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# Genetic Variation at the Th2 Immune Gene *IL13* is Associated with IgE-mediated Paediatric Food Allergy

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†On behalf of the Barwon Infant Study, the Melbourne Atopy Cohort study, the Peanut Allergen Threshold Study and the Probiotics and Peanut Oral ImmunoTherapy study.

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**Abbreviations**

**SNP**: Single nucleotide polymorphism
**OFC**: Oral food challenge
**SPT**: Skin prick test
***IL13***: *Interleukin 13*
**AIMs**: Ancestry informative markers
**IgE**: Immunoglobulin E

***Word count: 2,830***

**Background**: Food allergies pose a considerable worldwide public health burden with incidence as high as one in ten in 12-month old infants. Few food allergy genetic risk variants have yet been identified. The Th2 immune gene *IL13* is a highly plausible genetic candidate as it is central to the initiation of IgE-class switching in B cells.

**Objective**: Here we sought to investigate whether genetic polymorphisms at *IL13* are associated with the development of challenge-proven IgE-mediated food allergy.

**Method**: We genotyped nine *IL13* 'tag' single nucleotide polymorphisms (tag-SNPs) in 367 challenge-proven food allergic cases, 199 food sensitised-tolerant cases and 156 non-food allergic controls from the HealthNuts study. 12-month old infants were phenotyped using open oral food challenges. SNPs were tested using Cochran-Mantel-Haenszel test adjusted for ancestry strata. A replication study was conducted in an independent, co-located sample of four paediatric cohorts consisting of 203 food allergic cases and 330 non-food allergic controls. Replication sample phenotypes were defined by clinical history of reactivity, 95% PPV or challenge and *IL13* genotyping was performed.

**Results:** *IL13* rs1295686 was associated with challenge-proven food allergy in the discovery sample (P=0.003; OR=1.75; CI=1.20-2.53). This association was also detected in the replication sample (P=0.03, OR=1.37, CI=1.03-1.82) and further supported by a meta-analysis (P=0.0006, OR=1.50). However, we cannot rule out an association with food

sensitisation. Carriage of the rs1295686 variant A allele was also associated with elevated total plasma IgE.

**Conclusions:** We show for the first time, in two independent cohorts, that *IL13* polymorphism rs1295686 (in complete linkage disequilibrium with functional variant rs20541) is associated with challenge-proven food allergy.

**Key words**: food allergy, food sensitisation, *IL13*, interleukin-13, IgE, single nucleotide polymorphism

## Introduction

Interleukin-13 has a well-described role in mediating Immunoglobulin E (IgE) class switching, and is necessary for the development of T-helper type 2 (Th2) mediated allergic immune responses (1). IL-13 deficient mice exhibit impaired Th2 development and reduced Th2 cytokine production and IgE levels (1). In humans, polymorphisms at *IL13* have been studied in the context of asthma, eczema and atopy (2-10). *IL13* is one of the most replicated asthma and atopy genetic associations. Variants at *IL13* have been linked to elevated cord blood IgE (cbIgE) (11, 12) and elevated IgE at 12-months, 2, 4 and 8 years of age (13, 14). Of the 21 SNPs associated with cbIgE in the Yang *et al.* study an *IL13* polymorphism was one of the two SNPs with highest predictive accuracy for cbIgE (12). One well-studied variant, rs20541 (*IL13*+2044GA), produces an IL-13 gain of function amino acid substitution (Arg130Gln) variant with notably higher induction of downstream processes including CD23 monocyte expression and IgE class switching in B cells (15, 16). Another variant rs1800925 enhances IL-13 production in Th2 polarised cells and has been associated with asthma, eczema and sensitisation to foods (2, 17, 18). Liu *et al.*, identified epistatic effects of genetic variants of *IL13* and *IL4RA* on atopy and specific IgE production. These findings demonstrated that carriage of the TT genotype at rs1800925 of *IL13* was associated with sensitisation to foods, dependent on variants of *IL4RA* (18). Overall there is compelling evidence that *IL13* polymorphisms are associated with allergic disease. There is however a paucity of data regarding polymorphisms at *IL13* and the development of challenge-proven food allergy. We sought to comprehensively investigate a potential association between *IL13* genetic polymorphisms and the development of challenge-proven IgE-mediated food allergy in childhood from an Australian community-based population.

