

Original article

Genetic variation in CRTh2 influences development of allergic phenotypes

Background: Allergic disorders are characterized by an increase in the Th2 cytokines IL-4, IL-5 and IL-13, produced primarily by Th2 cells. These cells are marked by the expression of CRTh2 (chemoattractant receptor-homologous molecule expressed on Th2 cells), a receptor for prostaglandin D₂. As genetic variation plays a significant role in the predisposition for allergic disorders, we investigated the influence of single nucleotide polymorphisms (SNPs) in CRTh2. **Methods:** In a large study population of German children ($n = 4264$) from the International Study of Asthma and Allergy in Children (ISAAC II), six polymorphisms in CRTh2 were genotyped. Statistical analyses were performed using single SNP and haplotype analyses.

Results: Uncorrected associations among $-6373G > A$, $+1431G > C$ and $+1538A > G$ were observed with a number of allergic phenotypes ($P < 0.05$). After correction, association between $+1431C$ and specific IgE to food allergens remained significant ($P = 0.04$). Associations of haplotype (H)3 (containing $+1538G$) with reduced risk for asthma and H2 (containing $+1431C$) with increased risk for specific IgE to food allergens also remained significant after correction for multiple testing ($P = 0.004$).

Conclusions: Genetic variation within CRTh2 modifies the development of allergic sensitization and asthma in a population of German children.

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Allergic disorders such as asthma, allergic rhinitis and atopic dermatitis are mediated by T-helper type 2 (Th2) cells, a subset of CD4⁺ T lymphocytes polarized for expression of IL-4, IL-5 and IL-13, with little IFN- γ (1). Together these cytokines mediate allergic inflammation since IL-4 induces Th2 differentiation, both IL-4 and IL-13 initiate isotype switching to IgE and IL-5 is critical for eosinophil differentiation and survival (2). Following exposure, allergen crosslinking of IgE molecules on mast cells in peripheral tissues results in activation and release of a number of mediators, including Th2 cytokines, leukotrienes and prostaglandins (3). Prostaglandin D₂ (PGD₂) is released in the airways of asthmatics following allergen challenge and PGD₂ synthase transgenic mice over-expressing PGD₂ showed stronger inflammatory reactions in the lung than control mice upon antigen challenge [reviewed in (4)].

The effects of PGD₂ are through two G-protein coupled receptors, D prostanoid (DP)1 and DP2. PGD₂-DP1 signaling induces Gs- α -mediated release of intracellular cyclic adenosine monophosphate (5). DP2 is officially named GPR44 (6); however, is often referred to

as CRTh2 (chemoattractant receptor-homologous molecule expressed on Th2), as it is expressed by Th2, but not Th1, cells (7) as well as CD8⁺ Tc2 cells, eosinophils and basophils (8). *In vitro* PGD₂-CRTh2 signaling induces Gi- α -mediated calcium mobilization and selective chemotaxis of eosinophils, basophils and Th2 cells (8) as well as expression of IL-4, IL-5 and IL-13 (9). *In vivo* CRTh2-specific agonists increased eosinophil infiltration to the lungs following airway challenge and also exacerbated skin inflammation and IgE levels in a mouse model of atopic dermatitis (10, 11). In humans, the numbers of CRTh2⁺ T cells are elevated at sites of allergic inflammation such as the nasal mucosa and skin (12, 13). Therefore, release of PGD₂ from mast cells in mucosal tissues could stabilize the Th2 phenotype by signaling through CRTh2 (Fig. 1).

The gene encoding CRTh2 is located on chromosome 11q12-13 (6). To date, two single nucleotide polymorphisms (SNPs; rs11571288 and rs545659) in CRTh2 have been associated with increased risk for asthma in Chinese and African Americans (14, 15). To assess the influence of genetic variation across the CRTh2 locus in a European

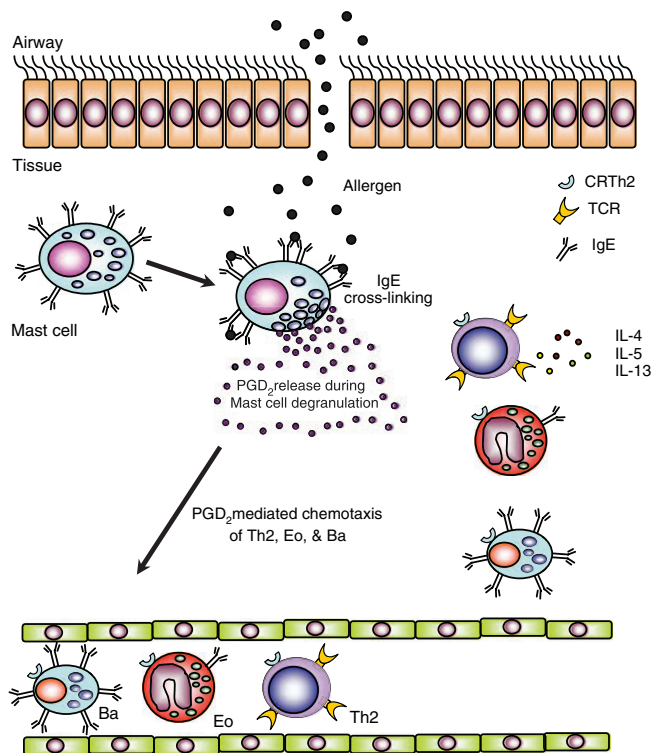


Figure 1. CRTh2 mediates inflammation at sites of allergen exposure. Allergen-crosslinking of IgE on mast cells results in release of PGD₂, a CRTh2 ligand. PGD₂-CRTh2 signaling mediates chemotaxis of Th2 cells, eosinophils and basophils to the tissue and expression of IL-4, IL-5 and IL-13 from Th2 cells.

population, we genotyped these and four other CRTh2 SNPs in a large cross-sectional population of German school children aged 9–11 years.

Methods

Selection of polymorphisms

Single nucleotide polymorphisms genotyped in this study were chosen from those provided by the Innate Immunity Programs for Genomic Applications, NHLBI Program for Genomic Applications (<http://innateimmunity.net>; accessed December 2007), identified by sequencing DNA of 23 cell lines from unrelated individuals of European American descent (Coriell Cell Repository, Camden, NJ, USA), except for CRTh2-6373G > A which was identified by HapMap (<http://www.hapmap.org/>). Five of the six SNPs (–2456C > T, –1883G > C, +1431G > C, +1538A > G, +3253T > C) were chosen as haplotype tagging SNPs (tagSNP) based on linkage disequilibrium (LD). SNPs with $r^2 \geq 0.8$ were considered to have a high degree of co-inheritance in Europeans. Instead of genotyping all the SNPs in the German samples, one was chosen that could effectively serve as a tag for the co-inherited SNPs and was therefore referred to as a 'tagSNP'. CRTh2-6373G > A was chosen based on *in silico* analysis (MatInspector; <http://www.genomatrix.de>) indicating its potential to alter putative transcription factor binding sites and/or CpG motifs, which may affect gene expression. Figure 2A shows the position of each SNP within the CRTh2 locus, minor allele frequency (MAF), LD data and chosen tagSNPs from

the Innate Immunity Programs for Genomic Applications (IIPGA). LD data are also represented in Haploview format (Fig. S1).

