ORIGINAL ARTICLE

Association of a *STAT 6* haplotype with elevated serum IgE levels in a population based cohort of white adults

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Background: Several studies have shown linkage of chromosome 12q 13–24 with atopy related phenotypes. Among candidate genes in this region is *STAT6* (signal transducer and activator of transcription), which is essential for Th2 cell differentiation, recruitment, and effector function.

Methods: We evaluated six polymorphisms of *STAT6* for evidence of associations with serum IgE levels and atopic diseases in a population based cross sectional cohort of 1407 German adults. Genotyping was performed using the matrix assisted laser desorption ionisation–time of flight mass spectrometry method. Haplotypes were estimated using the SAS/Genetics module, and population-derived IgE percentiles (50% IgE>53 kU/l, 66% IgE>99 kU/l and 90% IgE>307 kU/l) were modelled as outcome variables in haplotype trend regression analysis.

Results: All polymorphisms were genotyped successfully. Haplotype reconstruction revealed 8/64 possible haplotypes, reaching estimated frequencies of 1% or more. One polymorphism in intron 2 (rs324011) showed a significant association with total serum IgE (p = 0.015). A *STAT6* risk haplotype for elevated IgE showing odds ratios of 1.7 (p = 0.015) for IgE cut-off 100 kU/l, and 1.54 (p = 0.032), 1.6 (p = 0.025), and 2.54 (p = 0.007) for IgE percentiles 50%, 66%, and 90%, respectively was detected. The increased risk of this haplotype was confirmed by linear haplotype trend regression on log transformed IgE values (p = 0.007). Analysis further revealed a risk haplotype for specific sensitisation and a risk haplotype for asthma.

Conclusion: The data indicate that genetic variants within *STAT6* contribute significantly to IgE regulation and manifestation of atopic diseases.

Atopy is defined as familial tendency to develop characteristic IgE mediated allergic diseases of the skin and airways.[1] Epidemiological studies have shown a worldwide increase of the prevalence of atopic diseases (atopic eczema, allergic asthma, and rhinoconjunctivitis) over the last few decades, and they currently affect more than 10% of individuals in Western countries.[2 3]

The majority of patients with atopic diseases show sensitisation against environmental allergens, with high serum levels of total and allergen specific IgE, and positive skin prick test reactions.[4] Current pathophysiological concepts suggest interactions between susceptibility genes, the host's environment, and immunological factors.[5] T cells have been shown to be implicated in the pathogenesis of atopic diseases (reviewed by Rogmanani[6]), but their exact role is still not fully understood. Although in recent years it has become clear that the Th1/Th2 paradigm is insufficient to describe all immune response patterns associated with the development of atopy (reviewed by El Biaze *et al*[7]), a critical role for Th2 is is widely accepted.[8] In addition, at least in the majority of patients, IgE, eosinophils, and factors regulating IgE synthesis and eosinophil numbers and activity (namely Th2 cytokines such as interleukin (IL)-4, -5, -6, -9, -10, and -13) play a major role in driving atopy pathogenesis.[9]

Genetic susceptibility of IgE responsiveness is likely to be caused by a pattern of polymorphisms in multiple genes regulating immunological responses.[10 11]

Family and case−control studies have provided evidence that total serum IgE levels are largely determined by genetic factors that may be independent of specific IgE responses, and it has been suggested that total serum IgE levels are under stronger genetic control than atopic disease.[11–13] The

production of specific IgE is driven by Th2 cells that secrete IL-4 and -13 in response to antigen presentation. Binding of IL-4 and -13 to the IL-4R alpha chain recruits signal transducer and activator of transcription 6 (STAT6) into the receptor complex, activates germline transcription from the epsilon heavy chain gene locus, and, together with signals derived from the B cell surface molecule CD40, induces isotype switching in B cells and activates genes important for IgE synthesis.[14–16] Mice lacking STAT6 are unable to undergo class switching to IgE, exhibit an impaired proliferative response to IL4, and cannot differentiate naive T cells into Th2 cells.[17]

