A comprehensive analysis of the *COL29A1* gene does not support a role in eczema

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Background: Based on a recent positional cloning approach, it was claimed that the collagen 29A1 gene *(COL29A1)*, which encodes an epidermal collagen, represents a major risk gene for eczema underlying a previously reported linkage to chromosome 3q21. However, thus far, not a single replication attempt has been published, and no definitive functional data have been provided.

Objectives: We aimed to determine whether *COL29A1* polymorphisms contribute to eczema susceptibility and whether COL29A1 expression is altered in eczema.

Methods: We investigated the reported association of *COL29A1* variants with eczema, subtypes of eczema, and eczema-related traits in 5 independent and large study populations comprehensively phenotyped for allergic diseases: a set of 1687 German patients with eczema and 2387 population control subjects, a collection of 274 German families with eczema-diseases children, a cross-sectional population of German children (n = 3099), the Swedish population-based birth cohort Children Allergy and Milieu in Stockholm, an Epidemiologic Study (BAMSE) (n = 2033), and the European cross-sectional Prevention of Allergy—Risk Factors for Sensitization Related to Farming and Anthroposophic Lifestyle (PARSIFAL) study (n = 3113). An additional set of 19 *COL29A1* coding single nucleotide polymorphisms was analyzed in BAMSE and PARSIFAL. *COL29A1* expression was investigated by using *in situ* hybridization.

Results: We found no evidence for a relationship between *COL29A1* polymorphisms and eczema. The equivalence test rejected the hypothesis of association even excluding small effects. *In situ* hybridization carried out on biopsy specimens from lesional and nonlesional skin of patients with eczema and from healthy control subjects did not show any differences in the cellular distribution pattern of *COL29A1* expression at the mRNA level.

Conclusions: Our results suggest that *COL29A1* is unlikely to contain genetic variants that have a major effect on eczema or atopy susceptibility. (J Allergy Clin Immunol 2011;127:1187-94.)

Key words: *Atopic dermatitis, eczema, genetics,* Col29A1

Eczema (atopic dermatitis) is the most common chronic skin disease in infants and children, with prevalence rates of up to 20%, and one of the most common skin disorders throughout all ages. The disease frequently co-occurs with other atopic

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Abbreviations used

BAMSE: Children Allergy and Milieu in Stockholm, an Epidemiologic Study
COL29A1: Collagen 29A1 gene
FLG: Filaggrin gene
GLM: Generalized linear model
GWAS: Genome-wide association study
HWE: Hardy-Weinberg equilibrium
ISAAC: International Study of Asthma and Allergy in Childhood
LD: Linkage disequilibrium
MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight
OR: Odds ratio
PARSIFAL: Prevention of Allergy—Risk Factors for Sensitization Related to Farming and Anthroposophic Lifestyle
SNP: Single nucleotide polymorphism
SSC: Standard saline citrate
TDT: Transmission disequilibrium test
3′UTR: 3′ Untranslated region
WAO: World Allergy Organization

disorders, and in a substantial number of patients, eczema precedes the clinical manifestation of asthma and rhinitis.[1]

Eczema is a complex disease in which many genes act in concert with environmental factors to determine the phenotype.[2] The individual genetic factors are thought to be of low effect size and therefore hard to identify by means of linkage studies.[3,4]

However, with null mutations of the filaggrin gene *(FLG)*,[5-7] major risk factors for eczema could recently be identified in a region with reported linkage.[8] Filaggrin is an epithelial structural protein of key importance for epidermal differentiation and barrier function.[9,10] *FLG* mutations only partially explain the genetic linkage of eczema to the epidermal differentiation complex[11] and account for only 9% of the variability of susceptibility to eczema.[12] A genome-wide association study (GWAS) in patients with eczema has recently identified another susceptibility variant within the epidermal differentiation complex, which is located within the hornerin *(HRNR)* gene encoding the filaggrin-related protein hornerin.[13] These observations suggest that an inherited disturbance of the epidermal barrier is central to the pathogenesis of eczema.

The previously suggested linkage region for eczema and total serum IgE[14] on chromosome 3q21 was recently further investigated by using a positional cloning approach.[15] Fine mapping showed a significant association of several single nucleotide polymorphisms (SNPs) and a common haplotype in the collagen 29A1 gene *(COL29A1)* with eczema in families of European origin, and it was suggested that *COL29A1* is the gene underlying the linkage signal on 3q21 and represents a major novel susceptibility gene for eczema.[15] Because of its tissue-specific expression pattern[15] and its proposed role in maintaining epidermal integrity,[16,17] a role of this gene in eczema seems plausible.

However, thus far, no independent replication of these results has been provided. The cause of eczema is complex, and in recent years, many unreplicated associations have been reported with common genetic polymorphisms.[18] Comprehensive and straightforward replications of initial positive findings in independent studies, as is the case for *FLG*,[7] are essential in judging the validity of these associations.[19-21] We therefore investigated the previously reported *COL29A1* genetic variants in 5 large and independent, comprehensively phenotyped German, Swedish, and European population samples to clarify the potential role of *COL29A1* in eczema and related traits.

METHODS

Nomenclature

The terminology for atopic diseases is confusing, and terms such as eczema, atopic eczema, atopic dermatitis, childhood eczema, atopiform dermatitis, and flexural dermatitis are frequently used synonymously in the literature. In an attempt to standardize the nosology for allergic diseases, the nomenclature committee of the World Allergy Organization (WAO) recently published its recommendation for naming allergic diseases, such as asthma, eczema, and rhinitis.[22] In this article we try to follow these recommendations, although this study was performed before the WAO suggestions.

Study populations

We evaluated 5 independent study sets (Table I and Table E1 in this article's Online Repository at www.jacionline.org). All study participants were of European origin. The respective ethics review boards of the participating centers approved the study protocol, and informed consent was obtained from all probands, their legal guardians, or both.

Set 1 included 1687 patients with eczema and 2387 population control subjects, and set 2 consisted of 274 parent-offspring trios recruited through a child with active eczema. All probands from sets 1 and 2 were obtained from German University Hospitals (the University of Kiel, Technische Universität München, and the University of Bonn) and were of self-reported German ancestry. The dermatologist's diagnosis of eczema was made according to standard criteria in the presence of a chronic or chronically relapsing pruritic dermatitis with the typical morphology and distribution.[23,24] Subjects were classified as having asthma or allergic rhinitis when they reported a physician's diagnosis of asthma or hay fever, respectively. German control subjects of unknown phenotype were derived from the population-based Popgen Biobank.[25]

In sets 3 to 5 all health outcomes were reported by the parents in standardized and validated questionnaires. Nonaffected subjects were used as control subjects.

