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INVESTIGATIVE REPORT

Lack of Association between *Neuropeptide S Receptor 1* Gene (*NPSR1*) and Eczema in Five European Populations

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Eczema is often associated with development of allergic asthma. The *Neuropeptide S Receptor 1 (NPSR1)* gene has previously been associated with asthma and elevated serum IgE levels. The aim of this study was to investigate a potential association between the *NPSR1* gene and eczema in patients and healthy individuals from five different populations in Western Europe, in total 6275 individuals. Seven single nucleotide polymorphisms previously associated with allergic asthma were genotyped. The protein expression of *NPSR1* in the skin was studied using immunohistochemistry in six eczema patients and eight healthy individuals. No association was found between eczema and the seven single nucleotide polymorphisms in *NPSR1* in any of the populations, either independently or in combinations. In addition, no difference was detected in epidermal *NPSR1* expression between eczema patients and healthy individuals. These results strongly suggest that *NPSR1* is not involved in the pathogenesis of eczema. *Key words: atopic dermatitis; eczema; genetic association; G protein-coupled receptor for asthma susceptibility; neuropeptide S receptor 1.*

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Eczema, also known as atopic dermatitis (OMIM 603165) (1), commonly begins in infancy or early childhood, and is characterized by itchy, inflamed skin. It affects 10–20% of children in Western societies and shows strong familial aggregation (2). Eczema is associated with later development of allergic asthma and rhinoconjunctivitis in approximately 30–70% of the affected individuals (3). Eczema and other atopic manifestations share pathogenic and immunological features, such as elevated total serum IgE and/or allergen-specific IgE levels, even though the role of IgE in eczema still remains unclear (4). A candidate gene for asthma susceptibility and elevated total serum IgE levels was identified on chromosome 7p through positional cloning (5). Laitinen et al. (5) reported four risk haplotypes and three non-risk haplotypes in *Neuropeptide S Receptor 1 (NPSR1)* (also called *GPRA*, *GPR154*, GENE ID 387129) that showed significant association with asthma and/or high levels of total serum IgE in three independent populations. Later, other studies have partially replicated these observations (6, 7). In Chinese asthmatic patients, the amino acid changing single nucleotide polymorphism (SNP) (rs324981, Asn107Ile) showed association with airway hyper-responsiveness (8).

The *NPSR1* gene, coding for a G-protein-coupled receptor, undergoes alternative splicing resulting in two isoforms, A and B (5). Among multiple splice variants, only A and B produce receptors that reside on the cell surface (9). *In situ* hybridization with a NPSR1 specific antisense probe showed expression in the bronchus, the gastrointestinal tract and the skin (9) and polymorphisms in the *NPSR1* gene might therefore be a risk factor for different atopic phenotypes, such as eczema.

To investigate the role of *NPSR1* in eczema, we performed genetic association and expression studies. In total, we genotyped seven haplotype-tagging SNPs in the *NPSR1* gene in 6275 individuals (1848 eczema patients) from five Western European countries in order to determine if we could find any association with eczema. We also wanted to elucidate if we among our patients who were primarily included to have eczema we could find any association with other phenotypes often found together with eczema, such as asthma, rhinoconjunctivitis, allergen-specific serum IgE or elevated

total serum IgE. We also studied the expression pattern of the isoforms A and B in skin biopsies from eczema patients and healthy individuals.

MATERIALS AND METHODS

Study populations

Five independent samples with eczema patients and healthy controls from European populations were used for the genetic association analysis (Table I).

From Sweden, we used 406 multiplex families, with 572 sibling pairs and 30 half-sibling pairs affected with eczema, recruited during 1995 to 1997 in Stockholm, Sweden (in total 1440 individuals with successful genotyping in at least one marker) (10). Subjects were all classified as having eczema on the basis of clinical examination by dermatologists applying the UK Working Party's Diagnostic Criteria (11). Based on an interview by a dermatologist, subjects were classified as having asthma and/or rhinoconjunctivitis or neither.

