher prevalences were reported for doctor-diagnosed asthma.

Geographic differences were also observed for the Minor Allele Frequencies (MAF) of both polymorphisms. The lowest MAFs were observed in France (12% *TNFA-308* and 27% *LTA+252*) and the highest in the UK (21% *TNFA-308* and 41% *LTA+252*). The two polymorphisms were in strong linkage disequilibrium (X^2 = 7516.29, D' = 0.98, r^2 = 0.60, $p < 2.22e^{-16}$). Genotype distribution for both alleles was consistent with Hardy-Weinberg equilibrium in the control group ($p > 0.05$), except for the ECRHS centre in Grenoble, France ($p < 0.001$). Analyses presented in this paper include data from Grenoble. We repeated all analyses excluding this centre and results were only minimally modified (data not shown). We did not detect strong effetcs of population stratification in our sample, obtaining inflation factors around 1 (λ=1.05 in atopy, λ=1.06 in asthma and λ=1.30 in BHR).

Associations of *TNFA-308* with atopy, asthma symptoms and bronchial hyper-responsiveness adjusted for country (ECRHS) or centre (SAPALDIA), sex, age, BMI and smoking status are summarized in Table 3. A statistically significant association was found for asthma symptoms and *TNFA-308* heterozygous *G/A* genotype (OR = 1.38, p = 0.001) and for the *TNFA-308 A* allele (OR = 1.30, p = 0.001). An analysis of *TNFA-308* by study showed an increased risk of asthma symptoms in the ECRHS and not in SAPALDIA (Table 3 and Figure 1). The test for heterogeneity between the two studies was significant (Q = 5.92, df = 1, p = 0.015). In the ECRHS, a significant risk

increase for asthma symptoms was observed for the G/A, A/A genotypes and for the A allele (OR = 1.49, p = 6.3 x 10^{-5}). Stratification by country in the ECRHS showed no differences in risk (Figure 1; Q=2.66, df=8, p=0.95) and an increased risk (OR >1) was observed in all countries. Odds ratios for the random subsample of the ECRHS tended to be lower than those of the asthma enriched sample (data not shown). In SAPALDIA, no difference in effect of TNF-308 was observed between Latin and German speaking regions (Q=0.44, df=1, p=0.51). Exclusion of SAPALDIA subjects who were older than 45 years at baseline (so as to compare with a similar population structure as the ECRHS) did not affect the risk estimates for asthma. The OR for the A allele in all SAPALDIA subjects was 0.94 (p = 0.71) while the OR for SAPALDIA subjects less than 45 years was 0.89 (p = 0.57). The observed associations between *TNFA-308* and asthma symptoms were not modified by either sex or atopy. In both, ECRHS and SAPALDIA, *TNFA-308* was associated with a slight increase of BHR prevalence (OR A allele = 1.16, p = 0.03), with similar risks found in the two studies (OR A allele = 1.13 and 1.18 respectively). No significant associations were found for *TNFA-308* with atopy (Table 3). Odds ratios for the random subsample of the ECRHS tended to be lower than those of the asthma enriched sample (data not shown).

The strength of the association of *TNFA-308* genotypes was different for distinct asthma related phenotypes. Odds ratios for the A-allele and for distinct phenotypes are shown in Table 4. A positive association was found for most but not all phenotypes examined, although the differences between ECRHS and SAPALDIA remained with regard to phenotypes based on reported asthma symptoms.

Results for *LTA+252* are summarised in Table 5. Overall ORs tended to be lower than for *TNFA-308*. A statistically significantly increased risk for asthma symptoms was

observed for the heterozygous G/A genotype (OR = 1.21, p = 0.05), which was primarily driven by an association of the G-allele with asthma in the ECRHS sample (OR = 1.22, p = 0.01).

Haplotype analysis

The (*LTA+252/TNFA-308*) GA haplotype was positively associated with asthma symptoms (OR = 1.29, p = 0.003) when compared to the most common haplotype (*LTA+252 A / TNFA-308 G*). The prevalence of the *LTA+252 A / TNFA-308 A* haplotype was lower than 0.01 and could, therefore, not be tested for an association with asthma. No significant results overall were found for the *LTA+252 G / TNFA-308 G* haplotype (Supplementary material).