Study population and clinical characterization

Between 1995 and 1996, cross-sectional studies were conducted in Munich [International Study of Asthma and Allergies in Childhood II (ISAAC II)], in Dresden and in Leipzig to assess the prevalence of asthma and allergies in 7963 school children, attending the 4th class in all cities (age 9–11 years) using only standardized and validated study tools (16, 17). Within the study population, all children of German origin with DNA available were included in this analysis ($n = 1940$ in Dresden, $n = 1159$ in Munich, $n = 1165$ in Leipzig). Informed, written consent was obtained from all parents of children included in the study. All study methods were approved by the local ethics committees.

Asthma. Parental questionnaires for self-completion including the ISAAC core questions were distributed through the schools to the parents to assess respiratory health. The study methods for all phenotyping procedures have been previously described (16–19) and so we give only a short summary here: if 'asthma' was diagnosed by a doctor at least once or 'asthmatic, spastic or obstructive bronchitis' more than once the child was defined as having asthma. Wheeze was assessed by a positive answer to the ISAAC question 'Did you notice any wheezing in your child within the last 12 months (= current wheeze)' for Munich or Dresden. For Leipzig, wheezing was considered positive in children who reported recurrent wheezing at the time of investigation. Atopic asthma was defined as the concomitant co-occurrence of asthma with a positive skin prick test, while nonatopic asthma excludes a positive skin test. The control group for atopic and nonatopic asthma is always the nonasthmatics.

Hayfever and eczema. The definition of hay fever and eczema was also based on a parental report of doctor's diagnosis of the respective diseases in children from Munich and Dresden. Because the doctor diagnosis for eczema or atopic dermatitis was not available for Leipzig, for these children we used a combination of reporting as 'itchy dermal changes' and 'ever eczema' to define eczema.

Atopy and IgE. By skin prick test, the sensitivity to six common aeroallergens was assessed: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, *Alternaria tenuis*, mixed tree pollen and mixed grass pollen was tested. In Leipzig, a slightly different combination of common aeroallergens were used (*D. pteronyssinus*, cat, grass pollen, birch and hazel pollen and dog dander) and assessed by skin prick test with multitest device (Stallerkit, Stallergenes, France) (17). A child was considered atopic if a wheal reaction ≥ 3 mm occurred to one or more allergens after subtraction of the negative control.

Total serum IgE was measured using the Imulite system (DPC Biermann, Bad Nauheim, Germany). Specific IgE antibodies against inhalant allergens (local grass pollen, birch pollen, mugwort pollen, *D. pteronyssinus*, cat dander, dog dander, *Cladosporium herbarum*) and food allergens (egg white, milk, fish, wheat, peanut and soybean) were measured in a range between 0.35 and 100 IU/ml (Phadia, Uppsala, Sweden) in Munich and Dresden.

Current environmental smoke exposure was defined as any current environmental tobacco smoke exposure at the age of 9–11 years according to the information derived from parental questionnaires in Munich and Dresden or exposure to at least one daily smoked cigarette in Leipzig.

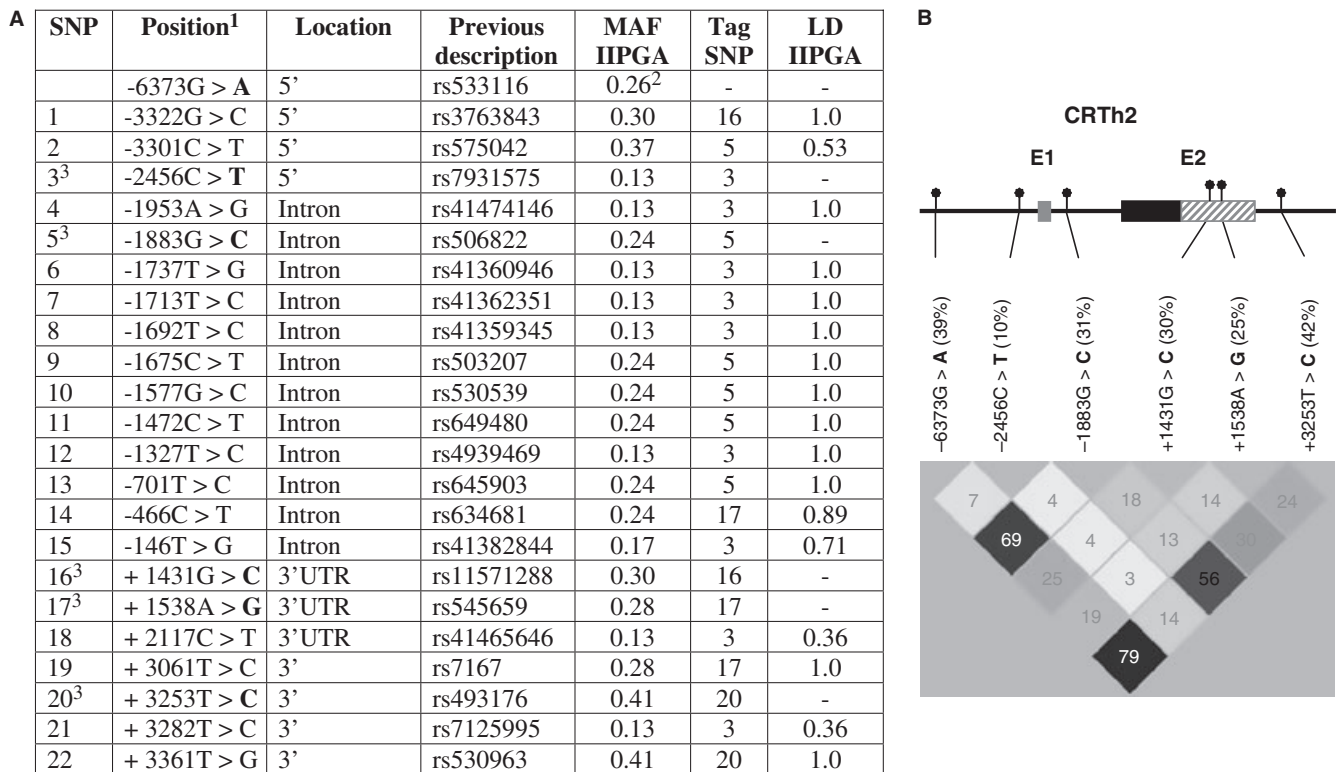


Figure 2. Single nucleotide polymorphisms in CRTh2. Minor allele frequency and linkage disequilibrium of CRTh2 SNPs in samples from the Innate Immunity Programs for Genomic Applications (IIPGA) (A) and children from Munich, Leipzig and Dresden ($n = 4264$; (B)). TagSNP indicates which SNP was genotyped, while LD IIPGA shows linkage disequilibrium (r^2) between the tagSNP and other SNPs throughout the locus. ¹Position relative to the ATG (+1). ²Minor allele frequency for CRTh2-6373G > A is PER-LEGEN data generated from the Coriell Cell Repository data set of 23 European Americans. ³Tagging SNPs, minor alleles are bold in column two. MAF, minor allele frequency; LD, linkage disequilibrium; E, exon; gray shading, untranslated exon 1; hatched box, 3' untranslated region.