From the cross-sectional PARSIFAL study (Prevention of Allergy Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle), samples from 3113 schoolchildren aged 5–13 years from five Western European countries were included (6). The PARSIFAL study was designed to investigate the role of different lifestyles and environmental exposures in farm children, children from Steiner schools (mainly from families with anthroposophic lifestyle) and two corresponding reference groups, to identify protective factors against the development of asthma and allergic disorders. In Austria, Germany, the Netherlands, and Switzerland farm children were recruited from schools in rural areas known to have a high percentage of farmers and in Sweden through the Farming Registry at the National Bureau of statistics. Children with anthroposophic lifestyle were recruited from classes in Steiner schools. The respective reference groups were recruited with similar methods from the same geographical areas. Information on environmental exposures and health endpoints were reported by the parents of the children, except for atopic sensitization (see below). Children ever diagnosed with asthma, or obstructive bronchitis more than once, were considered to have asthma. Children diagnosed with hay fever and who ever had had symptoms of hay fever, were considered to have rhinoconjunctivitis. The subjects were classified as having eczema when reporting ever having an intermittent itchy rash lasting at least 6 months and having a doctors' diagnosis of atopic eczema. The study design is described in detail elsewhere (12).

From Germany we used 224 families of Caucasian origin for eczema (complete trios with father, mother and child, and extended families) that had been recruited between January 2001 and December 2003 through an offspring with eczema (689 individuals) (13). Diagnosis of eczema was made on the basis of clinical examination by dermatologists applying the UK Working Party's Diagnostic Criteria (11). The subjects were classified as having asthma or rhinoconjunctivitis when they reported a physician's diagnosis.

The KORA (Cooperative Health Research in the Augsburg Region) S3 and S4 surveys are large population-based cross-sectional studies on adults performed from 1994 to 1995 and from 1999 to 2001 in the city and region of Augsburg, South Germany. KORA C represents an enriched sample of 1502 subjects out of the 4178 KORA S3 individuals, who had valid allergen-specific serum IgE results. Subjects were selected, stratified by age and sex, to provide 50% with and 50% without allergen-specific IgE to at least one of the allergens tested, and furthermore, so that within these groups 50% had reported symptoms of atopic diseases such as rhinoconjunctivitis, asthma or eczema. DNA was available from 1420 individuals. From KORA S4 ($n=4261$), 227 individuals with eczema and 227 matched controls were selected. The subjects were classified as having asthma, rhinoconjunctivitis or eczema when they reported a physician's diagnosis of asthma, rhinoconjunctivitis or eczema, respectively. The sampling frames and study designs have been described previously (13, 14).

IgE serology

In all populations in the study, allergen-specific IgE antibodies against a mixture of inhalant allergens were measured using ImmunoCAP System Phadiatop®FEIA (Phadia AB, Uppsala, Sweden). Additionally, in the PARSIFAL study and in the Swedish family material, antibodies against a mix of six common food allergens were measured using ImmunoCAP System™ Fx5 and

Table I. *Study populations (for reference to the studies, see Materials and Methods)*

	Swedish families $n=1440$[b]	PARSIFAL $n=2272$[b]	German families $n=689$[b]	KORA C $n=1420$[b]	KORA S4 $n=454$[b]	Total $n=6275$[b]
Age at inclusion (years), mean	29	9	18	49	44	
Country of origin (%)						
Germany		[a]	100	100	100	
Sweden	100	33.3				
Austria		30.5				
The Netherlands		22.0				
Switzerland		14.2				
Eczema (*n*)	1071	218	302	30	227	1848
Dermatologist's diagnosis of eczema (*n*)	1071		302			1373
Reported diagnosis of eczema (*n*)		218		30	227	475
Asthma (*n*)	450	202	117	136	42	
Rhinoconjunctivitis (*n*)	814	75	240	388	129	
Atopic sensitization[c] (*n*)	564	635	356	772	154	
Elevated total serum IgE[d] (*n*)	279	–	225	504	145	
Healthy individuals (*n*)		1365		348	58	1771

[a]Excluded from analysis due to Hardy-Weinberg equilibrium deviation.

[b]Denotes number of individuals with successful genotype in at least one marker.

[c]Defined as allergen-specific IgE $\geq$0.35 kU/l in Phadiatop and/or in Fx5.

[d]Individuals with total serum IgE levels above the 66th percentile in each population were defined as having an elevated level.

KORA: Cooperative Health Research in the Augsburg Region; PARSIFAL: Prevention of Allergy Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle.

ImmunoCAP System RAST®FEIA, respectively (Phadia AB). Atopic sensitization was defined as IgE ≥ 0.35 kU/l in Phadiatop and/or in Fx5.

Total serum IgE was measured in all subjects in the different cohorts (except in the PARSIFAL study) using the ImmunoCAP System™ IgE FEIA. Due to differences in age and reference values between the populations we analysed the trait "elevated total serum IgE" as a qualitative trait. Individuals with total serum IgE levels above the 66th percentile in each population were defined as having an elevated level.