## Methodology

**Discovery Phase Cohort**

This study included 722 Australian infants from the HealthNuts study, methodology of which is published elsewhere (19). Briefly, recruitment for HealthNuts took place between 2007 and 2011 for 5,276 12 month-old infants presenting for scheduled immunisations at council run clinics (19). All infants underwent a skin prick test (SPT) to peanut, egg white, sesame, and either cow's milk or shrimp (or both) for food sensitisation status during attendance at immunisation clinics. Those who had a wheal size of ≥ 1mm were invited to the Royal Children's Hospital for an open oral food challenge (OFC) using pre-determined objective diagnostic criteria (20). A random selection of 200 individuals with a negative SPT (no detectable wheal) to the food allergens underwent food challenges to confirm their status as negative controls. No individuals who originally had negative SPTs had positive OFCs. The main phenotypes of interest were any *food allergy* defined as having a positive SPT result (wheal ≥2mm) to peanut, egg white or sesame and clinical reactivity by OFC. Those individuals with a positive SPT (wheal ≥2mm) to peanut, egg white, cow's milk (or shrimp) or sesame but were asymptomatic by OFC were deemed *food sensitised tolerant*. *Non-food allergic* controls were those without a detectable SPT wheal to any foods at recruitment and had a subsequent negative OFC result in the clinic. Eczema was defined by parent reported doctor diagnosis or nurse observation on the day of OFC. Parents of study participants completed a questionnaire that included questions to parental country of birth, which were used to define ancestry strata. Individuals were classified as Caucasian if both parents were born in Australia, Europe, UK, Northern America or New Zealand (n=503). Individuals were classified as Asian if both parents were born in South East Asia (n=74). Those with one parent in each category were classified as mixed Asian-Caucasian (n=145). We validated these ancestry strata using a random sample of 344 individuals for which ancestry was also determined by genome-wide SNP typing and identity-by-descent cluster analysis, which revealed excellent (>90%) agreement between the parent-reported and genetically determined categories (**Supplemental methods**). Ten mL of blood was collected from 836 infants who attended clinics for IgE titers and genetic studies. Ethical approval was obtained from the Office for Children HREC (Human Research Ethics Committee) (CDF/07/492), the Department of Human Services HREC (10/07) and the Royal Children's Hospital (RCH) HREC (27047).

**Plasma IgE measures**

Total IgE was quantified by ImmunoCAP System FEIA (Phadia AB, Uppsala, Sweden) using plasma aliquots derived from blood samples.

**Tag-SNP selection**

To comprehensively measure polymorphisms at *IL13*, nine tag SNPs within a region of 91.5kb incorporating *IL13* and sequence ~5kb upstream and ~11kb downstream of the gene were incorporated into a multiplex genotyping assay (**Supplementary figure 1**). Tag SNPs were selected using HapMap data (HapMap Genome Browser Phase 1, 2 & 3 - data source: HapMap Data Rel 27 (Feb 09)) and the tagger function in Haploview to broadly cover 22 variants with linkage disequilibrium (LD) of $r^2 \geq 0.8$ (21). Primers for the multiplex assay were designed using Agena Bioscience MassArray Design 3.1 software (sequences available from the authors).

**Genotyping and quality control**

Genomic DNA derived from peripheral blood samples (n=836) were genotyped using Agena Bioscience iPLEX Gold chemistry and the MassARRAY mass spectrometer system according to manufacturer's instructions. Quality control of genotyping data was conducted in PLINK (22). Samples with a genotyping success rate of less than 95% were excluded (n=59), resulting in a final post-QC sample size of 722 in the HealthNuts discovery cohort of 12-month old infants; 367 food allergy cases, 199 food sensitised but tolerant cases and 156 non-food allergic controls (**Supplementary Table 1**). All nine *IL13* tag SNPs passed QC with a genotype call rate of $\geq$ 95% , there was no evidence of significant deviation from the Hardy Weinberg Equilibrium (HWE) ($p<0.01$).

**Discovery phase statistical analysis**

We compared allele frequencies between cases and controls using the Cochran-Mantel-Haenszel (CMH) test in the PLINK software (22), which controls for heterogeneity arising from population stratification using an "average" odds ratio. The total plasma IgE data were positively skewed and thus log transformed prior to analysis. The linear regression test in PLINK was used to test for an association between *IL13* variants and log transformed total plasma IgE, adjusted for food allergy as a covariate.