More than 10 regions have been identified as candidate regions influencing total serum IgE levels, atopy, and asthma.[18 19] Among the most frequently identified genomic regions is the long arm of chromosome 12 (12q13–24), where *STAT6* is located.[20] Various genome scans and studies restricted to chromosome 12q [21–30] have provided evidence for linkage of asthma related phenotypes with chromosome 12 markers. Significant associations with increased serum levels of IgE have been reported for single nucleotide polymorphisms (SNPs) in introns 2, 17, and 18 and for two SNPs in the 3' UTR of the *STAT6* gene in a previous family sibling pair study.[31] However, the primary trait examined within this affected relative study was asthma, and a rather low number of subjects was investigated. We aimed to validate the observations in a large population based cross sectional cohort of 1407 adults and to evaluate *STAT6*

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Abbreviations: IL, interleukin; RAST, radioallergosorbent test; SAP, shrimp alkaline phosphatase; SNP, single nucleotide polymorphism; STAT6, signal transducer and activator of transcription 6; TFB, transcription factor binding

polymorphisms with regard to associations with atopy related phenotypes.

For our analyses, we chose six polymorphisms spanning the whole *STAT6* gene (fig 1). The SNPs were selected on the basis of the above mentioned family study, completely screening all exonic and adjacent intronic regions and the probable promoter region of *STAT6*. The DNA variants analysed in the present study were the only ones that had been found to be associated with at least one atopic trait.[31]

MATERIALS AND METHODS
Study population
All subjects were recruited from the MONICA/KORA (Cooperative Health Research in the Augsburg Region) surveys, which are large population based cross sectional studies carried out every 5 years in the city and region of Augsburg, southwest Germany, since 1984. The objective and protocol of the MONICA surveys have been published earlier.[32 33] The study base of the MONICA/KORA surveys consists of all registered residents aged 25–74 years of the city of Augsburg, Germany, and two surrounding counties.

For analysing the influence of *STAT6* polymorphisms on IgE levels, we examined the KORA C study group. KORA C is based on a random, cross sectional sample stratified for age and sex studied in 1994/1995 (n = 4356) with a focus on allergic sensitisation and respiratory atopy. In the sera of these subjects, allergen specific IgE antibodies to common aeroallergens (grass and birch pollen, house dust mite, cat, and *Cladosporium*) were determined by the fluorescence enzyme immunoassay technique (CAP-FEIA; Pharmacia, Uppsala, Sweden). The cutoff point for radioallergosorbent test (RAST) reactivity was set at 0.35 kU/l. For KORA C, we aimed to recruit an enriched sample of 1600 subjects out of the 4178 individuals who had valid RAST results.

Subjects were selected, stratified by age and sex, so as to provide 50% with and 50% without a positive RAST, and, furthermore, so that within these groups 50% had given a positive answer to one of the following questions of the MONICA questionnaire: (*a*) ''Have you had asthma attacks within the last 12 months?'', (*b*) ''Do you have allergic rhinitis, e.g., hay fever?'', or (*c*) ''How much do you suffer from allergies?'' (positive = moderate/severe). To account for an estimated response of 63%, a sample of 2539 subjects was approached. Finally, 1537 subjects participated (60.5%), of whom 50.2% exhibited a positive RAST result, and 53.9% of this group and 43.1% of those with negative RAST result reported symptoms of atopy. Details of the sampling frame and study design of KORA C are given in fig 2.