Set 3 was a cross-sectional study consisting of 3099 German children aged 8 to 12 years performed in Munich and Dresden, Germany, as part of the International Study of Asthma and Allergy in Childhood (ISAAC), phase II.[26] Briefly, children whose parents reported a physician's diagnosis of endogenous or atopic dermatitis, asthma, or hay fever in the past were classified as having eczema, asthma, or allergic rhinitis, respectively.

Set 4 consisted of 2033 children from the Swedish population-based birth cohort BAMSE. In brief, between 1994 and 1996, 4089 newborn infants were recruited in the BAMSE study.[27] At 1, 2, 4, and 8 years of age, questionnaires focusing on symptoms related to atopic diseases and lifestyle factors were distributed to the parents of all children. Eczema and asthma were defined as parental report of a physician's diagnosis of atopic dermatitis or asthma at any of the 4 evaluation time points up to 8 years of age or a physician's diagnosis of rhinitis at the evaluation time points of 4 or 8 years of age.

Set 5 consisted of children from the cross-sectional PARSIFAL study. In total, 14,893 schoolchildren 5 to 13 years of age from 5 Western European countries were included,[28] and in the present study 3113 children with available DNA and consent for genetic analysis (1579 boys and 1534 girls) were analyzed. Children who had been given a diagnosis of atopic/allergic eczema were considered eczema cases. Children who ever had symptoms of seasonal rhinoconjunctivitis and had been given a diagnosis of seasonal rhinoconjunctivitis were considered allergic rhinitis cases. Children who had ever been given a diagnosis of asthma or obstructive bronchitis more than once were considered asthma cases.

In all sets skin prick testing (set 3, ISAAC) or serum measurements for total or specific IgE antibodies against a panel of aeroallergens (Sx1 CAP) and food allergens (FX5 CAP; Phadia, Freiburg, Germany; for BAMSE: Phadia AB, Uppsala, Sweden) were performed according to standardized procedures, as previously described.[26,29] A positive result of 0.35 kU/L or greater against at least 1 allergen or a wheal reaction of 3 mm or greater to 1 or more allergens after subtraction of the negative control was defined as "allergic sensitization." Atopic eczema was defined as eczema plus allergic sensitization according to the WAO's recommendation for naming allergic diseases.[22]

TABLE I. Characteristics of subjects in the different samples

	Set 1		Set 2	Set 3	Set 4	Set 5
	German eczema cases	German population control subjects	German eczema offspring	German cross-sectional (ISAAC)	Swedish birth cohort (BAMSE)	European cross-sectional (PARSIFAL)
$n_{cases}/n_{control\ subjects}$ (n families)	1687	2387	274	540/2454	567/1405	399/2650
Eczema (%)	1687 (100)	—	274 (100)	540 (18.0)	567 (28.8)	399 (13.1)
Atopic eczema (%)	1124 (79.2)	—	187 (71.6)	193 (6.7)	239 (16.8)	128 (4.1)
Asthma (%)	493 (32.7)	—	51 (30.0)	272 (8.9)	292 (15.0)	261 (8.5)
Rhinitis (%)	825 (54.0)	—	71 (39.2)	280 (9.2)	131 (6.4)	121 (4.0)
Sensitization (%)	1124 (79.2)	—	187 (71.6)	777 (25.9)	781 (38.4)	896 (28.8)
Male sex (%); $male_{cases}/male_{control\ subjects}$	743 (44.0)	1077 (45.1)	136 (49.6)	1561 (50.4); 249 (46.1)/1258 (51.3)	1047 (51.5); 296 (47.4)/724 (51.5)	1553 (49.9); 184 (46.1)/1333 (50.3)
Mean age (y [SD]) or range; $age_{cases}/age_{control\ subjects}$	22.2 (15.9)	38.6 (11.6)	9.4 (9.4)	9.6 (0.6); 9.6 (0.6)/9.7 (0.6)	<1-8	9.0 (1.8); 9.0 (1.9)/9.0 (1.8)
Mean IgE (SD); geometric mean	1519.0 (4303.1); 247.3 (8.4)		879.4 (2557.9); 89.0 (11.9)			

Percentages refer to individuals with available data.
—, Not available/unknown.

SNP selection and genotyping

For this study, 7 (rs13095825, rs16845861, rs10212372, A36603217 = rs57575291, rs10934938, rs4688761, and rs9883988) of the 8 SNPs (spanning 96 of the 139-kb genomic region) identified by Söderhäll et al[15] as having an association with eczema were genotyped for all 5 sample sets. The variant A36637742 could not be genotyped successfully and was therefore excluded. However, this variant was described to be in almost complete linkage disequilibrium (LD) with rs4688761 and rs10934938, which were among the variants investigated in the current study.[15] This was confirmed by genotyping the variant in 365 randomly chosen population control subjects from set 1 with a fluorescent-labeled PCR fragment on an ABI3730 automated sequencer (Applied Biosystems, Foster City, Calif) and in 96 randomly chosen subjects from set 3, in which cycle sequencing was carried out. All primer sequences and reaction conditions are available on request. In sample sets 4 and 5 the same 19 additional coding SNPs were genotyped as in the original study (see Table E5).[15] One of these SNPs (A36620315) was excluded because of a minor allele frequency of less than 1%. Two other SNPs (rs819085 and A36624476) significantly deviated from Hardy-Weinberg equilibrium (HWE) and were therefore excluded from analysis. In sets 1, 2, and 3 genotyping was performed with ligation-based SNPlex, as previously described.[13] In sets 4 and 5 genotyping for the *COL29A1* SNPs was performed by means of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom GmbH, Hamburg, Germany).

In addition, all DNA samples were genotyped for the most common *FLG* mutations: R501X, 2282del4, R2447X, and S3247X. For sets 1, 2, and 3, genotyping was performed as described previously in Weidinger et al.[26] In sets 4 and 5 the *FLG* mutations R501X, S3247X, and R2447X were genotyped with TaqMan allelic discrimination assays, and 2282del4 was genotyped with MALDI-TOF mass spectrometry. Primer sequences, amplification conditions, and allele sizes are available on request.

