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Biological and genetic interaction between Tenascin C and Neuropeptide S receptor 1 in allergic diseases

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Neuropeptide S receptor 1 (*NPSR1, GPRA 154, GPRA*) has been verified as a susceptibility gene for asthma and related phenotypes. The ligand for NPSR1, Neuropeptide S (NPS), activates signalling through NPSR1 and microarray analysis has identified Tenascin C (*TNC*) as a target gene of NPS-NPSR1 signalling. *TNC* has previously been implicated as a risk gene for asthma. We aimed therefore to study the genetic association of *TNC* in asthma- and allergy-related disorders as well as the biological and genetic interactions between *NPSR1* and *TNC*. Regulation of TNC was investigated using NPS stimulated NPSR1 transfected cells. We genotyped 12 *TNC* SNPs in the cross-sectional PARSIFAL study (3113 children) and performed single SNP association, haplotype association and *TNC* and *NPSR1* gene–gene interaction analyses. Our experimental results show NPS-dependent upregulation of TNC-mRNA. The genotyping results indicate single SNP and haplotype associations for several SNPs in *TNC* with the most significant association to rhinoconjunctivitis for a haplotype, with a frequency of 29% in cases ($P = 0.0005$). In asthma and atopic sensitization significant gene–gene interactions were found between *TNC* and *NPSR1* SNPs, indicating that depending on the *NPSR1* genotype, *TNC* can be associated with either an increased or a decreased risk of disease. We conclude that variations in *TNC* modifies, not only risk for asthma, but also for rhinoconjunctivitis. Furthermore, we show epistasis based on both a direct suggested regulatory effect and a genetic interaction between *NPSR1* and *TNC*. These results suggest merging of previously independent pathways of importance in the development of asthma- and allergy-related traits.

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INTRODUCTION

The neuropeptide S receptor (*NPSR1* also *GPRA* or *GPR154*) was recently identified as a susceptibility gene for asthma and high total serum IgE levels (1–6). The ligand for NPSR1, known as Neuropeptide S (NPS), activates signalling through NPSR1 (7,8). To identify target genes of NPS-NPSR1 signalling, a stable NPSR1-A overexpressing cell line was engineered and the effect of NPS stimulation on gene expression studied by microarray analysis (9). One of the upregulated genes, showing a robust >4-fold change, was Tenascin C (*TNC*).

TNC has been considered an important gene in asthma pathogenesis for over 10 years, because it is abnormally upregulated in asthmatic tissue (10). However, surprisingly little genetic evidence has been put forward to support this biomarker (11) despite the existence of genetic variants with potential functional differences. One genome-wide linkage study linked the region on 9q33, where *TNC* is located, to asthma (12) and recently a coding SNP (Leu1677Ile) was reported to strongly associate to adult asthma (13). The same region has, however, been considered as a candidate region for genome-wide studies searching for linkage to allergic rhinitis (14–16) even though no candidate genes have been investigated so far. TNC, also known as hexabrachion, is an extracellular matrix (ECM) protein functioning as an adhesion-modulating molecule with main biological roles in cell communication and signal transduction. TNC promotes weak connections with its conjugating proteins and is believed to be involved in cell migration and growth (17). The tenascin family of glycoproteins display highly restricted expression patterns and are found mainly during embryogenesis (18). In adult tissue, tenascins are only expressed in pathological states including inflammation, or in reparatory processes such as wound healing (17,18). Several previous studies have linked TNC expression to asthma and allergy both in mouse (19,20) and in human (10,21–25). Recently, *TNC* together with *NPSR1* were highlighted as two most interesting genes providing novel insights into asthma pathogenesis (11).

In complex disorders that have multiple susceptibility and protective genes, there is a possibility of risk modification depending on different allele combinations (epistasis or 'gene-gene interaction'). As both *NPSR1* and *TNC* have been implicated as risk factors for asthma, and since it has been suggested that TNC may be regulated by NPSR1 activation, our objective was to investigate the genetic role of *TNC* in asthma- and allergy-related disorders as well as the potential biological and genetic connection between NPSR1 and TNC. Using stable NPSR1 transfected cells, stimulated with NPS we investigated the mRNA expression pattern of TNC. We studied the genetic role of *TNC* and also the effect of different *TNC* and *NPSR1* allele combinations using the cross-sectional PARSIFAL (Prevention of Allergy-Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle) study. The PARSIFAL study includes children 5–13 years of age from five different European countries, well characterized for asthma- and allergy-related traits (26). The association of *NPSR1* SNPs to asthma- and allergy-related phenotypes in the PARSIFAL children has been reported earlier (3).

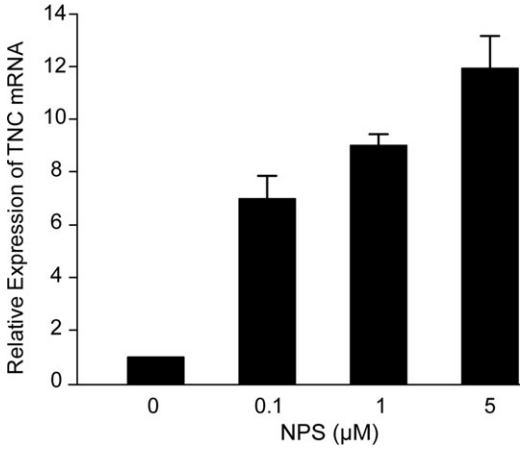

Figure 1. Dose-dependent expression levels of TNC-mRNA after NPS stimulation in concentrations 0.1, 1 and 5μM for 6 h of NPSR1-A transfected HEK 293H cells. The mRNA expression levels were measured by qRT-PCR in triplicates. Relative expression levels of TNC in the stimulated NPSR1-A cells were compared with the expression in unstimulated cells with GAPDH as endogenous control (mean ± SD).