Written and informed consent was obtained from all participants prior to the beginning of the study.
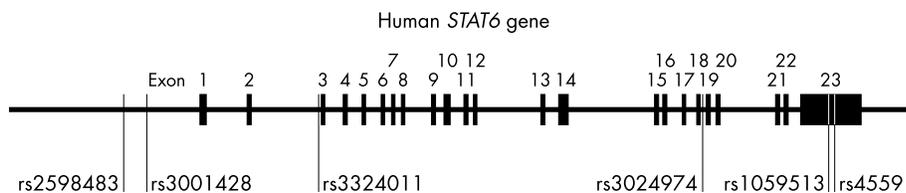
Phenotyping
All subjects had to complete a standardised questionnaire, which included demographic data and basis allergy questions of the ISAAC survey.[34] In addition, all probands were interviewed in a standardised manner to report on symptoms of atopic diseases, basic family data, and parental history of atopic diseases. Subjects were also asked to recall their highest level of education.

In all subjects a conventional lancet skin prick test (ALK-Scherax, Hamburg, Germany), and total and specific IgE measurements (ELISA; CAP-FEIA, Pharmacia, Uppsala, Sweden) were performed using a sample of common allergens (grass, birch, rye, mugwort pollen, *Alternaria*, *Cladosporium*, cat, dog, and *Dermatophagoides pteronyssinus*). Saline and histamine were used as controls. Results showing a positive reaction to saline or no reaction to histamine were excluded from analyses. Subjects were classified as having allergic rhinoconjunctivitis or asthma when they reported a physician's diagnosis of the relevant condition. Specific sensitisation was defined to be present if at least one of the specific IgE antibodies was positive (CAP class≥1, corresponding to ≥0.35 KU/l) or if a positive skin prick test reaction (wheal diameter≥3 mm) against at least one of the allergens tested was observed.
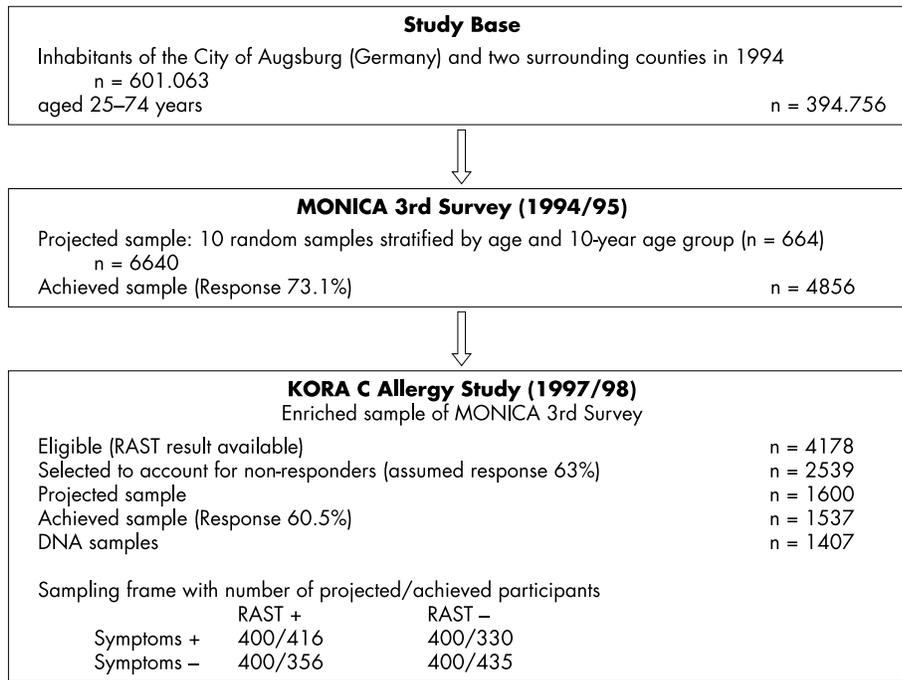
Based on all subjects with available DNA samples and existing IgE measurements in our dataset (n = 1407), IgE percentiles were calculated. Thus, for haplotype analysis, in addition to a cutoff point of 100 kU/l, the 50th (53 kU/l), 66th (99 kU/l) and 90th (307 kU/l) percentiles for total serum IgE were used as outcome variables.