Statistical analysis

All sets were formally tested for departure from the HWE assumption by using the χ^2 test. For low genotype frequencies, an exact test was used because the asymptotic distribution of the χ^2 test is inadequate.[30]

Association with qualitative traits in sets 1, 3, 4, and 5 was analyzed with logistic regression models, a well-known approach in the framework of generalized linear models (GLMs), with adjustment for sex (sets 3, 4, and 5) or age and sex (set 1). For *FLG* stratification, an indicator variable (mutation carriage, combined genotype, yes/no) was added to the linear predictor. Thus all reported odds ratios (ORs) are adjusted for respective covariates. GLMs deliver maximum likelihood estimates of the parameters and corresponding SEs and *P* values.[31] The analysis was carried out with R 2.9.1 software.[32] Analysis with a quasilikelihood model showed that the dispersion factor Φ was close to 1 in all models. Thus we did not adjust our models for overdispersion or underdispersion.

Set 1 used general population control subjects of unknown phenotype. In sets 3 to 5 unaffected subjects were defined as control subjects.

Log-transformed total IgE levels were analyzed by using linear regression.

For family-based association analysis (set 2), the transmission disequilibrium test (TDT) was used.[33] Parent-of-origin effects were investigated by means of an implementation in PLINK 1.07.[34] The maternal imprinting effect was tested with the method proposed by Weinberg.[35]

In the GLMs we used the same genetic model as for the case-control studies to be consistent with the analysis of family data in the original article and because the TDT[33] coincides with the optimal TDT-type test for an additive model.[36]

The following traits were analyzed in the single cohorts: sets 1 and 2, eczema, atopic eczema, eczema plus rhinitis, and eczema plus asthma; sets 3 and 5, eczema, atopic eczema, asthma, and allergic rhinitis; and set 4, eczema, atopic eczema, and asthma.

To estimate the level of heterogeneity between the subpopulations, we used the I^2 statistic.[37] Finally, we performed a meta-analysis for "eczema" as outcome with a random effects model, with ORs estimated by using a logistic regression adjusted for sex throughout all sets with the meta package implemented in R 2.9.1. Because the test of heterogeneity is underpowered when there are few studies and thus heterogeneity cannot completely be ruled out, we carried out a pooled analysis with adjustment for population effects. Because results were similar, we only report those from the pooled analysis.

Power calculations for the single-SNP analyses were performed with the genetic power calculator.[38] Because failing the significance level does not prove the absence of association, an additional equivalence test was performed with OR limits of 0.8 to 1.25 for all case-control studies (data sets 1, 3, 4, and 5) to test for statistical significance of the absence of associations between *COL29A1* SNPs and eczema. Using the *a priori* defined OR limits and the actual study size, we get critical allele frequencies by means of respective transformation, as shown in Table E4 in this article's Online Repository. The range within these frequencies is defined as the equivalence region. The equivalence test rejects the null hypothesis "existence of association" if the observed allele frequency in the cases falls

within this equivalence region. For statistical computation, we used the SAS macros provided by Wellek and Schumann.[39]

Haplotype statistics were performed with the 7 SNPs by using the software PLINK 1.07[34] and Haploview 4.1.[40] Because SNP A3667742 was not genotyped in all cohorts, sliding windows were used to ensure the analogy to the analysis presented in Söderhäll et al.[15]

Any *P* values given are uncorrected, 2-sided, and subject to a significance level of .05. Because a simple Bonferroni correction might be too conservative to account for multiple testing, for the purpose of interpretation, we only corrected for the number of SNPs tested but not the number of traits investigated. This results in a local significance level of .05/7 ≈ .007.

In situ hybridization

For *in situ* hybridization, skin biopsy specimens were taken from lesional and nonlesional skin in 4 patients with atopic eczema with a known genotype at rs4688761 (1 CC, 1 CT, and 2 TT) and from the volar upper arm in healthy control subjects with no history of atopic diseases or chronic inflammatory skin diseases. Biopsy specimens were fixed in 4% paraformaldehyde/0.1 mol/L sodium phosphate buffer (pH 7.4) for 30 minutes at room temperature and immersed in sterile 15% sucrose/1× PBS for 3 hours and 30% sucrose/1× PBS overnight at 4°C, followed by embedding tissue in OCT (Baxter Healthcare, Round Lake, Ill) at −80°C. Fresh sections (8 μm) were cut, dried, digested in proteinase K solution, postfixed, treated in acetic anhydride solution, and hybridized for 30 hours at 50°C with indicated probes in hybridization buffer containing: 5× standard saline citrate (SSC; pH 4.5), 50% formamide, 2% blocking powder (Roche, Mannheim, Germany), 5 mmol/L EDTA, 50 μg/mL yeast transfer RNA, 0.1% Tween 20, 0.05% 3(3-cholaminopropyl diethylammonio)-1-propane sulfonate, and 50 μg/mL heparin. Sections were then rinsed in 2× SSC and washed for 3 × 20 minutes at 61°C in 2× SSC/50% formamide. After washing and blocking, sections were incubated overnight at 4°C with alkaline phosphatase-conjugated anti-digoxigenin (1:2000 dilution in blocking solution, Roche), and detection was performed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution. Probes were generated from the 3′ untranslated region (3′UTR) sequence of *COL29A1*, as well as from a PCR product generated by using the primer sequences published by Söderhäll et al.[15]

RESULTS

For the current study, we first genotyped 7 SNPs (spanning 96 kb of the 139-kb genomic region) previously suggested as having an association with eczema[15] in 5 independent study sets thoroughly phenotyped for atopic diseases: a set of 1687 German patients with eczema and 2387 German population control subjects (set 1), a collection of 274 parent-offspring trios with eczema (set 2), a German cross-sectional study of 3099 schoolchildren (set 3), 2033 children from the Swedish population-based birth cohort BAMSE (set 4), and 3113 children from the European cross-sectional study PARSIFAL (set 5). Furthermore, an additional set of 19 coding *COL29A1* SNPs was studied in sets 4 and 5 to systematically cover the gene.

