

ICOS-gene variants are not associated with atopic disease susceptibility in European children

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The inducible co-stimulatory molecule, *ICOS*, is an important regulator of T cell differentiation and effector function. Previously, it was reported that two variants in the *ICOS* promotor region, g.1-1413G > A and g.1-693G > A, were associated with sensitization to airborne allergens, elevated serum IgE levels and Th2 cytokine production in a Hutterite population. The aim of this study was to evaluate these two and four other selected *ICOS* variants for association with atopic phenotypes in two large European prospective pediatric cohorts. We investigated subjects from the German Multicenter Allergy Study (MAS), which followed over 800 children with atopic family history from birth until 13 yr of age, and from the Early Treatment of the Allergic Child Study (ETAC), which collected DNA and clinical data of over 330 children with atopic dermatitis during their first 2 yr of life. We genotyped DNA from these children by melting curve analysis using fluorescence resonance energy transfer (FRET) probes. We could not confirm the previously reported association of g.1-1413G > A and g.1-693G > A with atopic phenotypes in our pediatric cohorts. Also four other *ICOS* variants at putative binding sites for transcription factors showed no association with atopic dermatitis, asthma, allergic sensitization and allergic rhinitis. Our data suggest that these *ICOS* variants do not play a major role in the development of atopy in European children.

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Atopic diseases are complex traits that are influenced by multiple genes as well as environmental factors. Several susceptibility loci for atopic predisposition have been identified by linkage analysis in certain populations. Recently, it was reported that two variants in the promotor region of the inducible co-stimulatory molecule, *ICOS*, (g.1-1413G > A and g.1-693G > A) were associated with sensitization to airborne aller-

gens, elevated serum IgE levels and Th2 cytokine production in a Hutterite population (1).

This on first sight was not totally unexpected since *ICOS* is an important regulator of T cell differentiation and effector function. The *ICOS*/*ICOS* ligand pathway seems to be most important for T/B cell cooperation regulating the production of IgG1, IgG2a, and IgE in mice (2), and the maintenance of the B cell memory pool in humans (3). Thus, *ICOS* as a key regulator of Th2 immune responses is an attractive candidate gene for atopic diseases.

The gene coding for *ICOS* spans over 25 kb and is divided into five exons (4). The promotor region upstream of the *ICOS* initiation codon

Abbreviations: ETAC, early treatment of the atopic child; FRET, fluorescence resonance energy transfer; *ICOS*, inducible co-stimulator; Ig, immunoglobulin; MAS, Multicenter Allergy Study; NF-κB, nuclear factor κB; RAST, radioallergosorbent test, Th, T-helper cell.

contains several binding sites for transcription factors, while no TATA box, CCAAT box or other initiator element is present (5). This and the fact that very few variants have been recorded in the *ICOS* coding sequence, while several genetic alterations have been detected in the promotor and intronic regions (1, 3, 5, 6), indicates that transcription of *ICOS* is mainly regulated by transcription factors. Therefore, and regulation of transcription rather than changes in the protein structure may affect *ICOS* modulated immune responses.

One of the previously reported variants in the *ICOS* promotor region, the g.1-1413G > A transition, was demonstrated to be located at a functional NF- κ B binding site, suggesting that its effect on *ICOS* transcription may be responsible for the observed atopic phenotype (1). Other variants located at putative regulatory sites of the *ICOS* gene have been described but not yet evaluated for association with atopic diseases: another putative binding site for NF- κ B [g.1-2144C > T (rs11889031)], one for Sp1 (g.1-2419T > C) in the *ICOS* promotor region, and two polymorphic sites located in putative regulatory sites of introns 3 and 4 [g.20065C > G (IVS3 + 126C > G) and g.22126G > T (IVS4-620G > T)] (5). In the present study, we evaluated these two risk-tagging as well as the four other selected variants in the *ICOS* gene for association with atopic phenotypes in two large prospective cohorts of children of European descent.

The German Multicenter Atopy Study (MAS) that has been described in detail in previous reports (7) included potential high-risk infants with cord blood IgE levels of ≥ 0.9 kU/l and/or ≥ 2 atopic family members. Cohort infants were brought in for follow-up visits at 1, 3, 6, 12, 18, and 24 months, with annual visits thereafter up to the age of 13 yr. Total and specific IgE antibodies against common food and inhalant allergens were analyzed at the age of 1, 2, 3, 5, 7 and 10 yr (CAP-RAST FEIA, Uppsala, Sweden). Phenotypes were defined as follows: *Current asthma*: one or more wheezing episodes during the previous 12 months. *Allergic sensitization*: specific IgE ≥ 0.7 kU/l against at least one out of nine food and inhalant allergens. *High IgE*: in childhood IgE levels underlie remarkable variation over time even in the absence of atopy, therefore high IgE was defined as total IgE above the 85th cohort percentile at $\geq$ two time-points. *Atopic dermatitis*: combination of dry skin and at least three out of nine typical signs (itching, erythema, red papules, vesicles, rhagades, excoriation, squamae, lichification, perifollicular

accentuation). The controls were defined as no sensitization at age 7 and 10 yr, no wheeze ever at age 10 yr, no runny nose without cold ever at age 10 yr and no current atopic dermatitis by age 7 yr. DNA samples collected at age 4 yr were available from 823 children.