Smoking effect

An association of *TNFA-308* (G/A, A/A genotypes compared to GG genotype) with asthma was significant among never smokers (OR = 1.33, p = 0.03) and ex-smokers (OR = 1.64, p = 0.01) while a non-significant excess risk was found for current smokers (OR = 1.20). This differential effect is only observed in ECRHS but not in SAPALDIA. In ECRHS, the effect of *TNFA-308* is significant among never smokers (OR = 1.50, p = 0.01) and ex-smokers (OR = 2.36, p = 0.0009). However, the p-value for interaction is not significant when examining never versus ever smokers (p = 0.52) or smoking status in three categories (p=0.23).

Results for Multifactor Dimensionality Reduction (MDR)

In the non-parametric MDR analysis we simultaneously detected multiple genetic loci and environmental exposures associated with asthma in the absence of a main effect.

We examined an increasing number of interactions starting from including singular effects up to 3-way interactions and tested the best fit of each combination of variables through permutation tests using 5000 replications. Among the 7 variables examined statistically significant results were found for a model including *LTA+252*, smoking and region (with 5000 permutations the permutation based $p < 0.001$). The best fit was for a model with two variables included smoking and region (permutation based $p < 0.001$).

Meta-analysis

The meta-analysis of *TNFA-308* was performed including the country-specific results from ECRHS countries and the two region-specific results from SAPALDIA as individual observations along with 15 published studies listed in Figure 1. Funnel plot for publication bias was not asymmetrical ($P > 0.10$) suggesting lack of publication bias in this meta-analysis. The ECRHS and SAPALDIA results were based on asthma symptoms. In total, 4,341 cases of asthma and 13,459 controls were examined (Figure 1). The combined odds ratio for asthma using a fixed effects model was 1.32 ($p < 0.001$) and for the random effects model was 1.35 ($p = 0.001$). Similar to a previous meta-analysis [35], we observed significant heterogeneity between the Caucasian populations ($Q= 56.57$, $df=17$, $p < 0.001$). Excluding the study by Albuquerque [26] that had a MAF of 31% that was different from all other studies, and the study by Shin [27] with different MAF than other Asian population studies (17%), the heterogeneity diminished considerably ($Q = 9.64$, $df = 15$, $p = 0.84$). The combined odds ratio in Caucasian populations after the exclusion of the study of Albuquerque [26] was 1.24 ($p < 0.0001$) for the fixed effects model and 1.29 ($p = 0.001$) for the random effects model. Heterogeneity was not significant in Asian populations although the ORs for a study in Korea [27] were markedly different from all other studies. Same risk was observed

when we include doctor diagnosed instead of asthma symptoms in the meta-analysis, with an odds ratio of 1.27 ($p < 0.001$).We also performed a meta-analysis of 10 previous studies on *LTA+252* and asthma [14,17,19,20,25,26,27,39,40,38,36] including the data from our study. The meta-analysis examined a total of 3,120 cases and 12,026 controls, with the combined odds ratio being 1.01 (95% CI, 0.99– 1.16, $p = 0.06$) for the fixed effects model and 1.08 (95% CI, 0.95–1.23, $p = 0.22$) for the random effects model.

Discussion

We performed a genetic association study on the *TNFA-308* and *LTA+252* genes with asthma and related phenotypes in two large European prospective cohorts. This is the largest association study of *LTA+252* / *TNFA-308* with subjects well phenotyped for atopy and respiratory symptoms using validated questionnaires and measures of lung function, bronchial hyper-responsiveness and atopy. We found a moderate but statistically significant association of the *TNFA-308 G/A* genotype and the *TNFA-308 A* allele with an increased asthma risk. The risks for BHR was also increased but to a lower extent, while no consistent associations were found for atopy. We verified our results with alternative non-parametric analyses and a meta-analysis of all published studies. Weaker associations were found for the *LTA* gene. Effects of the *TNFA* gene can be attributed to linkage disequilibrium with other asthma genes, to a main effect of *TNFA* or to an interaction and modification of the effect by environmental exposures.