Genetic analyses
Genotyping analyses were carried out by using the MassARRAY system (Sequenom, San Diego, USA). Briefly, genomic DNAs were amplified by PCR using HotStar*Taq* DNA polymerase (Qiagen, Hilden, Germany). Genotyping assays were carried out using 5 ng genomic DNA. PCR primers were used at 167 nM final concentrations for a PCR volume of 6 μl. The PCR conditions were a hot start at 95°C for 15 minutes, followed by denaturing at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 1 minute for 45 cycles, and finally incubation at 72°C for 10 minutes. PCR products were first treated with shrimp alkaline phosphatase (SAP; Amersham, Freiburg, Germany) for 20 minutes at 37°C to remove excess dNTPs and afterwards for 10 minutes at 85°C to inactivate SAP. ThermoSequenase (Amersham) was used for the base extension reactions. Extension primers were used at a final concentration of 5.4 μM in 10 μl reactions. The base extension reaction condition was 94°C for 2 min, followed by 94°C for 5 seconds, 52°C for 5 seconds, and 72°C for 5 seconds for 40 cycles. All reactions (PCR amplification, base extension) were carried out in a Tetrad PCR thermal cycler (MJ Research). The final base extension products were treated with SpectroCLEAN resin (Sequenom) to remove salts in the reaction buffer. This step was carried out with a Multimek 96 channel autopipette (Beckman Coulter), and 16 μl of resin/water suspension was added into each base extension reaction, making the total volume 26 μl. After rapid centrifugation (2000 rpm, 3 minutes) in an Eppendorf Centrifuge 5810, 10 nl of reaction solution was dispensed onto a 384 format SpectroCHIP (Sequenom) prespotted with a matrix of 3-hydroxypicolinic acid by using a SpectroPoint nanodispenser (Sequenom). A modified Bruker Biflex matrix assisted laser desorption ionisation–time of flight mass spectrometer (Sequenom) was used for data acquisitions from the SpectroCHIP. Genotyping calls were made in real time with MassArray RT software (Sequenom).

Figure 1 Gene structure of *STAT6* showing the location of all SNPs genotyped in this study.

Study Base

Inhabitants of the City of Augsburg (Germany) and two surrounding counties in 1994
n = 601.063
aged 25–74 years   n = 394.756

MONICA 3rd Survey (1994/95)

Projected sample: 10 random samples stratified by age and 10-year age group (n = 664)
n = 6640
Achieved sample (Response 73.1%)   n = 4856

KORA C Allergy Study (1997/98)
Enriched sample of MONICA 3rd Survey

Eligible (RAST result available)   n = 4178
Selected to account for non-responders (assumed response 63%)   n = 2539
Projected sample   n = 1600
Achieved sample (Response 60.5%)   n = 1537
DNA samples   n = 1407

Sampling frame with number of projected/achieved participants
	RAST +	RAST –
Symptoms +	400/416	400/330
Symptoms –	400/356	400/435

Figure 2 Design and sampling frame of the KORA C study.

For analysis of sequence binding sites MatInspector (release 7.0; Genomatix, Munich, Germany) was used.

Statistics

Descriptive statistics for quantitative and qualitative values are given by mean (SD) and absolute numbers or frequencies, respectively. For descriptive statistics, SPSS (version 11.5) was used. Association of quantitative traits with SNP genotype was tested with t tests for two independent samples or Satterthwaite tests using the SAS statistical software package (version 8.2). Deviation from Hardy-Weinberg equilibrium was tested with Pearson's χ^2 test statistic for any of the SNPs under consideration. Linkage disequilibrium was assessed with procedure ldmax in the Gold software package. Haplotype frequencies were estimated from genotype data using the EM algorithm.[35 36] To evaluate associations with quantitative and qualitative traits, haplotype trend regression models were applied including estimated probabilities of haplotypes in a linear and logistic regression approach as independent variables,[37 38] respectively. Further independent variables were highest school examinations, age, family size, and sex. Because smoking is an essential influence factor on IgE levels and atopic diseases,[39 40] smoking status was included as independent variable in multiple regressions.