Variant A36637742 (rs67372594) could not be genotyped successfully. However, this variant was described to be in almost complete LD with rs4688761 and rs10934938,[15] which were investigated in the current study. We confirmed this by typing 308 randomly chosen population control subjects from set 1 using a fluorescent-labeled PCR fragment. Analysis of LD pattern showed that SNP A36637742 is in strong LD with rs4688761 ($r^2 = 0.69$, $D' = 0.98$) and rs10934938 ($r^2 = 0.99$, $D' = 0.99$), both of which therefore serve as proxy variants. Mutational analysis of A36637742 by means of cycle sequencing in 96 randomly chosen subjects from set 3 supported these results (see Fig E1 in this article's Online Repository at www.jacionline.org).

The clinical characteristics of the subjects and the sample sizes for each trait within the 5 panels are shown in Table I. For all SNPs under investigation, the I^2 inconsistency metric (measuring the amount of heterogeneity not due to chance) was not significantly different from 0.

Genotyping success rates exceeded 91% for all SNPs in all sample sets. As reported in Table E1 in this article's Online Repository, *COL29A1* variants showed allele frequencies in all study sets similar to those reported in previous investigations in which this gene was also studied in cohorts with German and Swedish subjects[15,41] and similar to those in HapMap (HapMap genome browser phases I, II, and III). Two SNPs showed a slight deviation from HWE (A36603217 and rs10934938) in set 1 but not in other datasets. Both these SNPs are in strong LD and highly correlated with rs4688761 ($r^2 \geq 0.68$ and $D' \geq 0.98$), which did not show deviation from HWE.

Single-SNP association analysis

Considering multiple testing, univariate analysis did not show any significant associations between any of the SNPs and eczema in any of the study sets investigated nor in a pooled analysis and meta-analysis. Additional analysis of the combined sample set with a Markov chain Monte Carlo simulation procedure using the MCMCpack in R 2.9.1 did not show main differences; that is, no significance was found considering the percentiles of the resulting SNP-effect distributions. Results from the meta-analysis and pooled analysis were similar, and thus only those from the pooled analysis are shown (Tables II and III).

In sets 3 (ISAAC), 4 (BAMSE), and 5 (PARSIFAL) no associations were seen for the related traits asthma, allergic rhinitis (Table IV), and sensitization (data not shown). Within sets 1 (case-control population) and 2 (trios), no significant associations between *COL29A1* variants and the subphenotypes atopic eczema (eczema plus allergic sensitization), eczema plus asthma, and eczema plus rhinitis were observed (see Tables E2 and E3). No association were seen with total IgE levels (see Table E4). No age-genotype interactions were seen in set 1 (data not shown). Strong associations with *FLG* mutations were seen in all sets (set 1: OR = 4.65, $P = 9.67 \times 10^{-36}$; set 2: OR = 2.50, $P = 1.2 \times 10^{-4}$; set 3: OR = 3.01, $P = 2.58 \times 10^{-14}$; set 4: OR = 1.73, $P = .003$; set 5: OR = 3.01, $P = 4.37 \times 10^{-13}$). Additional adjustment for *FLG* as a covariate also failed to show any significant association between the genotyped *COL29A1* SNPs and the traits analyzed (data not shown).

Because parent-of-origin effects have been reported for *COL29A1* variants, as well as the linkage region,[14] we also tested for differences between maternal and paternal allele sharing in set 2. We were unable to confirm an excess of maternal transmissions (Table III). In the pooled analysis the equivalence test rejected the hypothesis of an association with eczema, even excluding small contributions of *COL29A1* to disease development defined by an equivalence region of an OR of 0.8 to 1.25 (see Table E5). A set of *COL29A1* coding SNPs additionally typed in BAMSE and PARSIFAL did not show any associations with eczema (see Table E6).

Haplotype analysis

The haplotype analysis revealed no significant differences of any haplotype frequencies between cases and control subjects (sets 1, 3, 4, and 5) and no significant overtransmission to affected

TABLE II. Association results of the SNPs in sets 1, 3, 4, and 5 and the pooled sample for eczema

	Set 1 (German case-control study)				Set 3 (ISAAC)				Set 4 (BAMSE)				Set 5 (PARSIFAL)				Pooled sample			
SNP	**OR**	**95% CI**		***P* value**	**OR**	**95% CI**		***P* value**	**OR**	**95% CI**		***P* value**	**OR**	**95% CI**		***P* value**	**OR**	**95% CI**		***P* value**
rs13095825	1.09	0.98	1.22	.127	0.99	0.86	1.14	.883	1.01	0.87	1.16	.940	1.07	0.91	1.25	.443	1.03	0.97	1.10	.344
rs16845861	1.02	0.89	1.17	.757	0.97	0.82	1.14	.695	1.05	0.88	1.24	.582	0.98	0.81	1.19	.848	1.01	0.94	1.09	.848
rs10212372	1.02	0.89	1.16	.797	0.94	0.80	1.12	.501	1.04	0.87	1.24	.664	0.94	0.77	1.14	.520	0.99	0.92	1.07	.822
A36603217	1.12	0.98	1.27	.100	1.08	0.92	1.28	.337	1.12	0.94	1.33	.191	1.10	0.91	1.32	.343	1.09	1.01	1.18	.021*
rs10934938	1.12	0.98	1.28	.099	1.08	0.91	1.27	.384	1.13	0.95	1.35	.182	1.12	0.92	1.35	.262	1.10	1.02	1.18	.017*
rs4688761	1.14	1.02	1.29	.026*	0.99	0.85	1.16	.930	1.08	0.93	1.27	.314	1.06	0.89	1.26	.519	1.06	0.99	1.14	.075
rs9883988	1.10	0.85	1.41	.471	0.97	0.72	1.31	.846	1.04	0.76	1.44	.796	1.27	0.89	1.80	.189	1.12	0.98	1.29	.106

OR, Adjusted odds ratio.
*Not significant after correction for multiple testing.