The two polymorphisms we examined are located in the major histocompatibility (MHC) class III region, near the HLA-B [5]. *TNFA-308 A* allele is in strong linkage disequilibrium with HLA A1, B8 and DR3 alleles [12], that are also associated with higher levels of

TNFA-308 [47]. Although some studies suggest that the association of *LTA+252* / *TNFA-308* is independent of MHC class II alleles [40,21], Moffat and Cookson [14] found that two haplotypes containing *TNFA-308A* allele (*LTA+252 G / TNFA-308 A / HLA-DRB1*3* and *LTA+252 G / TNFA-308 A / HLA-DRB1*2*), were associated with asthma and BHR, stronger than other haplotypes containing only *LTA+252 / TNFA-308* polymorphisms. Identification of the individual effects of *LTA+252* and *TNFA-308* is difficult due to the linkage disequilibrium. Our results indicated that *TNFA-308* was associated with several asthma related phenotypes, while less consistent results were observed for the main effects of *LTA+252*. Haplotype analysis showed that only the model with *TNFA-308 A* allele was associated with asthma, and that this association was equivalent to the model with *TNFA-308* alone. This evidence suggests that the associations observed for *LTA+252* in asthma and BHR may be due to its linkage with *TNFA-308*.

TNFA is a potent pro-inflammatory cytokine found in high concentrations in bronchoalveolar fluid from asthmatics [8,9,48]. *TNFA-308* is located in the promoter of *TNFA* gene and *in vitro* studies have reported an increased *TNF* gene transcription associated with the *TNFA-308* variant [12,49]. The *A* allele has been associated with increased expression [12] and secretion of *TNFA* [49], although this association is not uniformly supported [51,52]. We did not observe an association of the *TNFA* A-allele with atopy while we observed that for asthma symptoms and BHR. Furthermore we did not observe a modification of the association of *TNFA-308* and asthma by atopic status. One study [20] reported that *TNFA-308* was a risk factor for atopic asthma but not for non-atopic asthma, although a number of other studies did not find this interaction [23,27,40].

In our study, the pattern of the interaction between *TNF* polymorphisms and tobacco smoke was not clear. TNFA is central in acute cigarette smoke-induced inflammation and resulting connective tissue breakdown. Oxidative stress involved in inflammation is partly regulated by cytokines such as TNFA. *TNFA-308* effects on the inflammatory response to oxidative stress have been suggested in other studies in relation to exposure to ozone, occupational endotoxin and ETS, but results are inconsistent. Interaction of *LTA+252* and *TNFA-308* polymorphisms with environmental factors has been shown in several studies in relation to smoking, ozone and other environmental or occupational exposures although results have not been consistent [53,54,55,56].

This study had low power to evaluate differences between countries and regions due to the low MAF of the TNF-308 A-allele. We did not observe an effect of population stratificaction in our sample, although the number of markers tested could be insufficient to detect lower stratification in between European populations [57]. The association analyses of both markers revealed differences in risk between the ECRHS and the SAPALDIA cohorts for asthma symptoms while more consistent results were obtained for BHR and atopy. The study protocols of both studies were similar and phenotypes were defined using the same questions. Nevertheless, differences across the two cohorts tended to relate primarily to asthma phenotypes, defined by questionnaire, rather than to phenotypes derived from functional or biological tests. However, a study assessing the internal consistency of respiratory symptoms suggests that international comparisons are not affected by errors due to cross-cultural variations in the reporting of symptoms [58]. The major difference between the two studies was the inclusion in ECRHS of a subsample with subjects that reported respiratory symptoms in the initial screening questionnaire. This subsample represented more than 60% of the asthma cases in the ECRHS and leads to differences in symptoms prevalences when compared