The same covariates were included in logistic regressions testing associations of qualitative traits with SNP genotypes. Results are reported after applying backward elimination and stepwise variable selection. All haplotype analyses were implemented using the SAS/Genetics module.[38] Odds ratios are given with 95% confidence intervals (CI) and two sided p values in parentheses.

RESULTS

Within the KORA C study individuals (n = 1537), 8.8% (n = 136) were diagnosed with asthma, 2.0 % (n = 30) were suffering from atopic eczema, and 25.2% (n = 388) from allergic rhinoconjunctivitis. IgE serum levels of more than 100 kU/l were detected in 33.2% (n = 510). DNA samples were available from 1407 individuals. Between 1285 and 1356 samples (average call rate 95.1%) were genotyped successfully for the respective *STAT6* polymorphisms (fig 1). Allele frequencies and genotyping details are given in table 1. The allele distribution observed in our white population is

Table 1 *STAT6* polymorphisms, dbSNP accession number, localisation, allele frequency, call rates, and genotyping primers

SNP ID*	Location	Call rate	Allele	Allele frequency	Direction	PCR primer	Extension primer
rs2598483	5'flanking	95.5	G	0.90	F	ACGTTGGATGTACTGTCTGTGGATGTCTGC	CACGTGCTTTGTATGTGCTCC
			A	0.10	R	ACGTTGGATGTGTGCACATACGTGTTCACG	
rs3001428	5'flanking	96.4	C	0.95	F	ACGTTGGATGTGATTTCCAGAACCAGCTCC	CCACACACGTGCACTCATG
			T	0.05	R	ACGTTGGATGTTCAAACCCTCTGCTGCCTG	
rs324011	Intron 2	91.3	C	0.62	F	ACGTTGGATGGATGCCCCTGGTTTAAGGTG	ATAGCCCTCCTAGGGAC
			T	0.38	R	ACGTTGGATGCAGGGACCTCCCATAGATAG	
rs3024974	Intron 18	95.5	C	0.89	F	ACGTTGGATGTGACTGACCAAGGGTTGATG	GGGCTTAGTGCTTATCTG
			T	0.11	R	ACGTTGGATGAAGGTGAGTGTGGTGGTATG	
rs1059513	3'UTR	96.2	A	0.88	F	ACGTTGGATGAATTCCTGTTAGCCAGGTGG	ACGAAGAATCTCAGCCCT
			G	0.12	R	ACGTTGGATGCGTTCACACAGCTATACACG	
rs4559	3'UTR	95.2	A	0.63	F	ACGTTGGATGTGAACGTGTATGTACCTAGG	GCAACTAAGGTGCCAGCTATA
			G	0.37	R	ACGTTGGATGTGTAGCATATGTCAGAGAGGCC	

*SNP no. according to dbSNP (www.ncbi.nlm.nih.gov/SNP).

Table 2 *STAT6* haplotypes and haplotype frequencies with 95% confidence intervals (haplotypes with frequencies < 1% were not considered)

No.	Haplotype	Frequency (%)	95% CI
1	G-C-T-C-A-A	37.51	35.69 to 39.35
2	G-C-C-C-A-A	6.25	5.30 to 7.19
3	A-C-C-C-A-A	8.93	7.83 to 10.03
4	G-C-C-C-G-A	9.86	8.73 to 10.99
5	G-C-C-C-A-G	19.91	18.38 to 21.43
6	G-C-C-T-A-G	10.36	9.17 to 11.54
7	G-T-C-C-A-G	4.81	4.01 to 5.61
8	A-C-C-C-A-G	1.28	0.84 to 1.74

CI, confidence interval.

consistent with allele frequencies observed in previous studies.[29] [31]

Using multiple testing and a two sided significance level of 5%, none of the genotyped polymorphisms showed a significant deviation from Hardy-Weinberg equilibrium.