Since PARSIFAL and KORA C participants were not primarily recruited as eczema patients we excluded them when we analysed the phenotypes asthma, rhinoconjunctivitis, atopic sensitization and elevated total serum IgE levels in the context of eczema patients.

Ethics approval and informed consent were obtained in each participating study centre.

As healthy controls in the case-controls studies ($n = 1771$), individuals were selected who did not suffer from eczema, asthma, or rhinoconjunctivitis. Furthermore, the controls did not have elevated total serum IgE or atopic sensitization.

Genetic analysis

Seven SNPs between exons 2 and 3 within the *NPSR1* gene were genotyped: rs323917, rs323922, rs324377, SNP546333, rs324384, rs324396 and rs740347. These SNPs were selected on the basis of their risk or non-risk haplotype tagging properties and significant associations in previous studies (5–7). Genotypes were analysed using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (SEQUENOM Inc., San Diego, CA, USA) as described elsewhere (6, 15). Both the minor allele frequency of the markers and the haplotype frequencies were comparable to each other in the five different studies (Table II).

The rate of successful genotyping was above 90% for all study populations. Rs324384 could not successfully be genotyped in any of the three German patient materials (KORA C, KORA S4, or the family material from Germany).

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was evaluated for each SNP in all five populations using a χ^2-test as implemented in PLINK v1.01 (16). Association between genetic markers and disease status was analysed using the UNPHASED 3.0.10 program (17, 18) with the following analysis options; full model option, rare frequency threshold of 3%, uncertain haplotypes option (for the joint analysis and the three case-control studies) and uncertain and missing genotypes option for the two family materials. With individual haplotype option in the haplotype analyses, a score test of the difference in risk between haplotypes and all the others pooled together was performed, although the odds ratio (OR) is shown relative to a reference haplotype (the most frequent haplotype, H1). In the analysis of associated phenotypes in the context of eczema, the zero frequency threshold was set to 0.01, whereas in all the other analysis the default value was used. Significant *p*-values were corrected with permutation tests. Power analysis was performed using the Genetic Power Calculator (19) with model parameters set to calculate the power to detect a dominant effect.

Skin biopsy specimens and immunohistochemistry

Six adult eczema patients with positive atopy patch test (APT) reactivity to house dust mites (*Dermatophagoides pteronyssinus*) were included (two males, four females, age range 37–65 years). Four had elevated total serum IgE levels (498–13900 kU/l) and three of these had allergen-specific

Table II. *NPSR1 haplotypes and frequencies*

SNP	rs323917 [C/G]	rs323922 [G/C]	rs324377 [C/A]	SNP546333 [G/A]	rs324384 [T/C]	rs324396 [C/T]	rs74037 [G/C]	Haplotype frequencies					
								Joint	Swedish families	PARSIFAL	German families	KORA C	KORA S4
NT-000380	515224	522363	529556	546333	555608	563704	585883						
H1	C	G	C	G	C	T	G	0.320	0.305	0.325	0.332	0.295	0.324
H2	C	C	A	G	T	C	G	0.214	0.208	0.214	0.191	0.242	0.232
H3	C	G	C	G	C	C	G	0.240[a]	0.155	0.133	0.228[a]	0.244[a]	0.259[a]
H4	C	C	A	A	T	C	C	0.077	0.079	0.082	0.071	0.065	0
H5	C	C	A	G	T	C	C	0.058	0.055	0.049	0.089	0.055	0.046
H6	C	G	C	G	T	C	G	0.240[a]	0.092	0.102	0.228[a]	0.244[a]	0.259[a]
H7	G	C	A	G	T	C	G	0.061	0.070	0.062	0.046	0.058	0.046
MAF S	0.072	0.457	0.483	0.080	0.434	0.314	0.141						
MAF P	0.062	0.418	0.447	0.084	0.441	0.309	0.141						
MAF T	0.045	0.396	0.420	0.071	[b]	0.338	0.158						
MAF KC	0.061	0.409	0.444	0.077	[b]	0.317	0.141						
MAF KS	0.054	0.404	0.429	0.066	[b]	0.308	0.128						

[a]Joint haplotype frequency for H3 and H6, due to no genotyping of rs324384.
[b]Not genotyped in this patient material.
KORA: Cooperative Health Research in the Augsburg Region; PARSIFAL: Prevention of Allergy Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle; SNP: single nucleotide polymorphism.

serum IgE to *D. pteronyssinus* (ImmunoCAP, Phadia AB). Skin biopsies were taken from the APT sites (*D. pteronyssinus*) at 6, 24, 48 and 72 h after application and at 72 h after application with the vehicle alone and were snap-frozen (20). In addition, skin biopsies were obtained from 8 healthy donors. The protocol was approved by the regional ethics committee of Karolinska University Hospital, Stockholm, Sweden.