TABLE III. Results for *COL29A1* SNPs and eczema in the family sample set (set 2)

	All transmissions			Paternal			Maternal			Parent of origin	Maternal imprinting
SNP	**T**	**NT**	***P* value**	**T**	**NT**	***P* value**	**T**	**NT**	***P* value**	**P-POO**	***P* value**
rs13095825	127	109	.268	47	58	.283	62	69	.541	0.694	.550
rs16845861	92	84	.598	44	36	.371	48	48	1.00	0.509	.440
rs10212372	90	84	.705	44	37	.437	46	47	.917	0.523	.458
A36603217	85	70	.261	38.5	34.5	.640	46.5	35.5	.225	0.620	.577
rs10934938	83	70	.332	36.5	34.5	.812	46.5	35.5	.225	0.512	.457
rs4688761	109	98	.487	44.5	52.5	.417	64.5	45.5	.070	0.067	.016*
rs9883988	29	17	.105	13.5	5.5	.066	15.5	11.5	.441	0.348	.328

Maternal imprinting, Test for maternal imprinting proposed by Weinberg[35]; *P-POO*, test for parent of origin effect implemented in PLINK 1.07; *NT*, number of nontransmissions; *T*, number of transmissions.
*Not significant after correction for multiple testing.

TABLE IV. Association results of the SNPs in sets 3 (ISAAC), 4 (BAMSE), and 5 (PARSIFAL) for eczema-related traits

	Asthma												Allergic rhinitis							
	Set 3 (ISAAC)				Set 4 (BAMSE)				Set 5 (PARSIFAL)				Set 3 (ISAAC)				Set 5 (PARSIFAL)			
SNP	**OR**	**95% CI**		***P* value**	**OR**	**95% CI**		***P* value**	**OR**	**95% CI**		***P* value**	**OR**	**95% CI**		***P* value**	**OR**	**95% CI**		***P* value**
rs13095825	0.96	0.79	1.16	.682	0.94	0.78	1.14	.521	1.08	0.89	1.31	.464	1.09	0.91	1.32	.342	0.94	0.70	1.25	.661
rs16845861	1.07	0.86	1.33	.550	1.06	0.85	1.31	.611	1.07	0.86	1.34	.534	1.21	0.98	1.49	.076	0.92	0.66	1.29	.626
rs10212372	1.01	0.81	1.27	.901	1.02	0.82	1.27	.879	1.06	0.84	1.33	.609	1.09	0.88	1.36	.413	0.88	0.62	1.26	.480
A36603217	0.93	0.74	1.17	.551	1.16	0.93	1.44	.187	1.09	0.87	1.37	.460	1.07	0.87	1.34	.514	0.89	0.63	1.25	.499
rs10934938	0.94	0.75	1.18	.608	1.19	0.95	1.48	.124	1.09	0.86	1.38	.478	1.05	0.84	1.30	.668	0.89	0.63	1.27	.532
rs4688761	0.90	0.73	1.11	.320	1.07	0.88	1.31	.498	1.17	0.95	1.43	.140	1.06	0.87	1.29	.595	1.04	0.77	1.40	.803
rs9883988	1.29	0.89	1.86	.174	1.24	0.83	1.83	.290	1.22	0.80	1.87	.359	1.23	0.85	1.77	.265	0.71	0.33	1.53	.387

OR, Adjusted odds ratio.

offspring (set 2) by using haplotype windows of 5 and 4 SNPs, respectively (Table V). We could not replicate the association of eczema with the most frequent haplotypes in any of the cohorts, as described in the initial report.[15]

In situ hybridization of skin biopsy specimens from patients with eczema

The results described above prompted us to reinvestigate the expression pattern of COL29A1, which was reported to differ between eczematous and healthy skin.[15] To this end, we obtained biopsy specimens from both lesional and nonlesional skin from 4 patients with eczema with a known genotype at rs4688761, which in the initial study[15] had been described to display the strongest association, and 4 healthy control subjects. Using the identical PCR primers to amplify a COL29A1-specific template for cRNA synthesis, we obtained similar staining patterns with this probe in all samples. We therefore designed a second cRNA probe from the 3′UTR of the COL29A1 mRNA, which again revealed no difference in expression pattern or intensity among groups. Specifically, as indicated in Fig 1, we did not observe the absence of COL29A1 expression in the differentiated upper spinous and granular layers. Thus we could not confirm an abnormal cellular distribution pattern of COL29A1 expression in the differentiated outer epidermis of patients with eczema in either lesional or nonlesional skin.

DISCUSSION

In the present study we were unable to replicate recent findings from a fine-mapping study carried out in families of German origin and additional samples from Italy, Sweden, and The

TABLE V. Sliding window haplotype analysis in all sets using PLINK 1.07[34] and Haploview 4.1[40]

	Set 1 (German case-control study)			Set 2 (German families)				Set 3 (ISAAC)			Set 4 (BAMSE)			Set 5 (PARSIFAL)		
Haplotype*	Frequency, cases	Frequency, control subjects	*P* value	Frequency	T	NT	*P* value	Frequency, cases	Frequency, control subjects	*P* value	Frequency, cases	Frequency, control subjects	*P* value	Frequency, cases	Frequency, control subjects	*P* value
T G A A T . .	0.632	0.647	.175	0.617	129.9	121.7	.601	0.642	0.630	.447	0.623	0.631	.630	0.596	0.623	.141
. G A A T T .	0.694	0.706	.221	0.677	110.8	126.8	.302	0.696	0.687	.561	0.651	0.665	.392	0.666	0.679	.477
. . A A T T .	0.692	0.705	.204	0.677	111.3	127.1	.308	0.704	0.695	.545	0.651	0.670	.281	0.674	0.684	.562
. . . A T T G	0.736	0.752	.106	0.723	102.3	111.9	.513	0.739	0.738	.932	0.701	0.721	.215	0.713	0.728	.408

NT, Not transmitted; *T*, transmitted.
*Order of the haplotype SNPs: rs13095825, rs16845861, rs10212372, A36603217, rs10934938, rs4688761, and rs9883988.