In this study, we found that experimental NPS stimulation of NPSR1 transfected cells upregulated *TNC* mRNA in a dose-dependent manner. In the PARSIFAL study polymorphisms within *TNC* were genetically associated to childhood asthma- and allergy-related phenotypes, with the strongest association to rhinoconjunctivitis, and there appears to be significant epistasis between *NPSR1* and *TNC* altering the risk of disease.

RESULTS

NPS stimulation of NPSR1 regulates *TNC* mRNA expression in a dose-dependent manner

To validate and extend previous microarray results, suggesting a NPS-NPSR1 activation-dependent upregulation of *TNC* (9), we stimulated NPSR1-A transfected HEK-293H cells for 6 h with increasing concentrations of NPS (0.1, 1 and 5 μM). The mRNA expression levels of *TNC* were measured using quantitative reverse-transcriptase–PCR (qRT–PCR). The relative *TNC* expression of the NPS-stimulated HEK-293H cells, compared with unstimulated control HEK-293H cells, increased in a dose-dependent manner up to 12 times more at 5 μM NPS when compared with the unstimulated control (Fig. 1). We also validated the results in a human lung epithelial cell line (A549) transiently transfected with NPSR1-A. The result showed an 3.3-fold (± SD = 0.3) upregulation of *TNC* mRNA in cells stimulated with 0.1μM NPS for 6 h, compared with unstimulated control A549 cells. These results confirmed the regulation of *TNC* expression by NPSR1 signalling.

In vivo co-expression of NPSR1 and TNC in asthmatic bronchus

If there is biological connection in asthma between NPSR1 and TNC, the two proteins would likely be co-expressed in

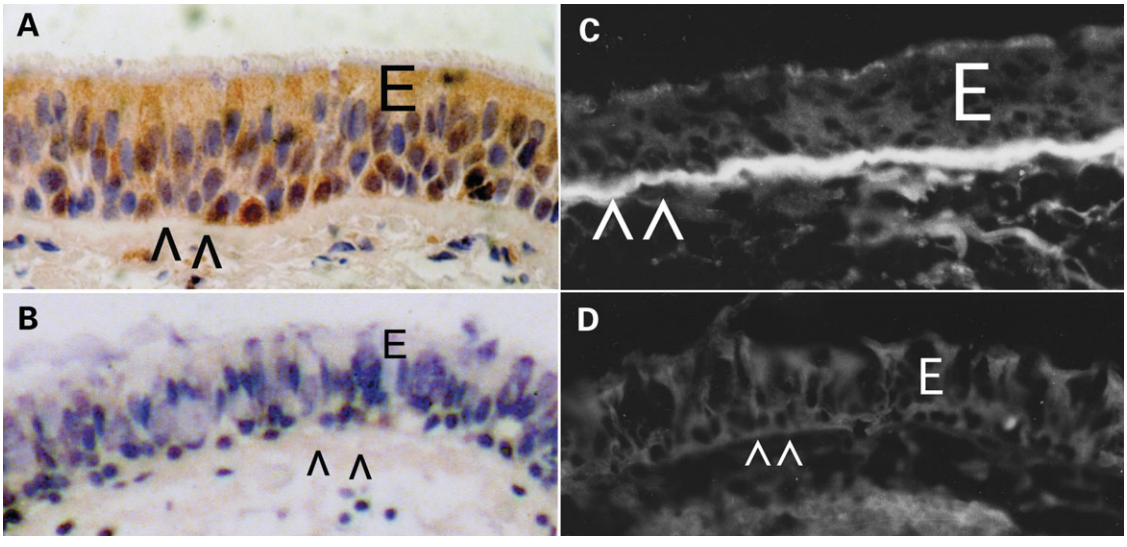

Figure 2. Bronchial biopsy specimens from at least eight asthmatic patients (exemplified in **A** and **C**) and eight normal control subjects (exemplified in **B** and **D**) were stained with antibodies against NPSR1-B (A and B) and TNC (C and D). NPSR1 immunoreactivity was detected in the epithelial cells in asthmatic patients (A), whereas no NPSR1 protein was detected in control subjects (B). TNC immunoreactivity was visible in the sub epithelial basement membrane layer in asthmatic patients (C) but not in control subjects (D). E, epithelium; arrowheads, basement membrane. Original magnification 250×.

the asthmatic airway. Bronchial biopsy specimens from healthy controls and asthmatic patients were stained with antibodies against NPSR1 and TNC, and the expression of both proteins was found elevated in asthmatic airways compared with healthy controls (Fig. 2). The two proteins were found in close proximity, with NPSR1 detectable in bronchial epithelial cells (Fig. 2A) and TNC in the subepithelial basement membrane zone (Fig. 2C).