Of the six SNPs genotyped in KORA C, polymorphism rs324011 showed a significant association with total serum IgE levels with geometric means for serum IgE (kU/l) 46.4 in CC wildtypes versus 56.6 in CT and TT genotypes (p = 0.015).

For haplotype analysis there were 1231 samples available for which genotyping was successful for each of the six *STAT6* polymorphisms. The EM algorithm showed 8 of 64 possible haplotypes, exceeding a frequency of 1%. The 95% CI values for frequencies are small, because of the large study population, indicating sufficiently high accuracy. The main haplotype GCTCAA had a frequency of 37.5% (table 2). For this haplotype we observed an increased risk of elevated IgE levels for all cutoff points examined, with odds ratios of 1.7 (95% CI 1.11 to 2.6, p = 0.015), 1.54 (95% CI 1.04 to 2.3, p = 0.032), 1.6 (95% CI 1.06to 2.4, p = 0.025) and 2.5 (95% CI 1.3 to 5.0, p = 0.007) for IgE cut-off (100 kU/l) and IgE percentiles 50%, 66%, and 90%, respectively. The increased risk of this haplotype is displayed in fig 3 and strongly confirmed by linear haplotype trend regression on log transformed IgE values with same covariates (p = 0.007).

Haplotype results are in accordance with the aforementioned association of SNP rs324011 (presence of T allele) with elevated levels of IgE. In particular, the effects of T allele frequencies of rs324011 on IgE levels with cutoff of 100 kU/l
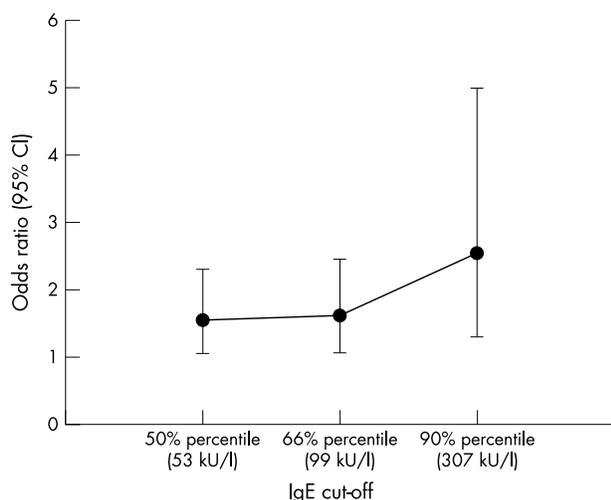
Figure 3 Odds ratios and 95% confidence intervals for IgE 50%, 66%, and 90% percentiles by the *STAT6* GCTCAA haplotype.

and IgE percentiles 50% and 90% in a multiple logistic regression analysis were significantly positive (p = 0.049, 0.045 and 0.013). This was also confirmed by multiple linear regression on log transformed IgE values (p = 0.016).

Analyses further revealed that haplotype GCCTAG is a significant risk factor for the development of specific sensitisation (OR = 2.0, 95% CI 1.1 to 3.9, p = 0.03), and haplotype ACCCAG is a significant genetic risk factor for asthma (OR = 2.17, 95% CI 1.8 to 255.5, p = 0.01). Correcting for IgE levels, the haplotype risk factor for asthma remained significant (p = 0.005).

No statistically significant associations were observed between single SNPs or SNP haplotypes in the *STAT6* gene and the prevalence of atopic eczema or hay fever (data not shown). In the case of atopic eczema, the lack of association might be due to the low prevalence within the study population.

DISCUSSION

This study analysed the effects of six polymorphisms in the *STAT6* gene on atopic phenotypes by using a population based cohort of 1407 German adults. One single polymorphism (rs324011) revealed to influence total serum IgE levels. Haplotype analysis showed the existence of a high risk *STAT6* haplotype for the development of elevated serum IgE levels. Furthermore, one *STAT6* haplotype was significantly associated with allergen specific IgE production, and another haplotype was significantly associated with an increased prevalence of asthma. These data indicate that genetic variants in the *STAT6* gene affect total and specific serum IgE levels and may thereby be involved in the pathogenesis of atopic diseases.