Secondly, we analyzed DNA samples of 334 children with atopic dermatitis in their first 2 yr of life from the Early Treatment of the Allergic Child Study ((ETAC) (8). Children from 12 European countries were enrolled in a randomized, double-blind, placebo-controlled trial of the efficacy of ceterizine treatment in the prevention of asthma. All children had a positive family history of atopy. Clinic visits occurred at screening, immediately before initiation of therapy and subsequently after 1 month, 3 months, and every 3 months thereafter during the 18-month treatment period. Blood samples were collected at 3, 12, 18, 24 and 36 months after the beginning of the study and total IgE and specific IgE to egg, milk, house dust mite, grass pollen, and cat were measured. Total and specific IgE levels of 0.35 kU/l and higher were considered as elevated were available.

We genotyped DNA samples by melting curve analysis using the LightTyper system (Roche Diagnostics, Mannheim, Germany). Polymerase chain reaction (PCR) conditions, oligonucleotide sequences used for PCR amplification, primers and FRET probe sequences are provided in Tables S1 and S2.

Chi-square tests were used to compare prevalences between groups in both studies. In case of small cell counts, Fisher's exact test was used instead. For the comparison of continuous variables between 2 groups, the non-parametric Wilcoxon rank-sum test was used. Differences were considered to be statistically significant when $p < 0.05$.

As described for the Hutterite population we found the two *ICOS* variants g.1-1413G > A and g.1-693G > A to be in linkage disequilibrium. The frequency of the risk-tagging allele g.1-1413A was 22% in the MAS and 18% in the ETAC cohort (Table 1), which is comparable to the Danish cohort in which these variants were first described (5), but lower than in the Hutterite population where it was 39%. In contrast to the Hutterite study, no significant differences in genotype frequencies were observed when children with atopic dermatitis, allergic rhinitis, polysensitization or asthma were compared to non-atopic controls (Table 2). Genotype distributions were not significantly different in children with longitudinally high IgE compared to those with low IgE phenotypes.

Table 1. Allele frequencies of the evaluated *ICOS* variants in various cohorts

Position	Regulatory element	Allele							
		MAS		ETAC		Danish study (5)		Hutterites (1)	
g.1-693	Unknown	G 0.78	A 0.22	G 0.82	A 0.18	G 0.78	A 0.22	G 0.61	A 0.39
g.1-1413	Functional NF- κ B binding site (1)	G 0.78	A 0.22	G 0.82	A 0.18	G 0.78	A 0.22	G 0.61	A 0.39
g.1-2144	Potential NF- κ B binding site (5)	C 0.93	T 0.07	C 0.93	T 0.07	C 0.92	T 0.08	nd	nd
g.1-2419	Potential Sp1 binding site (5)	T 0.77	C 0.23	T 0.79	C 0.21	T 0.78	C 0.22	nd	nd
g.20065	Intron 2 enhancer (5)	C 0.87	G 0.13	C 0.85	G 0.15	C 0.85	G 0.15	nd	nd
g.22126	Potential Sp1 binding site (5)	G 0.92	T 0.08	nd	nd	G 0.85	T 0.15	nd	nd

nd, not determined.

The other four alterations located at potential *ICOS* regulatory sites were not in linkage disequilibrium and were considered individually. The allele frequencies in our cohorts again were comparable to those in the initial report (Table 1).

The T allele at position g.1-2144, a potential NF- κ B binding site, showed a transient association with sensitization against grass pollen during the first year of life in the MAS cohort ($p = 0.00023$). However, specific IgE levels at that age were generally very low, and the mean IgE levels of children either homozygous for the mutation (TT, mean grass specific IgE 0.14 kU/l), or for the wildtype (CC, mean grass specific IgE 0.01 kU/l) and IgE levels of heterozygotes (CT, mean grass specific IgE 0.01 kU/l) all ranged below the first detection level, CAP1 (< 0.35 kU/l), making a clinical relevance rather unlikely. Similar results were obtained in the MAS cohort for the C allele at g.1-2419, located at a putative sp1 binding site, and the G allele at g.20065 (IVS3 + 126). If corrected for multiple testing, the association of g.20065G in the MAS as well as in the ETAC cohort showed no significance anymore (MAS $p = 0.107$, ETAC $p = 0.058$).