Genetic association of *TNC* alleles to childhood allergy- and asthma-related traits

With an attempt to replicate the previously suggested genetic association of *TNC* to asthma-related disorders, we genotyped children from the PARSIFAL study with 12 *TNC* SNPs tagging redundantly the previously associated 3′ part of the gene. The genotyping results indicated single SNP associations between several SNPs in *TNC* and current rhinoconjunctivitis, with the most significant associations to SNP rs3789873 intron 10, rs13321 Gln2008Glu and rs1330363 intron 15 (Table 1). Similar results were found when restricting the analysis to allergic rhinoconjunctivitis (i.e. a combination of current rhinoconjunctivitis and the presence of IgE antibodies to inhalant allergens), with the most significant association for rs3789873 (OR = 1.37, 95%CI = 1.09–1.74, $P = 0.008$). Weaker associations were found for rs2297181 intron 16, to doctor's diagnosis of asthma (OR = 1.29, 95%CI = 1.00–1.68, $P = 0.05$) (for a complete report, see Supplementary Material, Table S2A) and atopic sensitization (OR = 1.18, 95%CI = 1.00–1.39, $P = 0.05$). To test for a more specific asthma phenotype we analysed those having a doctor's diagnosis of asthma combined with an atopic sensitization, i.e. allergic asthma. These individuals showed a stronger association to rs2297181 (OR = 1.53, 95%CI = 1.10–2.13, $P = 0.01$). Haplotype analysis revealed three haplotype blocks (Fig. 3) with the most significant association in block 1, showing haplotype-specific association for the four-allele haplotype TGGT to current rhinoconjunctivitis (Table 2), and also showing associations to atopic sensitization (OR = 1.17, 95%CI = 1.02–1.33, $P = 0.03$) and allergic rhinoconjunctivitis (OR = 1.47, 95%CI = 1.15–1.87, $P = 0.002$). No significant association was found on the haplotype level to doctor's diagnosis of asthma (for a complete report, see Supplementary Material, Table S2B) but the more stringent phenotype allergic asthma revealed significant association for the five-allele haplotype AACCC in block 3 (OR = 1.52, 95% CI = 1.09–2.11, $P = 0.02$). We also tested for association to the extended haplotype grouping all 12 genotyped *TNC* SNPs. This full segment showed significant association for the haplotype TGGT_G_GA_GGCCG to current rhinoconjunctivitis (OR = 1.47, 95%CI = 1.17–1.84 p = 0.001), atopic sensitization (OR = 1.16, 95%CI = 1.02–1.33, $P = 0.03$) and allergic rhinoconjunctivitis (OR = 1.45, 95%CI = 1.13–1.86, $P = 0.004$). Doctor's diagnosis of asthma did not show significant association to an extended haplotype but allergic asthma showed significant association for the haplotype CGCC_G_GA_GACTC (OR = 1.44, 95%CI = 1.00–2.06, $P = 0.05$). No significant associations were found on either single SNP or haplotype level for the traits current wheezing or current atopic eczema.

NPSR1 and *TNC* alleles show epistatic effects on risks

Because TNC was regulated by NPSR1, and both *TNC* and *NPSR1* are independently associated to asthma- and allergy-related phenotypes, we evaluated potential epistatic effects between variants in the two genes ('gene-gene interactions') in the PARSIFAL study population. The association of *NPSR1* SNPs to asthma- and allergy-related phenotypes has been reported earlier for the PARSIFAL children (3) and we used these data to analyse joint effects of *TNC* and *NPSR1* variants. Evidence of significant interaction was

Table 1. Genetic association between *TNC* polymorphisms and current rhinoconjunctivitis in children from the PARSIFAL study

SNP[a]	Position in gene (NT_008470)	Minor allele frequency Cases, $n = 214$[d]	Controls, $n = 2849$[d]	Risk allele	OR (95% CI)[b]	*P*-value[b,c]
rs12351083 [T/C]	3′genome	0.28	0.30	T	1.10 (0.88–1.37)	0.387
rs1330362 [G/A]	3′genome	0.05	0.06	G	1.25 (0.78–2.01)	0.353
rs13321 [C/G]	Gln2008Glu	0.33	0.27	G	1.34 (1.09–1.66)	**0.006**
rs12347433 [T/C]	Arg1891Arg	0.27	0.29	T	1.11 (0.88–1.40)	0.389
rs2274750 [G/A]	Thr1781Ala	0.03	0.02	A	1.27 (0.71–2.26)	0.417
rs10817704 [G/A]	intron 17	0.03	0.05	G	1.76 (1.00–3.10)	**0.046**
rs2104772 [A/T]	Leu1677Ile	0.38	0.44	A	1.29 (1.05–1.59)	**0.014**
rs2297181 [G/A]	intron 16	0.11	0.12	G	1.07 (0.78–1.46)	0.681
rs1330363 [A/G]	intron 15	0.45	0.38	G	1.31 (1.07–1.60)	**0.008**
rs17819466 [C/T]	Thr1525Thr	0.12	0.13	C	1.01 (0.75–1.36)	0.961
rs7865462 [C/T]	intron 13	0.44	0.50	C	1.27 (1.04–1.55)	**0.018**
rs3789873 [C/G]	intron 10	0.32	0.25	G	1.41 (1.14–1.74)	**0.002**

[a]Nucleotides indicated in brackets [major/minor allele].
[b]Odds ratio (OR) with 95% confidence interval (CI) as well as *P*-value (χ^2 test) for differences in allele frequencies between cases and controls.
[c]*P*-values ≤ 0.05 are marked in bold.
[d]Numbers may vary slightly between SNPs depending on genotyping success for each SNP.

found between several of the *TNC* and *NPSR1* SNPs for atopic sensitization or doctor's diagnosis of asthma (Table 3). The coding TNC SNP rs2104772, Leu1677Ile, in combination with the NPSR1 SNPs rs323922 and rs324384, showed significant interaction for both these traits (Table 3). However, no significant interactions were seen between *TNC* and *NPSR1* for the outcome current atopic eczema and only few significant interactions were found for current rhinoconjunctivitis (rs12347433*rs324396; $P = 0.05$) or current wheezing (rs12347433*rs323917; $P = 0.05$, rs3789873*rs323917; $P = 0.02$, rs1330362*rs324384; $P = 0.03$, rs10817704* rs324384; $P = 0.01$). Due to lack of power, no interaction analyses were performed on combined phenotypes. The interaction analysis indicated that depending on the NPSR1 genotype, *TNC* variants can be associated with both an increased and a decreased risk of disease, as illustrated graphically in Figure 4 (for a complete report of odds ratios for all significant interactions in relation to atopic sensitization or doctor's diagnosis of asthma, see Supplementary Material, Table S3A and B).