with SAPALDIA. The magnitude of asthma risk in relation to *TNFA* genotypes in ECRHS after exclusion of the asthma enriched sample, tended to be to weaker although still positive, indicating that inclusion of the asthma enriched sample could not explain the differences in risk between the two studies. The slightly stronger association observed in the oversampled asthmatics from ECRHS could, possibly, reflect an underlying, stronger effect of the *TNFA-308* genotype with more severe forms of asthma and with asthma persistence as well as progression. There is, however, only very limited evidence supporting such an association [15,59]. Other potential differences, such as errors in genotyping could be discarded. Given the above, the most likely explanation for the absence of an association with self-reported asthma phenotypes in SAPALDIA is the lack of statistical precision due to a much lower number of cases as compared to ECHRS. The meta-analysis of all published studies reinforces our results of a positive association between *TNFA-308* genotype and asthma prevalence, although some geographical variability could be observed. An inverse association was observed in two studies only [26,27]. However, the combined estimate confirms the association of *TNFA-308* with an increased risk of asthma in European and Asian populations. Population stratification is a concern in large genetic association studies with heterogeneous population [57]. Subjects in our analysis were nearly entirely of European ancestry. But even within our study population differences in allelic frequencies were observed between countries with higher MAFs obtained in the UK. The two previous studies [26,27] that found an inverse association with asthma risk were outliers with regard to *TNFA-308 A* allele frequency. In our meta-analysis we observed a high variation on allelic frequency for *TNFA-308 A* allele between populations, with lower frequencies in those from Korea, Japan, China and Taiwan. One limitation of this meta-analysis is that the selection criteria were broad leading to the inclusion of different ages-groups and

definitions of asthma (e.g. children asthma, adult asthma, atopic asthma) while the biological mechanisms involved in each of these asthma-related phenotypes could be different. This is particularly important given the differences in risk observed in our study for different asthma phenotypes. There are limited published data on BHR so we could not do a meaningful meta-analysis for this endpoint.

Conclusions

In this large international population based prospective study, *TNFA-308* polymorphism was associated with a moderately increased risk of asthma and bronchial hyper-responsiveness while no association was found for atopy. These results were supported by a meta-analysis of published evidence.

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SAPALDIA.

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ECRHS

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Tables and Figures

Table 1. Descriptive table for ECRHS and SAPALDIA at baseline

	Both Cohorts	ECRHS		SAPALDIA	
		Asthma-Enriched sample	Random Sample	Age 18-44	All ages
Number of subjects	10736	796	3869	3572	6071
Gender (% males)	49	44	48	50	50
Mean age (years ± SD)	38±10	34±7	34±7	33±8	41±11
Mean BMI (kg/m^2± SD)	23.9±3.8	25.1±4.8	23.9±3.7	23.1±3.4	23.8±3.6
Smoking status(%)					
Never Smokers (%)	45.5	42.7	43.5	48	47.1
Ex Smokers (%)	22.4	18.6	21.7	19.6	23.3
Current Smokers (%)	32.1	38.7	34.7	32.5	29.6
Asthma Symptoms (%)	5.6	35.8	3.7	2.9	2.9
Physician diagnosed asthma (%)	8.8	38.7	6.7	6.2	6.2
Atopy (%)	21.5	48.4	24.7	22.9	19.8
FEV1% predicted (mean± SD)	106.6±14.6	100.3±16.4	105.8±13.3	107.1±13.4	107.8±14.9
FVC% predicted mean (± SD)	112.3±14.9	107.4±14.3	109.2±13.1	112.3±13.9	114.9±15.5
BHR (%)	12.5	37.8	11.7	9.2	9.9

Table 2. Symptoms prevalence at baseline and minor allele frequency by study site (in brackets, the prevalence in the random sample of ECRHS).