Genetic analysis

Genotyping was performed as previously described (20). Briefly, genomic DNA was extracted from whole blood by standard salting-out method and a modified primer extension preamplification was applied to reduce the amount of DNA necessary for the analysis. The MassArray system (Sequenom, San Diego, CA, USA) was used whereby a base-specific extension reaction was performed with Thermosequenase (Amersham, Piscataway, NJ, USA) and this reaction was dispensed onto a 384-format Spectro-Chip (Sequenom). A matrix-assisted laser desorption/ionization time-of-flight mass spectrometer, model Bruker Autoflex (Sequenom), was used for data acquisition. Genotyping calls were made in real time with MASSARRAY RT software (Sequenom).

Statistical analysis

Deviation from Hardy-Weinberg equilibrium (HWE) was analyzed using the chi-squared test, with expected frequencies derived from allele frequencies. Univariate associations between SNPs and dichotomous outcomes were investigated using the Armitage-Trend test in unadjusted analyses and the Wald-Test for sex and environmental tobacco smoke-adjusted models. We used the linear model in which the risk of a disease increases or decreases with an increasing/

decreasing number of alleles. Additionally, odds ratios (OR) with 95% confidence intervals of logistic regression models are given.

To check for tagging SNPs in our population, we used HAPLOVIEW (21). Haplotype analyses were performed where information about all SNPs were available. Haplotype frequencies were estimated using the expectation-maximization algorithm (22). To specify the effects of individual haplotypes, haplotype trend regressions (HTR) were performed where the estimated probabilities of the haplotypes are modeled in a logistic regression as independent variables (23).

To correct for multiple testing, we used a Bonferroni correction over all SNPs or the number of investigated individual haplotypes in every phenotype. If it is not marked you find the crude (unadjusted not corrected) P -values in the tables. All statistical tests were two-sided and a nominal level of significance ($\alpha = 0.05$) was used. Calculations were carried out with SAS (Version 9.1.3) and SAS/Genetics (SAS Institute, Cary, NC, USA).

Results

Genotyping and quality control

Genotyping of the six chosen CRTh2 SNPs was performed in the Munich, Dresden and Leipzig

populations. Figure 2B shows the (MAF) and LD data. Frequencies were highly similar to those reported by the IIPGA (Fig. 2A). Call rates exceeded 95% in the pooled sample. The complete results for allele frequencies and the test for HWE are available in Table S1A. Similar allele frequencies were observed for all SNPs using different genotyping assays in all three subpopulations, indicating the presence of genotyping errors to be extremely unlikely.

Linkage disequilibrium of CRTh2 and adjacent area

The genotyping strategy and selection of tagSNPs (−2456C>T, −1883G>C, +1431G>C, +1538A>G, +3253T>C) was based on LD data from IIPGA (Fig S1; Fig. 2A). Each of these tagSNPs was linked to at least one other SNP within the CRTh2 locus ($r^2 \geq 0.8$). Furthermore, LD between tagSNPs was low ($r^2 \leq 0.2$), except +3253T>C ($r^2 \leq 0.4$). CRTh2 is located on chromosome 11q12-13 (60 380 020–60 374 983), in the vicinity of FCERB (MS4A2; 59 612 713–59 622 593) and CC16 (SCFB1A1; 61 943 090–61 947 242), genes with SNPs previously reported to be associated with asthma and related phenotypes [reviewed in (24)]. Therefore, it was possible that associations observed with SNPs in the CRTh2 gene may originate from extended LD across the locus. However, when we examined 14 SNPs in FCERB, CC16-38A>G and the CRTh2 SNPs there was no significant LD ($r^2 < 0.2$, data not shown), making extended haplotype effects from these neighboring genes extremely unlikely.

Association studies with single SNPs

All six genotyped CRTh2 SNPs were examined individually for association to allergic disease (asthma, eczema and hayfever) and atopy. As associations with asthma

were observed, the subphenotypes of atopic asthma and nonatopic asthma as well as current wheeze were also analyzed. Clinical characteristics of this population are provided in Table S1B and have been described previously (25, 26). There was no significant selection bias for the phenotypes studied.

In the sample of 4264 children genotyped (Table 1), we found association between −6373G>A and increased risk for atopic asthma [OR 1.26 (1.00–1.57), $P = 0.045$], while +1538A>G was associated with reduced risk for asthma [OR 0.80 (0.66–0.96), $P = 0.020$] and eczema [OR 0.86 (0.74–0.98), $P = 0.029$]. +1538A>G showed consistent protective effect in this analysis with similar trends for all studied phenotypes and the covariate adjusted analyses showed nearly identical results (Table S1C). However, these associations did not remain significant after correction for multiple testing, although association between +1538A>G and asthma was close to the significance threshold (Table 1) and the effect was homogenous in all three cities (Table S1D). A significant association between +1431G>C and increased risk for specific IgE to food allergens in Munich and Dresden children was observed [OR 1.30 (1.07–1.58), $P = 0.007$], which remained significant after correction for multiple testing and covariate adjustment ($P = 0.042$, $P = 0.028$; Table S1C).

Haplotype analysis

Haplotype analysis was also performed for all six SNPs for the completely phased children ($n = 3831$). Four common ($\geq 3\%$) haplotypes were found (Table 2). Haplotype 3 (H3, G-C-G-G-G-T) containing +1538G was protective for asthma [OR 0.55 (0.37–0.82), $P = 0.004$], atopic asthma [OR 0.50 (0.28–0.89), $P = 0.020$], eczema [OR 0.71 (0.53–0.95), $P = 0.023$] and showed a trend for reducing the risk of specific IgE for food allergens [0.62 (0.39–1.0), $P = 0.049$] and current wheeze [OR 0.67

Table 1. Association of CRTh2 SNPs with atopic diseases, atopy or current wheeze in the whole sample ($n = 4264$)