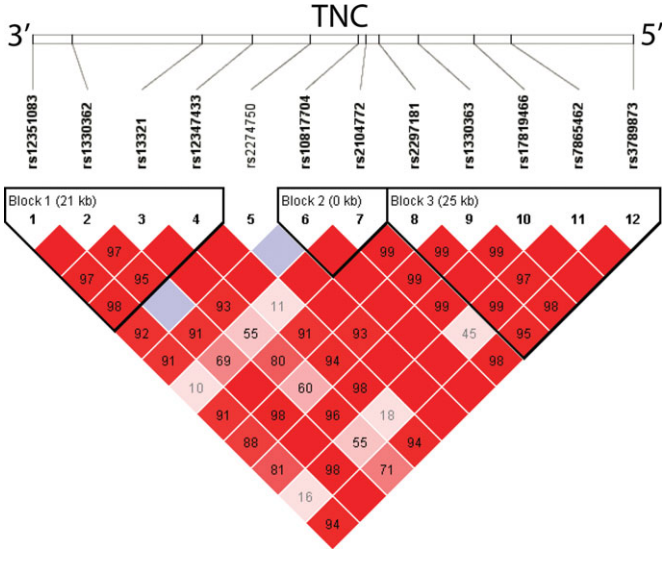

Figure 3. The 3′ 50 kb of TNC analysed in this study. The LD plot, as defined by Gabriel *et al*. (48), divides TNC in three haplotype blocks 1, 2 and 3. The studied SNPs in each block are indicated and also the relative position in the TNC gene (white bar). The numbers in each box correspond to the pair-wise linkage disequilibrium coefficient D′ between SNPs.

DISCUSSION

In asthma and allergy as in many other complex disorders, risk effects (estimated as ORs) of individual genes have turned out to be notably lower than the estimated total genetic risk. Therefore, it is possible that some combinations of risk genes or of risk genes and environmental exposures might interact and cause unexpected joint risk effects. To find such possible genetic interactions, it is reasonable to start evaluating combinations of already identified risk-modifying genes. A further useful consideration in searching for such epistatic effects might be any known biological interactions. When our previous microarray analysis suggested NPSR1-dependent regulation of TNC, and both genes had been independently associated with asthma or related phenotypes, it appeared well-motivated to hypothesize that the two genes might also show epistatic effect patterns.

By stimulating NPSR1 transfected HEK293H cells with NPS, we demonstrated that the upregulation of *TNC* mRNA is NPS dose-dependent, confirming and extending our previous suggestion of NPS-NPSR1-dependent regulation of TNC (9). We could also show that the NPS-NPSR1 pathway upregulates *TNC* mRNA in NPS-stimulated NPSR1 transiently transfected human lung epithelial cells. In a recent study of ovalbumin-induced bronchial asthma in *TNC*-deficient mice, a lack of TNC attenuated allergen-induced bronchial asthma (20). TNC has also been shown to be abundantly deposited in the basement membrane (BM) zone of asthmatic bronchi (10) and the same study indicates that the expression of TNC is higher in patients with severe and chronic asthma. Thus, TNC might be an important factor in the characteristic incomplete healing and remodelling of the asthmatic airways. As asthma and allergy prolong and get more severe

Table 2. Genetic association between *TNC* haplotypes and current rhinoconjunctivitis in children from the PARSIFAL study

Block	Haplotype	Estimated haplotype frequency		OR[a] (95% CI)	Empirical *P*-value[b]
		Cases, $n = 214$	Controls, $n = 2855$		
1	TGCT	0.39	0.43	0.84 (0.69–1.03)	0.117
	CGCC	0.27	0.29	0.89 (0.72–1.12)	0.329
	TGGT	0.29	0.21	1.49 (1.20–1.86)	**0.0005**
	TAGT	0.04	0.05	0.81 (0.50–1.30)	0.329
					[c]Global empirical *P*-value = 0.024
2	GA	0.62	0.56	1.29 (1.05–1.58)	**0.013**
	GT	0.35	0.38	0.84 (0.69–1.04)	0.117
	AT	0.03	0.05	0.57 (0.32–1.00)	**0.040**
					[c]Global empirical *P*-value = 0.032
3	GACTC	0.44	0.50	0.80 (0.65–0.97)	**0.049**
	GGCCG	0.31	0.25	1.39 (1.12–1.72)	**0.002**
	GGTCC	0.12	0.13	0.98 (0.72–1.32)	0.702
	AACCC	0.11	0.12	0.92 (0.68–1.26)	0.436
					[c]Global empirical *P*-value = 0.040

[a]ORs were calculated with the haplo.cc function included in the R package haplo.stats (http://www.R-project.org). Each analysis compares the designated haplotype to all others combined.
[b]Empirical *P*-value was estimated by randomized χ^2 tests (50 000 permutations) for differences in haplotype distribution in cases versus controls using the haplo.score function, haplo.stats. *P*-values ≤ 0.05 are marked in bold.
[c]The global empirical *P*-value was obtained from the Max-Stat simulation indicating the number of times a maximum score value for individual haplotypes exceeds the maximum value from the original data.