Study	Country	Sample Size	Atopy (%)	Asthma Symptoms (%)	BHR (%)	Physician Diagnosed Asthma	MAF[1] TNFA-308[2]	MAF[1] LTA+252[3]
ECRHS	All	4665 (3869)	29 (25)	9 (4)	12 (7)	16 (12)	0.16	0.32
	Norway	436 (436)	23 (23)	3 (3)	8 (8)	8 (8)	0.19	0.36
	Sweden	643 (457)	41 (30)	2 (6)	21 (8)	19 (8)	0.15	0.37
	UK	358 (289)	39 (34)	18 (7)	20 (8)	23 (17)	0.21	0.41
	Australia	341(248)	49 (42)	21 (7)	26 (13)	28 (21)	0.14	0.28
	Estonia†	228 (175)	22 (18)	4 (2)	5 (3)	-	0.15	0.29
	Germany	439 (439)	15 (15)	2 (2)	4 (4)	14 (14)	0.15	0.33
	Belgium	506 (455)	27 (24)	5 (2)	7 (5)	13 (9)	0.18	0.32
	France	549 (522)	28 (28)	7 (5)	13 (11)	14 (12)	0.12	0.27
	Spain	1165 (848)	22 (19)	6 (2)	9 (4)	13 (1)	0.15	0.29
SAPALDIA	All	6071	19.8	2.9	9.9	6.2	0.139	0.305
	German[2]	3308	19.7	2.9	9.8	6.6	0.137	0.307
	Latin[3]	2763	19.9	2.8	10	5.7	0.141	0.303

[1]In control subjects without asthma symptoms, physician diagnosed asthma, cough, phlegm, current wheeze or atopy
[2]German-speaking centres: Basel, Aarau, Davos, Wald;
[3]Italian- or French-speaking centres: Lugano, Payerne, Geneva, Montana
†Atopy in Estonia measured with specific IgE (No SPT available). Bronchial Hyperresponsiveness was not measured.

Table 3. Adjusted Association of TNF-308 genotype with atopy, asthma symptoms and bronchial hyper-responsiveness (BHR).

	G/G		G/A			A/A			A-allele				
	Ca/Co	OR	Ca/Co	OR (CI)	p-value	Ca/Co	OR (CI)	p-value	Ca/Co	OR (CI)	p-value	p-add[1]	p-addc[2]
Atopy													
All	1679/5516	1.0	567/1821	0.99 (0.89-1.11)	0.89	54/191	0.82 (0.60-1.13)	0.22	675/2203	0.96 (0.88-1.06)	0.44	0.52	0.53
ECRHS	801/2028	1.0	317/715	1.09 (0.93-1.29)	0.28	24/90	**0.59 (0.37-0.95)**	**0.03**	365/895	0.97 (0.85-1.12)	0.69	**0.04**	0.04
SAPALDIA	878/3488	1.0	250/1106	0.91 (0.78-1.06)	0.23	30/101	1.11 (0.72-1.70)	0.63	310/1308	0.95 (0.83-1.09)	0.48	0.40	-
Asthma Symptoms													
All	378/7195	1.0	175/2343	**1.38 (1.13-1.68)**	**0.001**	19/243	1.39 (0.83-2.32)	0.21	213/2829	**1.30 (1.11-1.53)**	**0.002**	**0.005**	**0.006**
ECRHS	248/2809	1.0	132/980	**1.59 (1.25-2.02)**	**0.0002**	17/110	**1.84 (1.04-3.26)**	**0.04**	166/1200	**1.49 (1.22-1.81)**	**6.3e-05**	**0.0003**	**0.0004**
SAPALDIA	130/4386	1.0	43/1363	1.03 (0.72-1.46)	0.89	2/133	0.51 (0.13-2.10)	0.35	47/1629	0.94 (0.69-1.29)	0.71	0.57	-
BHR													
All	686/5145	1.0	278/1655	**1.22 (1.05-1.42)**	**0.01**	27/178	1.08 (0.71-1.65)	0.71	332/2011	**1.15 (1.02-1.31)**	**0.03**	**0.04**	0.08
ECRHS	363/1975	1.0	146/688	1.12 (0.90-1.39)	0.31	18/73	1.32 (0.77-2.27)	0.31	182/834	1.13 (0.95-1.35)	0.18	0.41	0.43
SAPALDIA	323/3170	1.0	132/967	**1.32 (1.06-1.64)**	**0.01**	9/105	0.83 (0.42-1.67)	0.61	150/1177	1.18 (0.98-1.41)	0.09	**0.04**	-

Models are adjusted by age, sex, body mass index, country for ECRHS or study centre for SAPALDIA and smoking status

[1] P-value under additive model.

[2] P-value under additive model corrected by Genomic control.