In conclusion, we show here that none of the six evaluated variants in the *ICOS* gene was significantly associated with the development of

allergic phenotypes in our pediatric prospective cohorts, suggesting that genetic alterations in this region is unlikely to explain increased susceptibility for allergic disease in European children. Several aspects may very well explain these differences in the outcome compared to the Hutterite study: The observed accumulation of the risk tagging allele in the Hutterite population may reflect a selection process in this highly inbred population. Furthermore, environmental factors, such as exposure to allergens and viral or bacterial pathogens, may differ among our study and the Hutterite population. The involvement of co-stimulatory molecules in complex immune responses against a multitude of allergic and innate stimuli may have divergent effects on the development of the allergic phenotype. Also the mechanisms of adult sensitization as observed in the Hutterite population may differ from those in children.

To fully answer this and other related questions, genetic variants must be analyzed in case-control studies with standardized assessment of environmental factors, strict definition of phenotypes and recruitment of large study populations. This way characterized risk-tagging genes and genetic variants may help to establish novel strategies for prediction, prevention and treatment of allergic diseases.

Table 2. *ICOS* g.1-1413 genotype frequencies in atopy related phenotypes and non-atopic controls in the MAS cohort

Phenotype	n	Genotype frequencies		
		GG	GA	AA
Atopic dermatitis $\leq$ age 2 yr	185	0.65	0.31	0.03
Allergic rhinitis age 7 yr	81	0.65	0.32	0.02
Polysensitization age 7 yr	77	0.65	0.34	0.01
Polysensitization age 10 yr	89	0.64	0.34	0.02
Total IgE < 15 percentile at least two times at age 1, 2, 3, or 5 yr	97	0.54	0.38	0.08
Total IgE > 85 percentile at least two times at age 1, 2, 3, or 5 yr	87	0.67	0.30	0.03
Total IgE < 15 percentile at least two times at age 5, 7, or 10 yr	77	0.61	0.32	0.06
Total IgE > 85 percentile at least two times at age 5, 7, or 10 yr	82	0.67	0.29	0.04
Asthma age 7 yr	85	0.66	0.32	0.02
Non-atopic controls	125	0.62	0.34	0.04

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Supporting Information

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

Table S1. Oligonucleotide sequences used for PCR amplification

Table S2. Sensor and anchor probes for melting curve analysis

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Supplementary Table 1: Oligonucleotide sequences used for PCR amplification

Region of interest	Primer	Annealing Temperature
ICOS promotor (-693, -1413)	forward: 5'-GGAACCAGGGATTCTCACC-3'	60°C
	reverse: 5'-CTGAGACCCAGGAGCCC-3'	
ICOS promotor (-2144, -2419)	forward: 5'-GCACTTCCTGTGAGGTGAATGG-3'	56°C
	reverse: 5'-CCAGACAGTGAACTCTGGATTCC-3'	
ICOS intron 3 (g.20065)	forward: 5'-TGCCCACTTACTGGATTTCATGAC-3'	56°C
	reverse: 5'-GTA ACTCTCTGTAAGCCATCAAGTC-3'	
ICOS intron 4 (g.22126)	forward: 5'-CCACCCCTCAGTGGCTACAG-3'	52°C
	reverse: 5'-CATCTGTACACTTCTCAGAGGTCC-3'	

Primers against sequences flanking the regions of interest were designed based on the published nucleotide sequence (AF411059). PCR was performed using 0,5 U AmpliTaq Gold polymerase (Applied Biosystems), 400 µmol/l desoxynucleoside triphosphates, and 0,1 µmol/l of each primer in a total volume of 25 µl. Cycle conditions were as follows: initial denaturation for 12 min at 95°C, 40 cycles of 20 s denaturation at 95°C, 40 s of annealing at

the temperature specified in supplementary table 1, 90 s of primer extension at 72°C, and final extension for 2 min at 72°C.

Supplementary Table 2: Sensor and anchor probes for melting curve analysis

Variant	Anchor	Sensor
-693	LC640-GCAGGCTTTTAAACATTCTTGGTCTC-ph	GCAGGGATAGGGAGACCAC-FL
-1413	LC705-CCCTGCAGGATCAGCCTCCTC-ph	AGCTTCAGCAGCTAGGAGG-FL
-2144	GGTCTCCTATTTTCATGGTGGAAGGATACT-FL	LC640-AGGTCCTAGAAGAAATTCCAAGATAAT-ph
-2419	TTTTATGTCAAAGTCCTGTTTCAATGATCC-FL	LC640-TCCCATGCCTCACCTTCTCA-ph
20065	GTAATTTGACCAACAGGGAGACAATTCC-FL	LC640-TCCCCCAAGACATACCTAC-ph
22126	LC640-CCCCTTCTCCTGGGATCACCTCC-ph	TCACTCTCCCTGCCCCCTC-FL

Melting curve analysis to detect the SNPs was performed by using two pairs of fluorescence resonance energy transfer (FRET) probes (supplementary table 2) and the LightTyper (Roche Diagnostics). The donor probes comprise 5,6-carboxyfluorescein attached to 3'-o-ribose, and the acceptor probes were labeled with LightCycler Red 705 or 640 attached to the 5'-terminus.