	Asthma	Atopic asthma	Nonatopic asthma	Current wheeze*	Atopy	Eczema†	Hay fever	sIgE to food allergens‡
−6373G>A	1.14 (0.98–1.34)	1.26 (1.00–1.57)	1.06 (0.85–1.32)	1.12 (0.95–1.32)	1.03 (0.93–1.14)	1.07 (0.95–1.21)	1.01 (0.86–1.19)	0.98 (0.81–1.19)
Raw P -value	0.0965	0.0454 (0.2724§)	0.6242	0.1757	0.5474	0.2638	0.8773	0.8635
−2456C>T	1.03 (0.79–1.33)	1.17 (0.82–1.67)	1.02 (0.71–1.47)	1.07 (0.81–1.39)	0.88 (0.74–1.04)	0.97 (0.79–1.18)	0.94 (0.72–1.23)	0.99 (0.72–1.35)
Raw P -value	0.8496	0.3873	0.9230	0.6430	0.1349	0.7334	0.6564	0.9400
−1883G>C	1.09 (0.92–1.29)	1.14 (0.89–1.44)	1.03 (0.81–1.31)	1.05 (0.88–1.25)	1.04 (0.93–1.16)	1.06 (0.93–1.20)	1.01 (0.85–1.21)	0.92 (0.75–1.12)
Raw P -value	0.2974	0.2994	0.8143	0.6097	0.4972	0.4013	0.8695	0.4101
+1431G>C	1.07 (0.90–1.26)	1.00 (0.79–1.28)	1.13 (0.89–1.43)	1.17 (0.98–1.39)	1.06 (0.96–1.18)	1.12 (0.99–1.28)	1.10 (0.92–1.30)	1.30 (1.07–1.58)
Raw P -value	0.4516	0.9888	0.3148	0.0775	0.2617	0.0680	0.2928	0.007 (0.0420§)
+1538A>G	0.80 (0.66–0.96)	0.77 (0.59–1.02)	0.82 (0.62–1.07)	0.84 (0.69–1.02)	0.99 (0.88–1.11)	0.86 (0.74–0.98)	0.99 (0.83–1.20)	0.85 (0.68–1.07)
Raw P -value	0.0195 (0.1170§)	0.0677	0.1367	0.0835	0.8359	0.0291 (0.1746§)	0.9504	0.1664
+3253T>C	1.12 (0.96–1.31)	1.19 (0.95–1.49)	1.06 (0.85–1.32)	1.03 (0.87–1.22)	0.97 (0.88–1.07)	1.03 (0.91–1.16)	0.94 (0.80–1.10)	0.90 (0.75–1.09)
Raw P -value	0.1576	0.1288	0.6292	0.7103	0.5628	0.6615	0.4253	0.2722

Values represent OR (95% CI). Numbers in bold represent significant OR 95% CI and P values.

*Phenotypes are mixed: wheeze in the last 12 months in Munich/Dresden; wheeze problems still present in Leipzig.

†Phenotypes are mixed: dr. diagnosis of atopic dermatitis in Munich/Dresden – as ever eczema + as ever 'itchy dermal changes' in Leipzig.

‡Phenotype is available only in Munich/Dresden.

§Corrected P -values.

Table 2. Analysis of common (>3%) haplotypes in the pooled sample

			Frequency (%)			OR (HTR) and 95% confidence limits	<i>P</i> -value
Phenotype No. cases/controls		Haplotype*	Controls	Cases	Combined		HTR
Asthma 316/3440 (9.2%)	H1	A-C-C-G-A-C	29.71	31.91	29.90	1.226 (0.868–1.732)	0.2483
	H2	G-C-G-C-A-T	29.21	30.71	29.31	1.134 (0.802–1.603)	0.4782
	H3	G-C-G-G-G-T	25.48	20.05	25.04	0.550 (0.368–0.822)	0.0035†
	H4	A-T-G-G-A-C	7.70	8.22	7.75	1.153 (0.641–2.075)	0.6349
		All rare	7.90	9.11	8.00		
Atopic asthma 148/3440 (4.3%)	H1	A-C-C-G-A-C	29.71	31.66	29.79	1.202 (0.733–1.970)	0.4660
	H2	G-C-G-C-A-T	29.21	29.44	29.21	0.994 (0.602–1.640)	0.9816
	H3	G-C-G-G-G-T	25.48	19.19	25.23	0.502 (0.280–0.898)	0.0203
	H4	A-T-G-G-A-C	7.70	9.45	7.78	1.557 (0.710–3.417)	0.2691
		All rare	7.90	10.26	8.00		
Nonatopic asthma 152/3440 (4.4%)	H1	A-C-C-G-A-C	29.71	31.24	29.78	1.151 (0.705–1.878)	0.5736
	H2	G-C-G-C-A-T	29.21	31.88	29.31	1.276 (0.786–2.072)	0.3233
	H3	G-C-G-G-G-T	25.48	21.05	25.30	0.613 (0.350–1.071)	0.0855
	H4	A-T-G-G-A-C	7.70	7.90	7.71	1.052 (0.454–2.440)	0.9053
		All rare	7.90	7.94	7.90		
Current wheeze‡ 285/3922 (7.3%)	H1	A-C-C-G-A-C	29.81	30.26	29.85	1.050 (0.727–1.516)	0.7964
	H2	G-C-G-C-A-T	28.89	31.64	29.09	1.274 (0.888–1.829)	0.1885
	H3	G-C-G-G-G-T	25.53	21.91	25.25	0.672 (0.446–1.012)	0.0571
	H4	A-T-G-G-A-C	7.62	8.77	7.71	1.353 (0.742–2.466)	0.3239
		All rare	8.16	7.42	8.10		
Eczema§ 613/2941 (2.1%)	H1	A-C-C-G-A-C	29.32	30.73	29.56	1.139 (0.874–1.485)	0.3354
	H2	G-C-G-C-A-T	28.98	31.69	29.45	1.286 (0.992–1.667)	0.0578
	H3	G-C-G-G-G-T	25.57	22.54	25.05	0.712 (0.531–0.954)	0.0227
	H4	A-T-G-G-A-C	7.71	8.07	7.78	1.112 (0.709–1.742)	0.6445
		All rare	8.42	6.98	8.17		
Atopy 943/2746 (3.4%)	H1	A-C-C-G-A-C	29.58	30.33	29.78	1.075 (0.858–1.347)	0.5281
	H2	G-C-G-C-A-T	29.25	30.10	29.46	1.081 (0.864–1.351)	0.4959
	H3	G-C-G-G-G-T	25.33	24.37	25.09	0.905 (0.711–1.152)	0.4186
	H4	A-T-G-G-A-C	7.82	7.34	7.70	0.871 (0.588–1.291)	0.4920
		All rare	8.02	7.87	7.98		
sIgE to food allergens¶ 283/773 (36.6%)	H1	A-C-C-G-A-C	29.87	27.55	29.28	0.818 (0.539–1.242)	0.3459
	H2	G-C-G-C-A-T	29.17	36.20	31.01	1.796 (1.206–2.673)	0.0039†
	H3	G-C-G-G-G-T	25.19	20.96	24.05	0.623 (0.389–0.997)	0.0487
	H4	A-T-G-G-A-C	7.65	6.70	7.40	0.779 (0.377–1.607)	0.4987
		All rare					

*SNPs included in the haplotypes: –6373G>A/–2456C>T/–1883G>C/+1431G>C/+1538A>G/+3253T>C; OR and *P*-value of haplotype trend regression (HTR).

†Significant after correction for multiple testing.

‡Phenotypes are mixed: wheeze in the last 12 months in Munich/Dresden; wheeze problems still present in Leipzig.

§Phenotypes are mixed: dr. diagnosis of atopic dermatitis in Munich/Dresden – as ever eczema + as ever 'itchy dermal changes' in Leipzig.

¶Specific IgE from Munich and Dresden.