Table 4. Adjusted Association of TNF-308 genotype (A allele) with different asthma related phenotypes and their combinations, in the ECRHS and SAPALDIA cohorts

	ALL		ECRHS		SAPALDIA	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Atopy	0.97(0.88-1.07)	0.51	0.98(0.86-1.13)	0.82	0.96(0.84-1.09)	0.50
Asthma Symptoms	1.3(1.11-1.53)	0.001	1.49(1.23-1.81)	5.6e-05	0.94(0.69-1.29)	0.71
Bronchial hyper-responsiveness (BHR)	1.16(1.02-1.32)	0.03	1.14(0.95-1.36)	0.16	1.18(0.98-1.41)	0.09
Doctor diagnosed asthma	1.13(0.98-1.29)	0.09	1.37(1.15-1.63)	0.0004	0.84(0.67-1.05)	0.12
Asthma symptoms and atopy	1.25(1.02-1.54)	0.03	1.41(1.12-1.78)	0.003	0.78(0.47-1.29)	0.33
Asthma symptoms and BHR	1.25(0.98-1.61)	0.07	1.3(0.98-1.73)	0.068	1.05(0.62-1.77)	0.86
Wheeze, without a cold	0.97(0.85-1.09)	0.58	1.01(0.86-1.18)	0.92	0.9(0.72-1.11)	0.31

Models are adjusted by age, sex, body mass index, country for ECRHS or study centre for SAPALDIA and smoking status

Table 5. Adjusted Association of LTA-252 genotype with atopy, asthma symptoms and bronchial hyper-responsiveness (BHR).

	A/A		A/G			G/G			G-allele				
	Ca/Co	OR	Ca/Co	OR (CI)	p-value	Ca/Co	OR (CI)	p-value	Ca/Co	OR (CI)	p-value	p-add[1]	p-addc[2]
Atopy													
All	1005/3812	1	1011/3268	1.00 (0.91-1.11)	0.92	269/764	1.09 (0.93-1.28)	0.28	1549/4796	1.03 (0.96-1.11)	0.40	0.52	0.53
ECRHS	545/1445	1	538/1271	1.07 (0.92-1.23)	0.39	148/332	1.09 (0.87-1.36)	0.47	834/1935	1.05 (0.95-1.16)	0.35	0.51	0.52
SAPALDIA	460/2367	1	473/1997	0.95 (0.83-1.09)	0.49	121/432	1.10 (0.88-1.38)	0.41	715/2861	1.01 (0.92-1.12)	0.82	0.45	-
Asthma Symptoms													
All	255/4837	1	280/4231	**1.21 (1.00-1.45)**	**0.05**	65/1017	1.04 (0.77-1.41)	0.78	410/6265	1.08 (0.95-1.23)	0.26	0.44	0.13
ECRHS	165/1997	1	202/1750	**1.36 (1.08-1.72)**	**0.01**	58/455	**1.40 (1.00-1.96)**	**0.05**	318/2660	**1.22 (1.05-1.43)**	**0.01**	**0.02**	**0.02**
SAPALDIA	90/2840	1	78/2481	0.98 (0.72-1.34)	0.89	7/562	**0.40 (0.18-0.86)**	**0.02**	92/3605	0.80 (0.63-1.02)	0.08	**0.02**	-
BHR													
All	481/3431	1	425/3047	0.97 (0.84-1.12)	0.70	121/732	1.10 (0.88-1.37)	0.39	667/4511	1.02 (0.93-1.13)	0.65	0.60	0.64
ECRHS	271/1374	1	215/1269	**0.82 (0.67-1.00)**	**0.05**	76/325	1.09 (0.82-1.46)	0.54	367/1919	0.97 (0.85-1.12)	0.70	0.08	0.13
SAPALDIA	210/2057	1	210/1778	1.16 (0.94-1.42)	0.16	45/407	1.09 (0.77-1.53)	0.63	300/2592	1.08 (0.94-1.25)	0.28	0.39	-

Models are adjusted by age, sex, body mass index, country for ECRHS or study centre for SAPALDIA and smoking status

[1] P-value under additive model.

[2] P-value under additive model corrected by Genomic control.

Figure 1. Meta-analysis for TNF-308 and asthma symptoms including ECRHS and SAPALDIA