Immunohistochemical staining was performed on acetone-fixed, 6 μm thick, vertical cryostat sections. Affinity purified rabbit antibodies directed against NPSR1 isoforms A and B (5), respectively, were obtained from GeneOS Oy, Helsinki, Finland, and used at an IgG concentration of 5 μg/ml. We employed the avidin-biotin complex (ABC) method, using biotinylated secondary goat-anti-rabbit antibody and Vectastain® elite ABC kit (both from Vector Laboratories Inc., Burlingame, CA, USA) and developed with 3-amino-9-ethylcarbazol. Endogenous peroxidase activity was blocked with hydrogen peroxidase, and non-specific binding of the antibodies was reduced by incubating the sections with normal goat serum (Dako Cytomation A/S, Glostrup, Denmark) and avidin/biotin blocking kit (Vector Laboratories Inc.) prior to staining. Pre-immune rabbit serum and omission of the primary antibody were used as negative controls (Fig. 1). Positive staining with the rabbit antibodies directed against NPSR1 was blocked by pre-incubation of the antibodies with their corresponding peptide (GeneOS Oy, 25 μg/ml peptide to 5 μg/ml IgG) overnight, before it was added to the skin sections (Fig. 1).

RESULTS

Genetic analysis

All analysed markers were in HWE in the patient materials, except in the PARSIFAL group where two SNPs (rs324377 and rs323922) deviated from HWE ($p < 0.00007$ and $p < 0.0003$, respectively). As PARSIFAL contains individuals from five different countries