(0.45–1.01), $P = 0.057$]. The asthma effect holds even after correcting for multiple testing ($P = 0.004$).

H2 (G-C-G-C-A-T) containing +1431C showed increased risk for specific IgE to food allergens [1.80 (1.21–2.67), $P = 0.004$], and a trend for increasing the risk of eczema [OR 1.29 (0.99–1.67), $P = 0.058$]. The association with risk for specific IgE to food allergens remained significant after correction for multiple testing ($P = 0.004$).

Discussion

The CRTh2 pathway has been shown to play an important role the allergic response, mediating expression of Th2

cytokines and chemotaxis of eosinophils, basophils and Th2 cells (8). Although a number of SNPs are reported throughout the CRTh2 locus, this is the first study to examine a European population. Our data show that after correction for multiple testing, associations between +1431G>C and +1538A>G and phenotypes of allergic disease in German children were observed. Both these SNPs have been previously associated with increased asthma risk in Chinese American and African American populations (14, 15). However, comparison with our data from German children revealed interesting differences. First, the frequencies of the +1538G allele are very different in these populations. In Chinese American and African American populations, +1538G is the major allele (14, 15), while in Europeans it is the minor allele (Fig. 2).

Furthermore, we report the +1538G haplotype (H3) as protective against development of asthma while it was predisposing for asthma and near fatal asthma in Chinese and African Americans (14, 15). Despite divergent effects, collectively these studies suggest +1538A > G plays a role in the development of allergic disorders. Interestingly, a study in Japanese subjects failed to show association between CRTh2 SNPs, including +1431G > C or +1538A > G, and phenotypes of allergy and asthma (27). Differences may be due to environmental exposures, genomic architecture, synergy with other SNPs along the regulatory pathway or insufficient power to detect subtle changes in risk.

We also observed association between H2 (+1431C) and H3 (+1538G) and specific IgE to food allergens, indicating these haplotypes may influence allergic sensitization through the gut. As CRTh2 is a receptor for PGD₂ (7), a metabolite of the essential omega-6 polyunsaturated fatty acids, diet is the sole source of this mediator. Therefore, genetic variation within CRTh2 may result in differential responsiveness to dietary intake of foods containing omega-6 fat. This could initiate and/or perpetuate allergic sensitization, because PGD₂-CRTh2 signaling mediates expression of IL-4 and IL-13 (9), known inducers of isotype switching to IgE (2). In fact, a Th2 milieu has been observed in the intestine of subjects with food allergy (28), a condition which often co-occurs with asthma and eczema (29, 30). Furthermore, an association between serum levels of omega-6 fatty acid and hayfever has been observed (31). Recent work in mouse models suggests food allergy predisposes for sensitivity to other allergens such as house dust mite and subsequent allergic airway responses (32).

As LD between SNPs can vary substantially across ethnicities, altering haplotype structure, it is important to determine which SNPs are actually regulatory. +1431C and +1538G (which tag H2 and H3, respectively) are within the 3' untranslated region and one study has indicated they alter stability of CRTh2 mRNA and protein expression (14). *In silico* analysis also predicts +1431C may be regulatory because it creates putative binding sites for a number of transcription factors.

Another level of potential regulation is epigenetic modification, such as CpG methylation associated with gene silencing. +1538A > G creates a CpG motif and therefore contributes the potential for methylation at this site. In this study, we did find +1538G associated with protection from developing asthma; however, functional analyses are needed to determine whether methylation of this site coincides with this protective effect. Furthermore, although +1431C and +1538G are good candidates as regulatory SNPs, the IIPGA data indicate they are co-inherited with others throughout the gene (Fig. 2A). Therefore, further work is required to identify and characterize those which are regulatory.

We conclude that genetic variation across the CRTh2 gene may influence development of allergic disease. Characterizing these associations will help identify individuals likely to be responsive to anti-CRTh2 therapy, now being developed by a number of companies (33). As a cross-sectional study, these data are best used as hypothesis generating for future prospective and/or case-control studies, particularly to examine the relationship between CRTh2 SNPs and gene-environment interactions such as dietary exposure to omega-6 fatty acids.

Acknowledgments

Lisa Cameron developed the study, selected the SNPs for genotyping and wrote the manuscript; Martin Depner performed statistical analysis; Michael Kormann, Norman Klopp and Thomas Illig participated in genotyping; Erika von Mutius contributed to the collection of data and data analysis; Michael Kabesch supervised all experiments, participated in the development of the study design, collection of data, data analysis and wrote the final version of the manuscript. This work was funded by the national genome research network (NGFN) research grant NGFN 01GS 0429 and the German research foundation as part of the transregional collaborative research program TR22 'allergic immune responses of the lung', grant Z3. L.C. was additionally funded by the University of Alberta. All Authors declare that they have no competing financial or personal interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

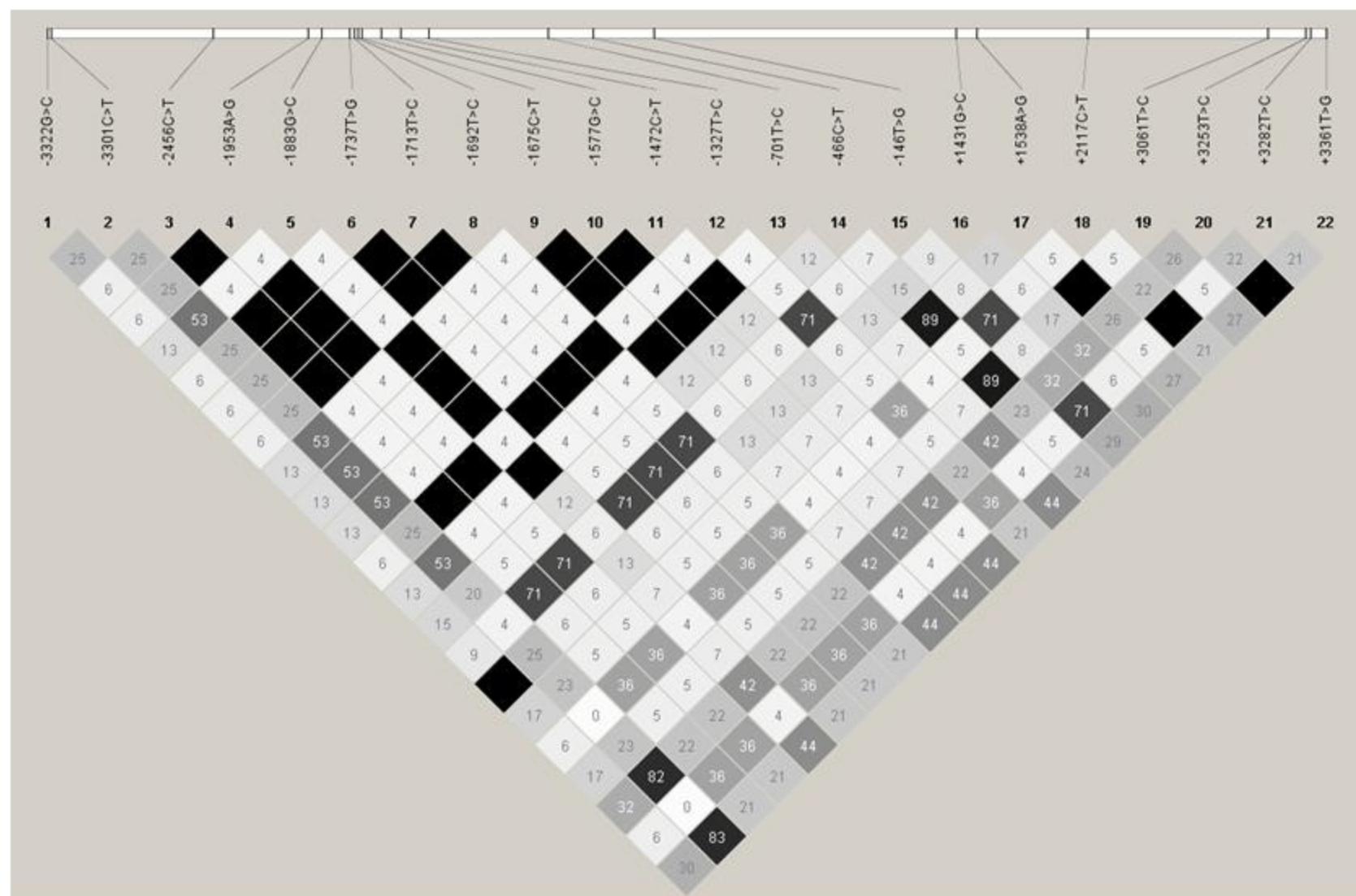
Figure S1. Haploview plot of linkage disequilibrium (r^2) for SNPs across the CRTh2 locus. All frequency data were from the Coriell Cell Repository data set for European Americans generated by the IIPGA (<http://innateimmunity.net>). Genotype data for each sample was unavailable for CRTh2-6373G>A and therefore could not be included in this analysis.