Table 3. Genotype–genotype interaction between *TNC* and *NPSR1* SNPs. Significant *P*-values[a] for interactions in either *atopic sensitization* (to the left) or *doctor's diagnosis of asthma* (to the right)

TNC SNPs[b,c]	*NPSR1* SNPs[b]						
	rs323917*	rs323922	rs324377	SNP546333*	rs324384	rs324396	rs740347*
rs12347433*	0.126: **0.041**						
rs10817704*					**0.030**: 0.346		
rs2104772		**0.009**: **0.032**	**0.004**: 0.066	0.163: **0.038**	**0.015**: **0.037**	0.833: **0.017**	
rs2297181*		0.460: **0.009**	0.321: **0.009**		0.892: **0.043**		
rs1330363					**0.040**: 0.078		
rs17819466*							**0.026**: 0.551
rs7865462					**0.038**: 0.796		
rs3789873*		0.145: **0.013**	0.145: **0.002**	0.953: **0.039**	**0.007**: 0.059		

Atopic sensitization: Doctor's diagnosis of asthma.
The *P*-values indicate whether the effect (OR) of one genotype is altered by effects of another genotype.
[a]*P*-values for departure from multiplicative interaction model were obtained by likelihood-ratio tests between the models with and without interaction term. *P*-values ≤ 0.05 are marked in bold and empty cells represents $P > 0.05$.
[b]Genotypes were defined as common homozygous, heterozygous and rare homozygous and a model-free dummy variable approach was used. Due to few rare homozygotes for some SNPs (indicated by a star *), they were combined with the heterozygous and a dominant genetic model was used.
[c]4 SNPs for *TNC* are not included because they did not show any significant interaction (rs12351083, rs1330362, rs13321, rs2274750).
Atopic sensitization, IgE ≥ 0.35 kU/L: cases $n = 893$, controls $n = 2203$.
Doctor's diagnosis of asthma: cases $n = 261$, controls $n = 2798$.

it is likely that more genes are involved and interact. The biological interaction, supported by the genetic interaction, between NPSR1 and TNC might be one of the factors leading the disease into a more severe direction and we therefore hypothesize that a NPSR1-driven upregulation of *TNC* mRNA might increase the severity of asthma- and allergy-related traits. This hypothesis is supported by the detection of NPSR1 and TNC upregulation in bronchial tissue in asthmatic airways, as well as by the proximity of the expression, where NPSR1 is over-expressed in the epithelial cells and TNC in the sub-epithelial basal membrane (Fig. 2). A possible mode of action might be that a stimulation and upregulation of NPSR1 on the surface of the epithelial cells enhances the epithelial cell production of TNC, and TNC is then secreted into the sub-epithelial basal membrane. Several studies have indicated upregulation of TNC by various Th2 cytokines (27–30). Our results extend this view by proposing upregulation through a possible new mechanism that might help to merge the roles of both NPSR1 and TNC in asthma- and allergy-related disorders.

Studying the PARSIFAL group of children, well characterized for asthma- and allergy-related disorders (26), we found that TNC is not only a biomarker for asthma and inflammation but also genetically associated to childhood rhinoconjunctivitis. Previously performed genome-wide linkage studies on allergic rhinitis have pointed towards the chromosomal region around 9q33, where *TNC* is located, but little is still known about underlying genetic mechanisms in

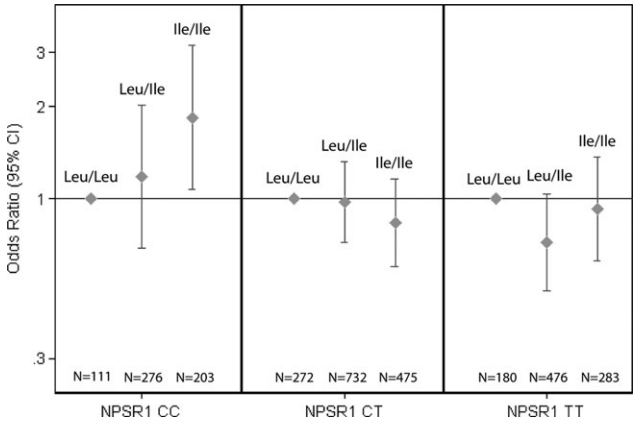

Figure 4. Effect of TNC rs2104772 (Leu1677Ile) genotypes Leu/Ile or Ile/Ile versus Leu/Leu in atopic sensitization (IgE≥0.35 kU/L), in relation to NPSR1 rs324384 genotypes. Compared with TNC allele Leu/Leu, the TNC Ile/Ile allele shows a significant increased risk effect if the individual carries the NPSR1 protective allele CC. If the individual carries the NPSR1 risk allele TT the TNC risk shows a tendency to diminish. Odds ratio (OR) for Leu/Ile versus Leu and Ile/Ile versus Leu, respectively (Leu/Leu = 1, baseline) given NPSR1 rs324384: 'CC' 1.18 (0.69–2.02) 1.84 (1.07–3.18), 'CT' 0.98 (0.72–1.32) 0.83 (0.60–1.16) and 'TT' 0.72 (0.50–1.04) 0.93 (0.63–1.37). N in the bottom of the picture correspondes to the number of subjects in each category. *P*-value for over-all interaction: 0.015.