Although the single polymorphism (rs324011) that revealed significant association with serum IgE levels is not located in the coding regions of the *STAT6* gene, it may have functional importance. It is located in intron 2, where two transcription factor binding (TFB) sites for NFκB are found close to each other, suggesting a regulatory function of this region in the transcription of *STAT6*. It may be speculated that the binding properties of NFκB to the TFB site are altered by the polymorphism. Although *STAT6* is known to be the key factor controlling the IgE germline gene promoter, it has been shown that other transcription factors must be present and functional. Importantly, evidence has been provided that NFκB interacts and synergises with *STAT6*.[41 42] Thus, the presence of two NFκB sites within the *STAT6* sequence may indicate the presence of a feedback regulation between NFκB and *STAT6* activation. However, expression analysis and transcription factor binding studies are needed to clarify the role of the polymorphism rs324011 in this putative interaction.

The data from our study are in concordance with a previous report indicating associations of *STAT6* polymorphisms with elevated serum IgE in a population of 108 sibling pair families examined for asthma as primary trait.[31] In our study, the influence of *STAT6* polymorphisms and haplotypes on IgE regulation has been examined for the first time in a large population based cross sectional cohort of adults.

It has been suggested that haplotype analyses may be of higher informative value for drawing associations between phenotypes and genetic variation than SNPs.[43] To assess the effects of haplotypes in our cross sectional study population of unrelated subjects, the 50th, 66th, and 90th percentiles for serum IgE levels were calculated as outcome variables. The 66th percentile corresponded to the widely used arbitrary cutoff of 100 kU/l.

Haplotypes were estimated according to standard methods using the EM algorithm. The frequent *STAT6* haplotype combination GCTCAA, which had a prevalence of 37.5%, revealed a strong association with high serum IgE levels. This

is strongly confirmed by linear haplotype trend regression using log transformed values of observed IgE levels as the quantitative dependent variable.

As the polymorphisms investigated in the present study cover the complete *STAT6* gene, it seems unlikely that relevant associations were missed. Linkage disequilibrium decreases even within the gene. Associations with elevated serum IgE clearly focus on rs324011. Haplotype GCTCAA is the only one with a T allele at position rs324011 (table 2). Thus the strongest contribution to the haplotype effect observed in this analysis for total IgE was made by the polymorphism rs324011, which was also significant in the single analysis. However, although rs324011 is significantly associated with serum IgE levels and is a good candidate for a functional SNP as it alters a TFB site, it is also possible that it is only in linkage diseqilibrium with the causal variant(s).

We further observed that haplotype GCCTAG is a significant risk factor for the development of specific sensitisation, and the haplotypic combination ACCCAG is a risk factor for asthma. However, asthma was not the primary trait examined within this study, and the number of individuals suffering from asthma was low (n = 136). In addition, no associations between *STAT6* SNPs or haplotypes and asthma were detected in a previous family based association study focusing on asthma.[31] In contrast, a strong association of a SNP within the 3'UTR of *STAT6* with mild atopic asthma characterised by presence of specific IgE or high total IgE was reported in a Japanese population of adults.[44] Furthermore, most of the positive linkage results to 12q13–24 have been reported for asthma and only to a minor extent for IgE levels. Thus, further studies with sufficient numbers of cases are needed to clarify the role of *STAT6* as an asthma candidate locus on 12q.

In conclusion, our data indicate that *STAT6* gene polymorphisms and haplotypic combinations significantly contribute to the regulation of total serum IgE levels, which have been shown to be under strong genetic control in segregation analysis. One polymorphism located in intron 2 of the *STAT6* gene seems to be mainly responsible for the observed association results. Owing to its localisation in the centre of a NFκB transcription factor binding site, this polymorphism may exert a functional role. In addition, *STAT6* polymorphisms possibly also influence specific IgE production and manifestation of atopic diseases such as asthma.

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