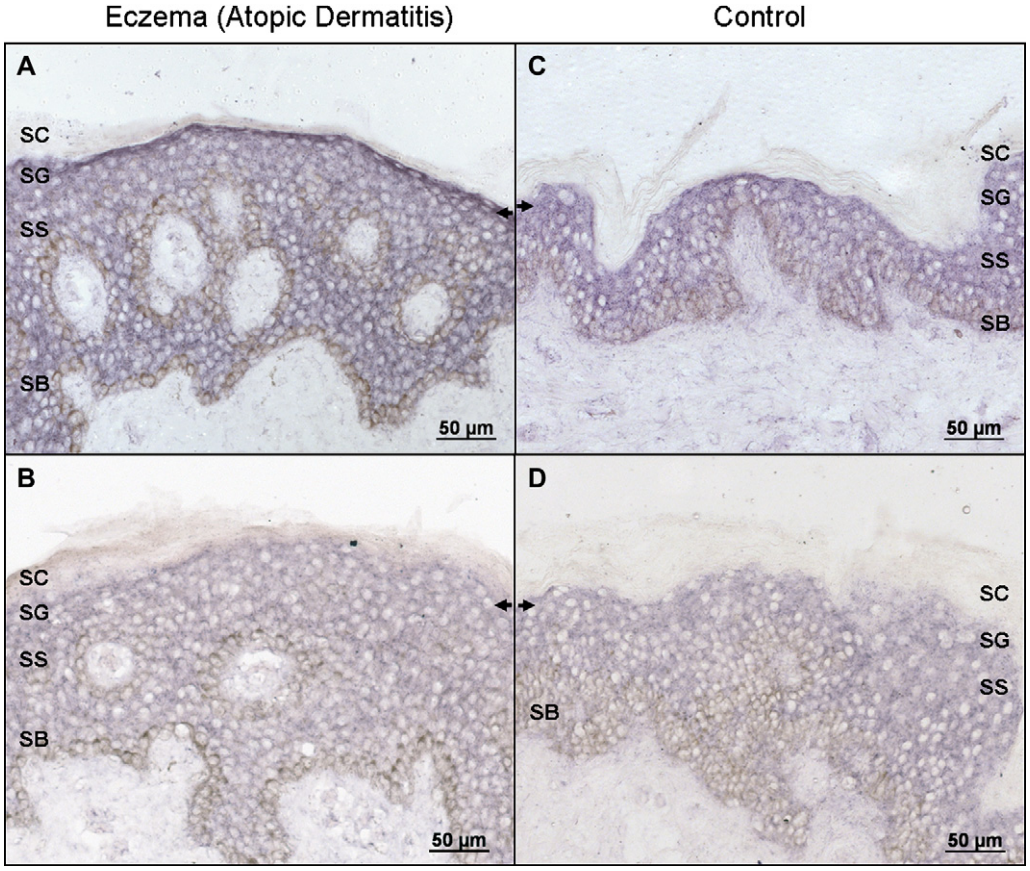

FIG 1. Gene expression analysis of *COL29A1* in patients with eczema (**A** and **B**, *left*) and healthy (**C** and **D**, *right*) skin. *COL29A1* mRNA was detected by using the 3′UTR antisense probe (Fig 1, *A* and *C*, *upper panels*) and a probe generated to the sequences published in Söderhäll et al[15] (Fig 1, *B* and *D*, *lower panels*). Both probes show an almost identical staining pattern. Stratum basale *(SB)*, stratum spinosum *(SS)*, stratum granulosum *(SG)*, and stratum corneum *(SC)* are indicated.

Netherlands,[15] which described 8 SNPs and 1 common haplotype in *COL29A1* to predispose to eczema. *COL29A1* is located on human chromosome 3q21 within a region for which linkage with eczema and suggestive linkage with total IgE levels had been found in a screen carried out in families of European origin.[14] In a positional cloning approach using the families from the original linkage scan, this susceptibility region was narrowed down to 1 haplotype block, and *COL29A1* was suggested to represent the disease-causing gene, with 8 SNPs showing strong associations with eczema and a maternal transmission pattern to affected children in both the discovery panel and an independent set of families. Reportedly, the association of SNP rs4688761 with eczema accounted for most of the linkage signal.[15] In the current study this SNP association was not replicated.

In our study we examined the same set of SNPs previously reported to increase eczema risk[15] in several large-scale and well-characterized study sets of almost equivalent ethnicity (German) and additional pan-European datasets, which included both childhood and adult populations. First, we conducted an adequately powered case-control study (1687 cases and 2387 population control subjects) to test for association between eczema and the

COL29A1 variants. We did not observe any association between any of the variants or haplotypes and eczema. Next we performed family-based tests of association in 274 parent-offspring trios for eczema. No evidence for excess transmission of any *COL29A1* alleles or haplotypes was obtained. Neither could we confirm parent-of-origin effects as reported in both the original linkage scan and the subsequent fine-mapping study. Consistent with these observations, we did not observe any association between *COL29A1* variants and eczema in a large German cross-sectional study of more than 3000 schoolchildren, a large European cross-sectional study of more than 3000 children, and a population-based Swedish birth cohort of more than 2000 children. Pooled analysis of all study sets for "eczema" as outcome yielded no significant associations after correction for multiple testing.

Because of the marked heterogeneity of the eczema phenotype, we also analyzed subphenotypes, such as atopic eczema, eczema plus asthma, and eczema plus rhinitis, without obtaining significant associations. Likewise, none of the *COL29A1* SNPs showed significant associations with any of the atopic traits asthma, allergic rhinitis, sensitization, and total IgE. This also helps to interpret findings from a recent small-scale GWAS using pooled samples, in which a suggestive signal from *COL29A1* on increased levels of IgE against specific allergens was picked up. However, in the same study the authors were not able to replicate the association,[42] and neither could we.

In addition, an equivalence test, which was carried out to evaluate the statistical significance of the absence of associations between *COL29A1* SNPs and eczema, supports the results of no association. Finally, we investigated an additional set of all *COL29A1* coding SNPs in sets 4 and 5,[15] again without obtaining evidence for association with eczema.

Our results from *in situ* hybridization are in contrast to those of the original study, which had reported an abnormal cellular distribution of COL29A1 mRNA in skin biopsy specimens of patients with eczema.[15] The expression pattern we obtained was identical in lesional and nonlesional skin of patients with eczema and skin from healthy control subjects, and in particular did not show a differential expression in the stratum spinosum. Because we used the same probe as in the initial report and in addition a second different probe to detect *COL29A1*, it is unlikely that this difference can be explained by technical means, although the protocols we used differed slightly from that used in the initial study.[15] Also, this excludes the detection of different splice variants from *COL29A1*, particularly because our probe (Fig 1, *A* and *C*) binds to the 3′UTR of *COL29A1* mRNA. To increase the chance for a positive result and to reduce the risk of false-negative results, we had stratified our samples by explicitly selecting carriers of risk genotypes in *COL29A1* for expression experiments. However, no differences were observed.