Table S1. (A) Pooled sample; HWE in Dresden, Leipzig and Munich. (B) Clinical characteristics of the German

sample. (C) Association of CRTh2 SNPs with atopic diseases, atopy or current wheeze in the whole sample ($n=4264$), adjusted and unadjusted results. (D) CRTh2 SNPs significantly associated with atopic diseases, atopy or current wheeze in the whole sample ($n=4264$), pooled and stratified for city.

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CRTh2 SNPs and allergy, Supplementary Figure 1



CRTh2 SNPs and allergy

SUPPLEMENTARY TABLES

Supplementary Table 1a - Pooled sample

		MAF		p HWE						
Dresden/ Leipzig/Munich (n=4264)	Call Rate	PGA	MDL	no asthma	all	Freq.	WT	HT	PO	1 st PCR primer; 2 nd PCR primer; Extension primer
-6373G>A						obs	1557	1862	638	ACGTTGGATGGCAATCGTCGCAGAAACAAC; ACGTTGGATGCTCTGTTGTTTAAAGCTGCC; TTGTTCTGACAGCCCGA
	95.1%	0.26	0.39	0.0286	0.0389	exp	1526	1924	607	
-2456C>T						obs	3337	698	50	ACGTTGGATGAAAAGCACACCCCTAGTCAC; ACGTTGGATGAGACTGCAGAAGTCCAGAGG; GTGCACGTGAAAAAATC
	95.8%	0.13	0.10	0.0198	0.0503	exp	3326	720	39	
-1883G>C						obs	1976	1698	394	ACGTTGGATGTTTGAATCCCAGCTCCTCAG; ACGTTGGATGTTTACAAGCAGTCTGGGTGG; GTGGGAAGATGACACCAGCA
	95.4%	0.24	0.31	0.3383	0.2943	exp	1962	1726	380	
+1431G>C						obs	2059	1631	392	ACGTTGGATGTGATGTGCCTGTGAATCACC; ACGTTGGATGAAGTGCAGACTCTCAGTACC; AGTACCCGGCCCCGGCCT C
	95.7%	0.30	0.30	0.0272	0.0089	exp	2024	1701	357	
+1538A>G						obs	2271	1488	274	ACGTTGGATGAAGCCAGTGCGGCAGGTTCA; ACGTTGGATGTTCCGACTCCACTGCCCTAG; CCTAGAGTGGGAAGTCTATTA
	94.6%	0.28	0.25	0.1170	0.1551	exp	2254	1522	257	
+3253T>C						obs	1370	1924	752	ACGTTGGATGTTCTGTCCCACACAGACCTG; ACGTTGGATGCAAACCCATGGCTCCAGAAC; CCACCCGACCGGGGCTCCTC
	94.9%	0.41	0.42	0.0787	0.0954	exp	1344	1976	726	

MAF, minor allele frequency; *HWE*, Hardy-Weinberg equilibrium; *PGA*, Program for Genomic Applications; *Freq.*, frequency, obs=observed, exp=expected; *WT*, wildtype; *HT*, heterozygous; *PO*, polymorphism

Supplementary Table 1a - HWE in Dresden

Dresden (n=1940)	<u>Call R%</u>	<u>MAF</u>	p HWE	Freq.	WT	HT	PO	p HWE (no asthma)
CRTH2 -6373G>A	97.5%	0.38	0.6786	obs exp	721 717	887 896	284 280	0.7320
CRTH2 -2456C>T	98.8%	0.09	0.0213	obs exp	1594 1586	299 316	24 16	0.0167
CRTH2 -1883G>C	98.0%	0.31	0.9777	obs exp	902 902	816 815	184 184	0.9827
CRTH2 +1431G>C	97.6%	0.29	0.0188	obs exp	972 951	740 782	182 161	0.0368
CRTH2 +1538A>G	96.7%	0.26	0.9573	obs exp	1040 1040	713 714	123 123	0.9476
				obs	641	877	347	
CRTH2_+3253T>C	96.1%	0.42	0.1247	exp	625	909	331	0.2027

Supplementary Table 1a – HWE in Leipzig

Leipzig (n=1165)	<u>Call R%</u>	<u>MAF</u>	p HWE	Freq.	WT	HT	PO	p HWE (no asthma)
CRTH2 -6373G>A	93.7%	0.38	0.1080	obs exp	431 418	490 515	171 158	0.1028
CRTH2 -2456C>T	92.6%	0.10	0.6788	obs exp	867 866	199 202	13 12	0.4776
CRTH2 -1883G>C	93.4%	0.30	0.4240	obs exp	542 536	444 455	102 96	0.6301
CRTH2 +1431G>C	93.3%	0.28	0.5204	obs exp	564 560	432 441	91 87	0.4371
CRTH2 +1538A>G	93.1%	0.27	0.1986	obs exp	580 572	415 432	90 82	0.2124
CRTH2_+3253T>C	93.2%	0.42	0.1896	obs exp	376 365	508 529	202 191	0.1077