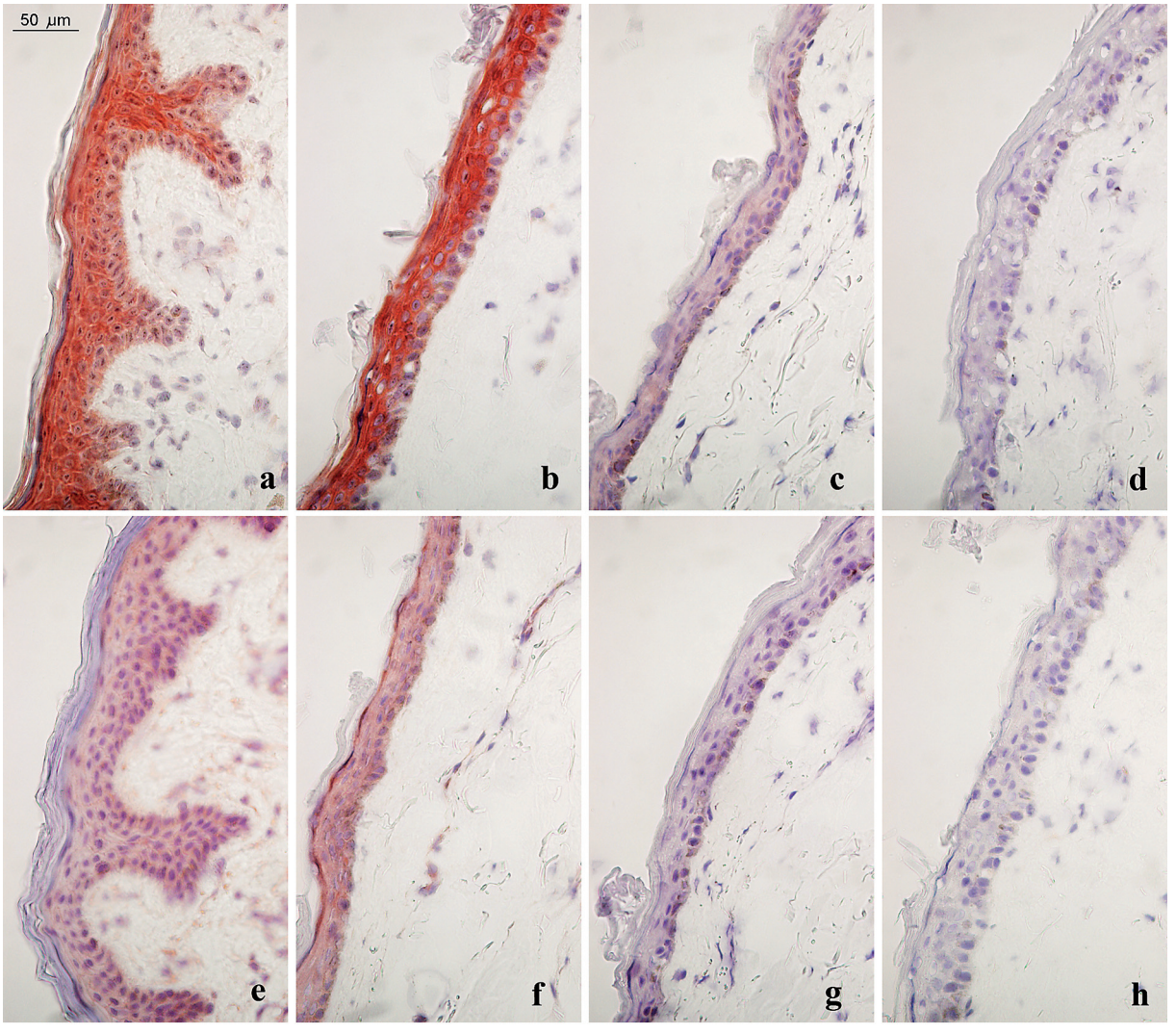

Fig. 1. Epidermal *NPSR1* expression does not differ between eczema patients and healthy individuals. Immunohistochemical staining on biopsy specimens from atopy-patch-tested skin 48 h after application of *Dermatophagoides pteronyssinus* extract from an eczema patient (65-year-old female, SCORAD 50, total serum IgE 13900 kU/l and with a high level of *D. pteronyssinus* specific IgE (>99 kU/l)) (a and e) and a healthy control (female, 47 years old) (b–d and f–h). Sections (a) and (b) are stained with rabbit anti-NPSR1 isoform A (5 μg/ml), (e) and (f) with rabbit anti-NPSR1 isoform B (5 μg/ml). No background was seen in staining with pre-immune serum (10 μg/ml IgG) for isoform A (c) or isoform B (g). The specificity of the epidermal NPSR1 staining was verified by complete blocking with the corresponding peptides (d) for isoform A, and (h) for isoform B), see Materials and Methods.

Table III. *Lack of association to eczema for 7 SNPs in the NPSR1 gene*

		Swedish families	PARSIFAL	German families	KORA C	KORA S4	Joint
SNP	Minor allele	*P*	*P*	*P*	*P*	*P*	*P*
rs323917	G	0.11	0.72	0.22	0.09	0.68	0.06
rs323922	C	0.53	0.20	0.40	0.91	0.60	0.75
rs324377	A	0.92	0.17	0.48	0.64	0.61	0.60
SNP546333	A	0.32	0.65	0.15	0.39	0.96	1.00
rs324384	C	0.42	0.16	[a]	[a]	[a]	[a]
rs324396	T	0.07	0.08	0.76	0.64	0.75	0.45
rs74037	C	0.91	0.75	0.45	0.09	0.76	0.44

[a]Not genotyped in this patient material

P: global *p*-value; SNP: single nucleotide polymorphism; KORA: Cooperative Health Research in the Augsburg Region; PARSIFAL: Prevention of Allergy Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle.

in Europe, we tested HWE in the different subgroups and found that the non-atopic individuals in the German subgroup in PARSIFAL was not in HWE regarding these SNPs ($p < 1.2 \times 10^{-7}$ and $p < 2.4 \times 10^{-7}$, respectively). We therefore excluded the German subgroup in the PARSIFAL material from further analysis.

We found no association with eczema for any of the seven SNPs in any of the five different populations or in a joint analysis with all materials pooled together (Table III). There was also lack of association with any of the seven common haplotypes of *NPSR1* in the different patient materials (data not shown) and also when pooling the materials into a joint analysis (Table IV). In this joint analysis the power was more than 93% for detecting a factor with an allele frequency >0.13 (H4 or H5) and an OR of 1.4.