COL29A1 (also named *COL6A5*[16,17]) encodes the collagen VIα5 chain, a member of the collagen protein superfamily, which is considered important for maintaining extracellular matrix structure and function[43] and has been suggested to play an important role in keratinocyte cohesion.[15] Because findings on *FLG*, the strongest and most widely replicated eczema risk gene, implicate that the structural integrity, functional integrity, or both of the epidermis is a key factor in the development of eczema and subsequent respiratory diseases,[7] *COL29A1* appeared to be a plausible candidate gene. Our current study, which is both relatively large and the first published study to also investigate effects on atopic phenotypes other than eczema, such as asthma and rhinitis, found no significant associations.

Careful replication studies in independent populations of sufficient sample size are of crucial importance and are considered the gold standard to validate genotype-phenotype associations.[44] However, significant results found in association studies of complex diseases often fail in replication trials.[45] There are numerous potential reasons for a lack of replication, such as insufficient statistical power, a lack of precise comparability of the phenotype of interest, population stratification or population-specific effects, or analysis of different variants.[44] Regarding the conflicting results obtained in the initial[15] and current studies, we think that it is unlikely that the lack of replication can be ascribed to any of these causes. In the current study we analyzed the same genetic variants using subjects of the same ethnic background, age range, and ascertainment criteria as the population used for the mapping of *COL29A1*. Even allele frequencies in our populations were very similar to those in the original study. Finally, additional coding *COL29A1* SNPs typed in sets 4 and 5 also failed to show associations with eczema (see this article's Online Repository).

A *post hoc* power calculation indicated that for an SNP with a minor allele frequency of 20% under an additive model, the pooled sample had 99% and 90% power to detect a true OR of 1.25 or greater for eczema and atopic eczema as outcomes. The power to detect an OR of 1.5 was greater than 99% for both outcomes.

Our study suggests that *COL29A1* is unlikely to contain a major locus modulating eczema risk in populations of Western European origin. In addition, it is unclear whether the 3q21 locus is a major eczema risk locus because it has not been truly replicated, and the region was not identified in a recent large-scale GWAS on eczema[13] carried out in subjects of the same ethnic background as the original linkage study. In that GWAS a susceptibility variant on chromosome 11q was discovered, which was recently replicated in an independent study[46] but needs further validation and replication.

In conclusion, we have performed an extensive replication study on *COL29A1* variants as potential risk factors for eczema in 5 independent study samples. Our findings show that it is unlikely that genetic variants in *COL29A1* have a major effect on eczema risk in populations of European origin. Furthermore, we could not confirm an abnormal cellular distribution of *COL29A1* mRNA in skin biopsy specimens of patients with eczema.

Key messages

- ***COL29A1* does not represent a risk gene for eczema (atopic dermatitis).**
- ***COL29A1* expression does not differ between eczema lesions and healthy skin.**
- **Careful replication studies in independent populations of sufficient sample size are of crucial importance and the gold standard to validate genotype-phenotype associations.**

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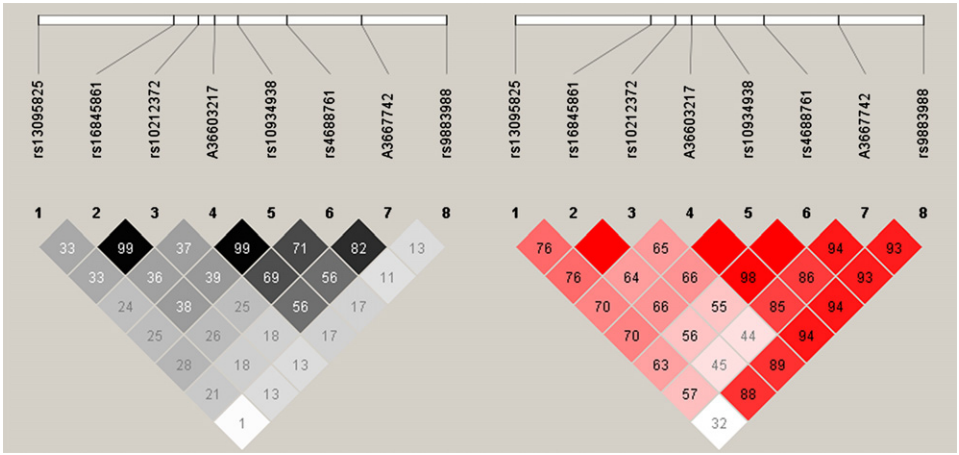

FIG E1. LD of the *COL29A1* SNPs measured by r^2 and D′ analysis with 308 population control subjects from set 1.

TABLE E1. MAF and HWE *P* value for all SNPs in all cohorts

		Set 1			Set 2			Set 3		Set 4		Set 5	
		German control subjects		German eczema cases	German parents		German eczema offspring	German cross-sectional (ISAAC)		Swedish birth cohort (BAMSE)		European cross-sectional (PARSIFAL)	
SNP	Minor allele	MAF	HWE	MAF	MAF	HWE	MAF	MAF	HWE	MAF	HWE	MAF	HWE
rs13095825	C	0.32	0.247	0.33	0.34	0.92	0.32	0.33	0.67	0.35	0.94	0.35	0.31
rs16845861	A	0.19	0.085	0.20	0.21	0.75	0.21	0.20	0.18	0.21	0.96	0.21	0.70
rs10212372	G	0.20	0.116	0.20	0.20	0.67	0.21	0.20	0.40	0.20	0.36	0.20	0.44
A36603217	G	0.18	0.016	0.19	0.18	0.35	0.20	0.20	0.59	0.20	0.57	0.20	0.96
rs10934938	G	0.18	0.032	0.19	0.19	0.38	0.20	0.20	0.75	0.20	0.28	0.20	0.88
rs4688761	C	0.25	0.084	0.26	0.27	0.997	0.28	0.26	0.43	0.29	0.52	0.28	0.65
rs9883988	A	0.04	0.052*	0.05	0.05	1.00*	0.06	0.05	0.36*	0.05	0.21*	0.04	1.00*

MAF, Minor allele frequency.
*Exact test for HWE in SNPs with low genotype frequencies.