rhinoconjunctivitis and few studies trying to identify candidate genes have been performed up to date (14–16,31–34). We propose *TNC* as a candidate gene for childhood rhinoconjunctivitis based on the association seen to several SNPs in the *TNC* gene. TNC has previously been indicated to have a role in upper airway disease (35). We found a risk associated with changing the *TNC* rs2104772 A allele Leu1677 into Ile1677, the same risk allele previously associated to adult asthma (13). The TNC protein is made up of epidermal growth factor (EGF)-like repeats, fibronectin type III (FN-III) domains and a terminal region of a fibrinogen domain. *In vivo*, six of these polypeptide chains assemble in the N-termini to form a hexabrachion (18). Previously performed protein structure modelling of TNC revealed that the amino acid replacement Leu1677Ile, situated in the Fn-III domain, could affect the structural stability and this was suggested to alter the molecular elasticity of TNC (13). Intriguingly, the Leu1677Ile variant also showed the most pronounced role in gene–gene interaction with *NPSR1*. Yet a coding SNP rs13321, changing Gln2008 into Glu, showed significant association to current rhinoconjunctivitis. SNP rs13321 is positioned in the fibrinogen domain which is situated at the most distal part of the hexabrachion and influences the interaction with other proteins (18). An amino acid change here could possibly lead to an altered ability of TNC to interact with conjugating proteins.

By permuted haplotype analysis, adjusted for potential confounders, we could confirm the single SNP association of *TNC* to current rhinoconjunctivitis, both on an individual haplotype level and on a global level for each haploblock. We showed that both the 1677Ile variant and the 2008Glu variant were associated to an increased risk for childhood rhinoconjunctivitis, and that 2008Glu was involved in the risk-associated haplotype TGGT ($P = 0.0005$). Both on single SNP and haplotype level we could see weaker associations to *TNC* in children with atopic sensitization and on single SNP level to doctor's diagnosis of asthma. As *TNC* previously has been associated to adult asthma in a Japanese population (13), we might have expected to see a stronger association to asthma in our material. One explanation for the weak association might be due to the less severe asthmatic phenotype in the PARSIFAL children compared with the more severe and prolonged disease in the Japanese population. This is strengthened by our analysis on allergic asthma, which can be considered as a more specific form of asthma, showing stronger association than asthma alone. It has also been proposed that there is a link in children between the development of upper and lower respiratory tract disorders (36) and that rhinitis is a risk factor for adult-onset asthma (37). Thus, it is possible that the same *TNC* SNPs are associated with rhinoconjunctivitis in children and asthma in adults.

When investigating *TNC* and *NPSR1* SNP interaction, several significant interactions were seen, particularly for doctor's diagnosis of asthma and atopic sensitization. The observed pattern was that most interactions were found between *TNC* rs2104772 (Leu1677Ile) and several of the *NPSR1* SNPs and between *NPSR1* rs324384 and several of the *TNC* SNPs. For doctor's diagnosis of asthma, *TNC* SNPs rs2297181 and rs3789873 were also found to interact with several of the *NPSR1* SNPs. This interaction pattern can in part be explained by the tight LD between the SNPs, which might not be independent of each other. The tagging SNPs in haploblock 2 (rs2104772, Leu1677Ile) and in haploblock 3 (rs3789873), that showed association to current rhinoconjunctivitis, also showed a distinct involvement in interaction with *NPSR1* for doctor's diagnosis of asthma and atopic sensitization.

TNC and *NPSR1* showed a complex interaction depending on the combination of genotypes (Fig. 4 and Supplementary Material, Table S3A and B). Apparently, *TNC* genotypes can be associated with both increased and decreased risk of disease, depending on *NPSR1* genotype status. This phenomenon of risk effects being reversed has been described earlier by Lin *et al*. (38) and also discussed by Hersh *et al*. (6), suggesting that the opposite effect of the SNPs, frequently observed in replication studies, might be due to gene–gene and/or gene–environment interactions. Here we can show that gene–gene interaction plays a role in reversing *NPSR1* and *TNC* risk genotypes. It would be interesting to interpret the effect of the interactions *in vivo*, but due to low endogenous *NPSR1* expression we have been working with stably or transiently transfected cell systems. Because the associating *NPSR1* SNPs are intronic and likely regulatory, their interaction effects cannot be addressed in transfected cell models.

The PARSIFAL study has a unique design representing western European children selected on the basis of farming and anthroposophic lifestyles and their respective reference groups from rural and sub-urban/urban communities, and includes exposures relating to different environments known to influence the development of asthma- and allergy-related disorders (39,40). Thus we cannot exclude that the environment plays a role here as well. In this study we have focused on the overall genetic association and gene–gene interaction effect across the total sample set rather than investigating the specific gene–environment effects. Although

further studies of the mechanisms behind the *TNC* and *NPSR1* interaction are needed, the observed *TNC* and *NPSR1* interaction suggests that certain variants of the genes interact and affect the development of asthma and allergy.

Asthma is a complex disorder and it is now well established that many genes are involved genetically in modifying its risk. Current evidence suggests that asthma risk genes act in a context-dependent manner, i.e. gene–gene interactions or gene–environment interaction (41). In the interaction analysis, the combined *TNC* and *NPSR1* risk effect was clearly different compared with *TNC* and *NPSR1* individual risk effect, with a much clearer interaction for atopic sensitization and doctor's diagnosis of asthma than for rhinoconjunctivitis. Thus, polymorphisms in *TNC* might play a more independent role for development of upper airway disease such as rhinoconjunctivitis, whereas for asthma, the interplay with other genes, like *NPSR1*, becomes more prominent.

In summary, we here show that the asthma candidate gene *TNC*, previously thought of chiefly as a phenotypic marker for inflammation, also contributes to the genetic risks for asthma and allergy in childhood. We provide an example of gene–gene interactions that is supported both by evidence of a direct biological regulatory effect of one gene by the other, and by joint analysis of combined genetic risk effects. Our results suggest that NPSR1, its ligand NPS and TNC are members of a new pathway for the development of asthmatic and allergic disorders.