In the analysis of atopic phenotypes in the context of eczema, we found rhinoconjunctivitis to be significantly associated with the minor allele of polymorphism rs324396 in *NPSR1* (OR 1.25 (1.06–1.47, $p < 0.03$ corrected after 1000 permutations). No significant association was found with *NPSR1* and any of the other phenotypes in the context of eczema even if the power to detect these was over 80% in our material.

Immunohistochemistry

In both eczema patients and healthy controls, a strong epidermal expression of isoform A was found, although this was weaker in the basal proliferating keratinocytes. Isoform B showed a weak but homogenous epidermal expression in all individuals (Fig. 1). None of the isoforms showed an appreciable difference in epidermal *NPSR1* expression between eczema patients and healthy controls, between eczema patients with high or low total serum IgE levels, or between different time-points after atopy patch testing with allergen or vehicle application.

DISCUSSION

Asthma and eczema can both be manifestations of atopic diseases and share some pathogenic and immunological features, such as the tendency to develop allergen-specific IgE against common allergens in our environment. The association of *NPSR1* and asthma and raised IgE originally described by Laitinen et al. has been replicated in several studies (6–8). Genetic association has also been found to other allergic phenotypes, such as atopic sensitization and allergic rhinoconjunctivitis (6). The NPSR1 protein is found to be expressed in the bronchus, the gut and the skin (5, 9). Recently, inflammatory bowel disease has also been shown to associate with one haplotype in *NPSR1* (H2) and with the functional SNP (rs324981, Asn107Ile) (21). In this study we wanted to elucidate whether any of the SNPs in the *NPSR1* gene also were associated with eczema, and if the NPSR1 protein expression was altered in skin with eczema compared with healthy skin.

We found no association with eczema for any of the analysed SNPs in *NPSR1* or the common haplotypes. Our results suggest that genetic variants in *NPSR1* do not influence the susceptibility for eczema, thus confirming the results found by a German study, that found no association of polymorphism in rs232922,

Table IV. *Lack of association of NPSR1 haplotypes with eczema in all five materials. No values are significant.*

Haplotype	Case	Control	Case frequency	Control frequency	OR	95% CI
H1	549	1287	0.325	0.317	1.0	
H2	370	871	0.219	0.215	1.05	0.89–1.23
H3/H6	415	982	0.246	0.242	0.99	0.85–1.16
H4	123	314	0.073	0.077	0.95	0.76–1.20
H5	96	224	0.057	0.055	0.91	0.71–1.17
H7	95	255	0.056	0.063	0.87	0.67–1.13

OR: odds ratio; CI: confidence interval.

with eczema (22) and a group from UK who reported lack of association with any of the haplotypes H1–H7 in *NPSR1* and adult eczema (23).

Considering the number of eczema patients in this study (1848) and the estimated power of 93%, it is probable that a true association with eczema would have been found, assuming that the impact of *NPSR1* gene variants would be of a similar magnitude in eczema and asthma. However, we could not discriminate the haplotype H6 from H3 in the joint analysis and can therefore not exclude an association of the haplotype H6 with eczema. Furthermore, the impact of the functional SNP rs324981 on eczema susceptibility was not evaluated in this study, but its association is unlikely given its strong association with the risk/non-risk haplotypes.

We also showed that the two isoforms, NPSR1-A and NPSR1-B, are expressed in the epidermis of both healthy individuals and eczema patients, but with no apparent difference between patients and controls. The APT-induced eczema was used here as an experimental model of eczema (20, 24), and in addition lesional and non-lesional eczema skin was stained with comparable results (data not shown). The ligand identified to interact with NPSR1 is a 20-residue peptide called Neuropeptide S (NPS). Studies have shown strong expression of NPS in the brain (25) and also in bronchial and colonial epithelia (9), but not in the skin (23). Absence of expression of the ligand in the skin might help explain the lesser importance of NPSR1 in the eczema pathogenesis, but as NPS is co-expressed in all other tissue with NPSR1, its absence may also be a technical artefact.

Regarding the atopic phenotypes, the only significant association with *NPSR1* we found in the context of eczema was with rs324396 and rhinoconjunctivitis. No other phenotype was associated with *NPSR1* in the context of eczema.

In conclusion, we report a lack of genetic association of seven *NPSR1* polymorphisms with eczema in five European eczema patient materials. In addition, we found the NPSR1 isoforms A and B to be expressed in the epidermis of healthy individuals and eczema patients, but with no difference in expression. Taken together, these findings suggest that the *NPSR1* gene is not a susceptibility gene for eczema.

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