Supplementary Table 1a - HWE in Munich

Munich (n=1159)	<u>Call R%</u>	<u>MAF</u>	p HWE	Freq.	WT	HT	PO	p HWE (no asthma)
CRTH2 -6373G>A	92.6%	0.40	0.0687	obs exp	405 391	485 514	183 169	0.0312
CRTH2 -2456C>T	94.0%	0.10	0.6779	obs exp	876 875	200 203	13 12	0.4921
CRTH2 -1883G>C	93.0%	0.30	0.2043	obs exp	532 523	438 456	108 99	0.1807
CRTH2 +1431G>C	95.0%	0.32	0.2261	obs exp	523 514	459 476	119 110	0.5220
CRTH2 +1538A>G	92.5%	0.22	0.2320	obs exp	651 644	360 374	61 54	0.0898
CRTH2_+3253T>C	94.5%	0.43	0.9130	obs exp	353 354	539 537	203 204	0.9213

HWE was used as a quality control instrument for genotyping. It is well known that deviation from HWE at an uncorrected α -level of 0.05 may occur in very large datasets (like that used here) by chance when multiple HWE analyses are performed. To check if there is a systematic genotyping error, we had analyzed HWE in all three populations genotyped in the study with the same genotyping assay (pooled - Munich, Dresden and Leipzig). The deviation from HWE does not replicate in the different populations and in fact, allele frequencies are only slightly different in the three populations, indicating that these slight HWE deviations may be due to study size rather than to technical genotyping problems or population stratification.

Supplementary Table 1b. Clinical Characteristics of the German Sample

	Total study population		Genotyped	
	N=7940 ¹		N=4264	
	n ²	[%]	n ²	[%]
Male gender	3879/7655	50.67%	2151/4257	50.53%
Age ³	9.7 (0.60)		9.7 (0.60)	
Asthma	650/7750	8.39%	350/4185	8.36%
Atopic asthma ⁴	218/7318	2.98%	164/3999	4.10%
Non atopic asthma ⁴	229/7329	3.12%	167/4002	4.17%
Current wheeze ⁵	587/7616	7.71%	317/4093	7.74%
Hay fever	639/7743	8.25%	336/4174	8.05%
Atopic dermatitis ⁶	923/5417	17.00%	540/2994	18.0%
Eczema ⁶	1200/7349	16.33%	679/3960	17.15%
Atopy (+ve skin test)	1474/5866	25.13%	1056/4110	25.69%
BHR	462/3821	12.09%	300/2468	12.16%

¹ In Leipzig for 2311 of 2334 children data available, 2612 children of Munich, 3017 children of Dresden

² n affected / n with data available; ³ presented as mean and standard deviations ($\pm$ SD); ⁴ 4185 children of the genotyped population in the definition of the asthma variable are composed by 3835 controls without asthma, 164 atopic asthmatics, 167 non-atopic asthmatics and 19 asthmatics for whom the atopy status was not defined. In the table the percentage of all cases for the well defined variable is reported, i.e. atopic asthma 164/(3835+164) and non-atopics 167/(3835+167). We did the same for the total study population. ⁵ Variable is pooled: wheeze in the last 12 months in Munich/Dresden; wheeze problems still present in Leipzig. ⁶ Because doctor diagnosis of atopic dermatitis is not available in Leipzig, atopic dermatitis is reported only for children of Dresden and Munich (first variable) and for the pooled variable (second variant), which is called eczema and is defined by a combination of reporting as “itchy dermal changes” and “ever eczema” in the Leipzig children.

Supplementary Table 1c. Association of CRTh2 SNPs with atopic diseases, atopy or current wheeze in the whole sample (N=4,264), adjusted¹ and unadjusted results

	Asthma	Atopic Asthma	Non-atopic Asthma	Current Wheeze ²	Atopy	Eczema ³	Hay fever	sIgE to food allergens ⁴
	OR (95%-C.I.) of logistic regression p-value, adjusted and unadjusted analyses (adjusted for sex and environmental tobacco smoke)							
-6373G>A	1.14 (0.98-1.34)	1.26 (1.00-1.57)	1.06 (0.85-1.32)	1.12 (0.95-1.32)	1.03 (0.93-1.14)	1.07 (0.95-1.21)	1.01 (0.86-1.19)	0.98 (0.81-1.19)
raw p-value	p=0.0965	p=0.0454	p=0.6242	p=0.1757	p=0.5474	p=0.2638	p=0.8773	p=0.8635
Corrected p		p=0.2724						
Adjusted ¹	1.13 (0.96-1.33)	1.25 (0.99-1.57)	1.04 (0.83-1.30)	1.14 (0.96-1.35)	1.04 (0.93-1.15)	1.07 (0.95-1.21)	1.04 (0.88-1.22)	0.98 (0.81-1.19)
adjusted p	p=0.1330	p=0.0564	p=0.7531	p=0.1366	p=0.5017	p= 0.2487	p=0.6744	p=0.8623
-2456C>T	1.03 (0.79-1.33)	1.17 (0.82-1.67)	1.02 (0.71-1.47)	1.07 (0.81-1.39)	0.88 (0.74-1.04)	0.97 (0.79-1.18)	0.94 (0.72-1.23)	0.99 (0.72-1.35)
raw p-value	p=0.8496	p=0.3873	p=0.9230	p=0.6430	p=0.1349	p=0.7334	p=0.6564	p=0.9400
adjusted ¹	1.03 (0.79-1.34)	1.18 (0.82-1.69)	1.02 (0.70-1.48)	1.08 (0.82-1.41)	0.88 (0.74-1.05)	0.96 (0.78-1.17)	0.95 (0.72-1.25)	0.99 (0.73-1.36)
adjusted p	p=0.8358	p=0.3697	p=0.9228	p=0.5974	p=0.1628	p= 0.6630	p=0.6917	p=0.9716
-1883G>C	1.09 (0.92-1.29)	1.14 (0.89-1.44)	1.03 (0.81-1.31)	1.05 (0.88-1.25)	1.04 (0.93-1.16)	1.06 (0.93-1.20)	1.01 (0.85-1.21)	0.92 (0.75-1.12)
raw p-value	p=0.2974	p=0.2994	p=0.8143	p=0.6097	p=0.4972	p=0.4013	p=0.8695	p=0.4101
adjusted ¹	1.08 (0.91-1.28)	1.13 (0.88-1.44)	1.01 (0.79-1.29)	1.06 (0.89-1.27)	1.04 (0.93-1.17)	1.06 (0.93-1.21)	1.03 (0.86-1.23)	0.92 (0.75-1.13)
adjusted p	p=0.3682	p=0.3333	p=0.9471	p= 0.5172	p=0.4542	p= 0.3512	p= 0.7347	p=0.4095
+1431G>C	1.07 (0.90-1.26)	1.00 (0.79-1.28)	1.13 (0.89-1.43)	1.17 (0.98-1.39)	1.06 (0.96-1.18)	1.12 (0.99-1.28)	1.10 (0.92-1.30)	1.30 (1.07-1.58)
raw p-value	p=0.4516	p=0.9888	p=0.3148	p=0.0775	p=0.2617	p=0.0680	p=0.2928	p=0.007
Corrected								p=0.0420
adjusted ¹	1.09 (0.92-1.29)	1.01 (0.79-1.29)	1.17 (0.92-1.48)	1.18 (0.99-1.41)	1.06 (0.95-1.18)	1.11 (0.98-1.26)	1.06 (0.89-1.27)	1.33 (1.09-1.61)
adjusted p	p=0.3322	p=0.9402	p=0.1981	p=0.0632	p= 0.3334	p= 0.1084	p= 0.4925	p= 0.0046
corr. adjust. p								p= 0.0276
+1538A>G	0.80 (0.66-0.96)	0.77 (0.59-1.02)	0.82 (0.62-1.07)	0.84 (0.69-1.02)	0.99 (0.88-1.11)	0.86 (0.74-0.98)	0.99 (0.83-1.20)	0.85 (0.68-1.07)
raw p-value	p=0.0195	p=0.0677	p=0.1367	p=0.0835	p=0.8359	p=0.0291	p=0.9504	p=0.1664
corrected p	p=0.1170					p=0.1746		
adjusted ¹	p=0.78 (0.64-0.95)	0.75 (0.57-1.00)	0.81 (0.62-1.06)	0.80 (0.66-0.98)	0.99 (0.88-1.12)	0.87 (0.75-1.00)	1.01 (0.84-1.22)	0.85 (0.68-1.06)
adjusted p	p=0.0139	p=0.0492	p=0.1277	p=0.0332	p= 0.9336	p= 0.0500	p= 0.9162	p=0.1478
corr. adjust. p	p=0.0834	p=0.2952		p=0.1992		p=0.3000		
+3253T>C	1.12 (0.96-1.31)	1.19 (0.95-1.49)	1.06 (0.85-1.32)	1.03 (0.87-1.22)	0.97 (0.88-1.07)	1.03 (0.91-1.16)	0.94 (0.80-1.10)	0.90 (0.75-1.09)
raw p-value	p=0.1576	p=0.1288	p=0.6292	p=0.7103	p=0.5628	p=0.6615	p=0.4253	p=0.2722
corrected p								
adjusted ¹	1.12 (0.95-1.31)	1.20 (0.96-1.50)	1.04 (0.83-1.31)	1.05 (0.89-1.25)	0.97 (0.88-1.08)	1.03 (0.91-1.16)	0.96 (0.81-1.13)	0.90 (0.74-1.09)
adjusted p	p=0.1662	p=0.1143	p=0.7350	p=0.5395	p= 0.6408	p= 0.6255	p= 0.6040	p=0.2685