MATERIALS AND METHODS

Cell culture, RNA isolation and cDNA synthesis

Stable human epithelial kidney (HEK) -293H, NPSR1-A over expressing cell line has been described elsewhere (42). The cells were cultured in 293 SFM II medium (Gibco/Invitrogen) supplemented with penicillin/streptomycin and constantly cultured under puromycin selection (0.8 μg/ml) (Sigma-Aldrich, St Louise, MO, USA). For dose–response experiments, cells were seeded at 1×10^6 cells/ml and stimulated with 0.1, 1 or 5 μM NPS (SFRNGVGTGMKKTSFQRAKS) (MedProbe, Oslo, Norway) for 6 h. Unstimulated control samples were collected in parallel. Human epithelial lung cells (A549) (a kind gift from Dr Sam Okret) were cultured in F-12/D-MEM medium (1:1; Gibco/Invitrogen) supplemented with fetal calf serum, L-glutamine and penicillin/streptomycin. Cells were seeded at 0.2×10^6 cells/ml before transient transfection (Lipofectamin^{TM}2000 Reagent, Invitrogen, Carlsbad, USA) with NPSR1-A constructs (42). Twenty-four hours after transfection the cells were stimulated with 0.1 μM NPS for 6 h. Unstimulated control samples were collected in parallel. All cells were cultured at 37°C in a humified 5% CO_2 incubator. Total cellular RNA was isolated with the RNAeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was for HEK-293H cells performed with TaqMan reverse transcription reagents (Applied Biosystems, Rotkreuz, Switzerland) using random hexamers and for A549 cells performed with iScript (Biorad, Hercules, USA) with a oligo (dT) and random primer mix, according to the manufacturer's protocol.

Quantitative real-time PCR

The mRNA expression was measured with qRT–PCR using SYBR® Green for TNC (primers: fwd TGCGGTCCAGTTGAGTCTGA and rev TGTGAAGGCATCCACTGAACA) and TaqMan® for the GAPDH control (primers and probe from Applied Biosystems). For A549 cells HPRT (primers: fwd TCAGGCAGTATAATCCAAAGATGGT and rev AGTCTGGCTTATATCCAACACTTCG) was also used side-by-side with GAPDH as an endogenous control to verify the stability of GAPDH. The PCR assay was performed in a total volume of 25 μl, containing 1:10 diluted cDNA template, 12.5 ul SYBR® Green PCR Master Mix (Applied Biosystems), 200 nM of each primer and 100 nM of GAPDH probes, using 7500 Fast Real-Time PCR system (Applied Biosystems) with the following reaction conditions: 50°C for 2 min and 94°C for 10 min; following 45 cycles of 92°C for 14 s and 1 min at 60°C. A dissociation stage was added to the SYBR® Green reactions to confirm primer specificity. All assays were carried out in triplicate. Relative quantification and calculation of the range of confidence was performed with the comparative ΔΔCT method (43). Results are shown as relative expression compared with unstimulated cells and GAPDH (Applied Biosystems) was used as an endogenous control.

Immunohistochemistry

Bronchial biopsy specimens were obtained from at least eight adult normal control subjects and eight asthmatic patients. The severity of asthma was from mild-to-moderate based on their medication, lung function results and bronchial hyper responsiveness to histamine. The patients had no respiratory tract infections or asthma exacerbation within 4 weeks before taking the biopsies and no smoking history for at least 2 years. All patients gave their informed consent for the study. The biopsies were, for TNC stainings, snap-frozen in liquid nitrogen before embedded in Tissue Tek, ornityl carbamyl transferase as previously described (10) and for NPSR1 stainings, formalin fixed and paraffin-embedded. Immunostaining was performed using a monoclonal antibody 100EB2 for TNC (44) before incubated with FITC-coupled sheep anti-mouse IgG (Jackson Immunosearch Laboratories, West Grove, PA). Sections were then examined under a Leitz Aristoplan fluorescence microscope. For NPSR1, polyclonal rabbit antibodies for the −B isoform (1) were used together with the ABC method (Vectastain Elite ABC kit, Vector Laboratories, Burlingham, CA). Negative controls were obtained by omission of the primary antibody, by replacement with an irrelevant mAb or staining with preimmune sera.

Study design

The cross-sectional PARSIFAL study includes 14 893 school children 5–13 years from five western European countries (26). The PARSIFAL study was originally designed to investigate the role of different lifestyles and environmental exposures in farm children, Steiner school children (who often have an anthroposophic lifestyle) and two corresponding rural and urban/suburban reference groups, respectively.

The aim was to identify protective factors for development of asthma and allergic disorders, and hence, the PARSIFAL children are well characterized for these traits. In the present study 3113 children with available DNA and consent for genetic analysis (1579 boys and 1534 girls) were included. Ethical approval for the study, including genetic analyses, was obtained in each country according to law.

All health outcomes were reported in questionnaires by the parents, except for atopic sensitization, which was assessed from blood sampled from the children. *Current rhinoconjunctivitis* symptoms were defined as sneezing, runny nose, nasal block-up and itchy eyes in the child during the last 12 months without having a cold at the same time. A doctor's *diagnosis of asthma* was considered to be present for children reporting ever having been diagnosed with asthma, or with obstructive bronchitis more than once. *Current wheezing* was defined as at least one episode of wheezing during the last 12 months and *current atopic eczema* if the child had ever had an itchy rash intermittently for at least 6 months and, in addition, reported an itchy rash at any time during the last 12 months. *Atopic sensitization* was defined as at least one allergen-specific serum IgE test ≥ 0.35 kU/L against a mixture of common inhalant (Phadiatop®) and common food allergens (fx5®) (ImmunoCAP™, Phadia AB, Uppsala, Sweden), respectively. *Allergic rhinoconjunctivitis* was defined as current rhinoconjunctivitis as described earlier, in combination with sensitization to inhalant allergens (Phaditop®) and *allergic asthma* was defined as those having a doctor's diagnosis of asthma in combination with sensitization to inhalant allergens.