TABLE E2. Results of association analysis for several eczema-related traits in set 1 using an additive model adjusted for sex and age

	Atopic eczema			Eczema + asthma			Eczema + rhinitis		
SNP	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**
rs13095825	1.15	1.02-1.30	.023*	1.03	0.87-1.22	.723	1.08	0.95-1.24	.244
rs16845861	1.07	0.92-1.23	.395	1.05	0.86-1.27	.630	1.00	0.85-1.17	.993
rs10212372	1.06	0.91-1.22	.466	1.03	0.85-1.25	.766	0.99	0.84-1.16	.862
A36603217	1.15	1.00-1.33	.053	1.09	0.90-1.32	.399	1.12	0.96-1.31	.162
rs10934938	1.15	1.00-1.33	.052	1.09	0.90-1.32	.392	1.12	0.96-1.31	.156
rs4688761	1.17	1.03-1.33	.019	1.09	0.91-1.30	.350	1.15	1.00-1.32	.052
rs9883988	1.11	0.84-1.47	.449	1.04	0.72-1.50	.827	1.05	0.78-1.42	.743

*Not significant after correction for multiple testing.

TABLE E3. Results of association analysis for several eczema-related traits in set 2

	Atopic eczema			Eczema + asthma			Eczema + rhinitis		
SNP	**T**	**NT**	***P* value**	**T**	**NT**	***P* value**	**T**	**NT**	***P* value**
rs13095825	81	76	.750	26	22	.665	32	26	.511
rs16845861	52	62	.399	20	15	.499	24	22	.883
rs10212372	53	60	.572	20	15	.499	25	22	.770
A36603217	50	53	.844	16	13	.710	22	16	.417
rs10934938	49	52	.842	16	13	.710	21	16	.511
rs4688761	65	72	.608	23	15	.256	31	21	.212
rs9883988	13	20	.296	7	4	.546	5	4	1.00

NT, Number of nontransmissions; *T*, number of transmissions.

TABLE E4. Results from association analysis with log-transformed IgE in set 1

	Adjusted for sex and age		Adjusted for sex, age, and *FLG*	
	Estimate	***P* value**	**Estimate**	***P* value**
A36603217	0.003	.975	0.008	.933
rs10212372	−0.014	.884	−0.006	.952
rs10934938	0.005	.959	0.012	.895
rs13095825	−0.016	.840	0.007	.932
rs16845861	−0.008	.929	0.003	.978
rs4688761	−0.014	.867	0.004	.960
rs9883988	−0.035	.839	−0.020	.909

TABLE E5. Results of association analysis and equivalence tests for eczema in the pooled sample (sets 1, 3, 4, and 5) using a dominant model adjusted for cohort effects

SNP	OR	95% CI	C1	C2	X	Ed
rs13095825	1.02	0.93-1.11	1646	1819	1724	Yes
rs16845861	1.03	0.94-1.12	1046	1206	1131	Yes
rs10212372	1.00	0.92-1.10	1019	1176	1094	Yes
A36603217	1.12	1.02-1.22	1024	1182	1140	Yes
rs10934938	1.12	1.02-1.22	1008	1164	1123	Yes
rs4688761	1.08	0.99-1.18	1338	1512	1448	Yes
rs9883988	1.12	0.97-1.30	259	298	293	Yes

Ed, Equivalence decision; *X*, observed number of minor alleles in cases.

TABLE E6. Results from association analysis of additional SNPs with eczema in BAMSE and PARSIFAL (sets 4 and 5) using an additive model

					Set 4 (BAMSE)*				Set 5 (PARSIFAL)†			
SNP	**Allele**	**Amino acid change**	**Amino acid position**	***COL29A1* location**	**MAF**	**OR**	**95% CI**	***P* value**	**MAF**	**OR**	**95% CI**	***P* value**
a36590333	T/C	Met/Thr	56	Exon 3	0.021	1.16	0.73-1.86	.532	0.022	0.93	0.53-1.61	.784
rs1453241	G/A	Glu/Lys	455	Exon 5	0.209	1.04	0.87-1.23	.671	0.201	0.98	0.80-1.18	.806
a36602721	T/G	Val/Gly	669	Exon 6	0.005	0.43	0.13-1.48	.182	0.006	1.58	0.64-3.86	.319
rs16827168	A/G	His/Arg	805	Exon 6	0.023	0.88	0.55-1.43	.609	0.029	1.06	0.67-1.68	.794
rs11917356	A/G	Asp/Gly	982	Exon 7	0.141	1.12	0.91-1.36	.283	0.145	1.18	0.95-1.45	.129
rs1353613	C/G	Ile/Met	1114	Exon 8	0.017	1.36	0.81-2.29	.250	0.011	0.72	0.31-1.68	.452
a36611838	G/A	Val/Ile	1276	Exon 9	0.056	1.04	0.77-1.40	.814	0.054	1.41	1.03-1.91	.030‡
rs12488457	C/A	Pro/Thr	1280	Exon 9	0.279	1.08	0.93-1.27	.313	0.273	1.12	0.95-1.33	.188
rs1497312	C/G	Cys/Ser	1477	Exon 17	0.228	1.08	0.91-1.27	.386	0.231	1.15	0.96-1.37	.129
rs35886424	G/C	Thr/Ser	1539	Exon 20	0.128	1.05	0.85-1.30	.640	0.133	0.98	0.78-1.24	.887
rs16827497	C/T	Pro/Ser	1589	Exon 23	0.219	1.08	0.91-1.29	.367	0.212	1.10	0.91-1.33	.327
a36645464	A/C	Glu/Asp	1750	Exon 33	0.051	1.07	0.79-1.46	.651	0.046	0.70	0.46-1.06	.091
a36654694	C/T	Arg/Cys	2120	Exon 35	0.001	1.65	0.27-9.89	.585	0.001	1.10	0.13-9.14	.933
rs322117	A/G	Asn/Ser	2560	Exon 40	0.149	0.87	0.72-1.07	.188	0.145	0.95	0.76-1.18	.618
a36685870	G/A	Ala/Thr	2589	Exon 40	0.052	0.81	0.59-1.13	.216	0.050	1.03	0.73-1.44	.888
rs11355796	DEL/T	Stop/Leu	2591	Exon 40	0.224	0.98	0.83-1.15	.773	0.206	0.87	0.72-1.07	.189

MAF, Minor allele frequency.
*Adjusted for sex.
†Adjusted for sex and age.
‡Not significant after correction for multiple testing.