¹ adjusted for sex and environmental tobacco smoke; data presented as OR of logistic regression (95% Confidence Interval), Bonferroni corrected p-values are given in the case that the uncorrected p-value reach significance ($\alpha \leq 0.05$); ² Phenotypes are mixed: wheeze in the last 12 months in Munich/Dresden; wheeze problems still present in Leipzig; ³ Phenotypes are mixed: dr. diagnosis of atopic dermatitis in Munich/Dresden – as ever eczema+ as ever “itchy dermal changes” in Leipzig; ⁴ Data only available in Munich or Dresden

Supplementary Table 1d. CRTh2 SNPs significantly associated with atopic diseases, atopy or current wheeze in the whole sample (N=4264), pooled and stratified for city

	Asthma	Atopic Asthma	Non-atopic Asthma	Current Wheeze¹	Atopy	Eczema²	Hay fever	sIgE to food allergens³
+1431G>C	1.07 (0.90-1.26) p=0.4516	1.00 (0.79-1.28) p=0.9888	1.13 (0.89-1.43) p=0.3148	1.17 (0.98-1.39) p=0.0775	1.06 (0.96-1.18) p=0.2617	1.12 (0.99-1.28) p=0.0680	1.10 (0.92-1.30) p=0.2928	1.30 (1.07-1.58) p=0.0070
Dresden	1.06 (0.82-1.37) p=0.6803	0.95 (0.64-1.39) p=0.7807	1.18 (0.82-1.69) p=0.3799	1.29 (1.01-1.65) p=0.0373	1.04 (0.89-1.21) p=0.6413	1.09 (0.91-1.31) p=0.3404	1.00 (0.79-1.26) p=0.9814	1.25 (0.96-1.62) p=0.0991
Leipzig	1.12 (0.78-1.60) p=0.5410	1.13 (0.68-1.86) p=0.6445	1.00 (0.58-1.70) p=0.9870	0.96 (0.62-1.47) p=0.8397	0.99 (0.79-1.23) p=0.8926	1.24 (0.94-1.64) p=0.1346	0.89 (0.58-1.38) p=0.6141	- - -
Munich	1.01 (0.76-1.34) p=0.9503	0.95 (0.64-1.43) p=0.8155	1.10 (0.75-1.60) p=0.6299	1.08 (0.79-1.48) p=0.6166	1.19 (0.97-1.45) p=0.0973	1.09 (0.87-1.37) p=0.4535	1.40 (1.03-1.89) p=0.0303	1.35 (1.02-1.80) p=0.0350
+1538A>G	0.80 (0.66-0.96) p=0.0195	0.77 (0.59-1.02) p=0.0677	0.82 (0.62-1.07) p=0.1367	0.84 (0.69-1.02) p=0.0835	0.99 (0.88-1.11) p=0.8359	0.86 (0.74-0.98) p=0.0291	0.99 (0.83-1.20) p=0.9504	0.85 (0.68-1.07) p=0.1664
Dresden	0.93 (0.70-1.23) p=0.6048	1.05 (0.70-1.57) p=0.8175	0.94 (0.63-1.42) p=0.7827	0.79 (0.60-1.05) p=0.1096	1.05 (0.88-1.24) p=0.6081	0.96 (0.79-1.17) p=0.6663	1.10 (0.86-1.41) p=0.4351	0.92 (0.69-1.23) P=0.5649
Leipzig	0.66 (0.44-0.99) p=0.0408	0.52 (0.28-0.97) p=0.0349	0.73 (0.41-1.29) p=0.2740	0.94 (0.62-1.43) p=0.7824	0.95 (0.76-1.19) p=0.6650	0.88 (0.65-1.19) p=0.4072	1.10 (0.72-1.68) p=0.6616	- -
Munich	0.80 (0.57-1.12) p=0.1858	0.72 (0.44-1.18) p=0.1900	0.80 (0.50-1.26) p=0.3263	0.90 (0.62-1.29) p=0.5540	0.92 (0.73-1.16) p=0.4805	0.71 (0.54-0.94) p=0.0153	0.77 (0.52-1.13) p=0.1818	0.81 (0.57-1.15) p=0.2399

Data presented as *OR of logistic regression* (95% Confidence Interval), *p values* with Armitage Trend test.

¹ Phenotypes are mixed: wheeze in the last 12 months in Munich/Dresden; wheeze problems still present in Leipzig;

² Phenotypes are mixed: dr. diagnosis of atopic dermatitis in Munich/Dresden – as ever eczema+ as ever “itchy dermal changes” in Leipzig; ³Data only available in Munich or Dresden