SNP selection and genotyping

Eighteen SNPs were selected, localized in the mid to the 3′ end of *TNC* based on a previous study (13) and HapMap CEU LD-pattern (http://www.hapmap.org/), using the Tagger algorithm implemented in Haploview, with the LD correlation parameter set to 3.2 $r^2 > 0.8$ (http://www.broad.mit.edu/mpg/haploview/) (45). Based on association results from the literature (rs2104772) (13) and/or coding properties (rs13321, rs12347433, rs2274750, rs17819466) five SNPs were forced into the Tagger selection. Thirteen SNPs were the picked for genotyping since they captured and tagged more than two nearby SNPs or obtained coding properties (Supplementary Material, Table S1).

Primers for multiplex PCR and extension reactions were designed by the SpectroDesigner software (Sequenom GmbH, San Diego, CA, USA) (Supplementary Material, Table S1). PCR and extension reactions were performed according to manufacturer's standard protocols. The SNP analysis was performed by MALDI-TOF mass spectrometry (matrix-assisted laser desorption/ionisation-time of flight; Sequenom GmbH). Each assay was validated using 24 unrelated Caucasians and 3 CEPH DNA samples as well as 14 trios from the CEU population. Based on no significant deviation from Hardy–Weinberg equilibrium ($P > 0.05$ using χ^2 test) and a genotyping success rate above 95% (with the exception of rs3789873 which was accepted with a genotyping success rate of 88%), 12 SNPs were accepted after the validation run and used for genotyping. Due to a plate failure, 360 individuals had missing data for rs12347433. Five of the 12 genotyped SNPs are situated in introns, five in coding regions and two in the 3′ genomic region. SNP selection and genotyping of *NPSR1* in the PARSIFAL material is given elsewhere (3) but briefly seven tagging SNPs (rs323917, rs323922, rs324377, SNP546333, rs324384, rs324396 and rs740347) reported in the Laitinen *et al*. study (1) where chosen in the conserved haplotype block of *NPSR1*. The SNP analysis was performed as described earlier.

Statistical analyses

Allelic association was analysed for cases versus controls in Haploview 3.2 (45) using χ^2-test. Odds Ratios (OR) and 95% Confidence Intervals (CI) were calculated. Block-wise inheritance of the 12 markers was estimated using the LD measure D' in Haploview 3.2 (45). Haplotype association was tested in the statistical software 'R' (http://www.R-project.org) (46) using the haplo.score algorithm implemented in the haplo.stats package (47). A permutation test procedure where haplotypes were treated as fixed while the disease association status (case or control) is randomized was used to estimate both the empirical *P*-value for each haplotype frequency and the global empirical *P*-value for the whole haplotype set distribution. The proportion of 50 000 such randomized χ^2-tests where a stronger association was found than in the actual data provided the empirical *P*-value of the observation. Adjustment was made for the covariates country-of-origin, sampling group (farm children, Steiner school children and the corresponding reference groups) and sex. Haplotype frequencies in cases and controls, as well as association OR and 95% CI were estimated with the haplo.cc function (haplo.stats) without adjustment for covariates. The frequency of each haplotype in cases versus controls was compared with the combined frequency of all other assigned haplotypes in cases versus controls in each respective block. All analyses of the haplotype block structure grouping all 12 SNPs were made in Haploview 3.2.

A multiple logistic regression model was used to test for gene–gene interaction between *TNC* and *NPSR1* SNPs by adding an interaction term between the genotypes of interest, using STATA (Statistical Software, Version 8.0, Collage Station, TX, and USA). *TNC* and *NPSR1*genotypes were defined as common homozygous, heterozygous and rare homozygous. A genetic model-free coding with indicator (dummy) variables for heterozygote and defined common homozygote genotypes (versus rare homozygote) was used as a first approach to investigate the pattern of interaction between *TNC* and *NPSR1* genotypes. For those SNPs where the rare homozygotes were too few, they were combined with the heterozygotes and a dominant genetic model was used. *P*-values for departure from a multiplicative interaction model on the OR scale were obtained by likelihood-ratio tests between the models with and without interaction term. The global significance of the interaction was tested by a permutation test where the genotypes were held constant and the disease association status was permuted 1000 times. The regression models were adjusted for the potential confounders; country-of-origin, sampling group and sex.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. C.O.-P., E.M., S.B., A.L., R.L., J.R., E.v.M., G.D., M.W. and M.v.H. declare no competing interests; A.S. was appointed to a full-time professorship in Clinical Allergy Research by Karolinska Institutet of Stockholm, Sweden in November 1995; this was a donation given at one occasion for 15 million SEK from Kabi Pharmacia, Uppsala, Sweden and the annual proceeds cover the salary for A.S. and the salary for one administrative assistant/research assistant until retirement and A.S. has no obligation to the donator; J.V., L.A.L. and J.K. are coinventors on patents related to the discovery of NPSR1 as an asthma susceptibility gene; G.P. received $38 000 in 2002 and $10 000 in 2003 from AstraZeneca as research grant for epidemiological registry studies of comorbidities in lung cancer patients; F.N. is employed by AstraZeneca (AZ), holds some AZ shares and AZ also supports his academic part-time adjunct position at Karolinska Institutet.

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