**Replication phase**

Candidate gene associations were replicated in an independent paediatric sample from multiple Melbourne based studies with comparable food allergy measures; 132 non-allergic controls and 36 food allergic cases from the Barwon Infant Study (BIS) (23), 198 non-allergic controls and 57 food allergic cases from the Melbourne Atopic Cohort (MACs) (24), 72 food allergic cases from the Peanut Allergen Threshold Study (PATS) (25) and 38 food allergic cases from the Probiotic and Peanut Oral Immuno-Therapy study (PPOIT) (26). Demographics and clinical characteristics are presented in **Supplementary Table 2**). Ethical approval was provided by Office for Children HREC for PATS (HRECApp32166A and 2012P002475). The RCH Human Research and Ethics Committee HREC 27086Q (PPOIT). Mercy Maternity Hospital Ethics Committee (R88/06) for MACS with 18-year follow-up, including collection of DNA, was approved by the Royal Children's Hospital (HREC 28035); BIS: Barwon Health Human Research Ethics Committee HREC (10/24). The definition of phenotypes for the majority of the sample was similar to the discovery study, OFC (BIS, PPOIT, PATs) or clear history of reactivity within 1-2 hours in addition to SPT (>2mm) or sIgE (>0.35 kUA/L) sensitisation (PPOIT, PATs). The widely accepted 95% PPV (SPT > 95% PPV) (27) for diagnosing clinical allergy was utilised for MACs samples. Non-food allergic cases were defined with negative SPT to a panel of foods in the MACs study and with OFC in addition to negative SPT wheal in the Barwon Infant Study. Eczema was defined by history of doctor-diagnosis or nurse-observation at clinic. Individuals with an unclear phenotype below the 95% PPV (n=60) were removed from analysis. Assay design and genotyping were carried out as for the discovery phase. Ancestry strata were genetically determined (described below) using a panel of 49 ancestry informative markers (AIMs) derived from a panel described in Bousman *et al.*, 2013 (28). Ancestral population clusters were determined in PLINK using Identity-by-state (IBS) distance clustering resulting in 463 individuals of European descent, 51 of mixed European-Asian descent and 15 of Asian descent (post-QC). Consistent with the discovery phase, individuals with a genotyping success rate of less than 95% were excluded (n=71). All SNPs pass the 95% genotyping call rate and none significantly deviated from HWE (P<0.01). After QC there were 533 phenotyped and genotyped individuals (203 food allergic cases and 330 non-food allergic controls).

The replication cohort genotyping data were analysed in PLINK using logistic regression models adjusted for population stratification using the first and second principal components (PCs). Meta-analysis of discovery and replication samples was conducted in

PLINK with random and fixed effects models to assess any heterogeneity of effect sizes between studies (570 food allergic cases and 486 non-food allergic controls).

**Results**

Following genotyping and quality control, 367 out of 722 (50.8%) participating infants with blood samples in the discovery phase of the study had clinical food allergy. Egg allergy was the most common type of food allergy (89.3%), followed by peanut allergy (36.2%) whilst allergy to sesame was less common (6.7%). Food sensitised or food allergic infants were more likely to be male with one or more Asian parents. Eczema rates were higher in the food allergy group (56.4%) (**Supplementary Table 1**).

***IL13 variant rs1295686 is associated with challenge-proven food allergy***

Allele and genotype frequencies between discovery phase food allergy cases (n=367) and non-allergic controls (n=156) were tested using the CMH clustering test adjusted for ancestry strata. *IL13* variant rs1295686, which is in complete LD ($r^2$ =1) with a previously described functional variant rs20541 (Gln144Arg), was associated (nominal P<0.05) with challenge proven food allergy (P=0.003; OR=1.75; CI=1.20-2.53, **Table 1. A.**). A sensitivity analysis of infants without eczema (174 food allergic cases and 90 non-food allergic controls) reproduced this association suggesting it was independent of eczema co-morbidities (P=0.008; OR=1.82; CI=1.17-2.83, **Supplementary Table 3**). We also adjusted for atopic morbidities using logistic regression, which did not materially alter the pattern of association (**Supplementary Table 4**). There was weak evidence of an association between rs1295686 (P=0.06; OR=1.48; CI=0.98-2.23) and rs1295687 (P=0.05; OR=2.19; CI=1.01-4.76) with the food sensitised tolerant phenotype (n=199) (**Table 1. B.**). There was no evidence that any of the variants tested increased the risk for food allergy when comparing food allergic cases (n=367) to food sensitised tolerant children (n=199) (**Table 1.C**)

**SNPs in *IL13* are associated with variations in total plasma IgE**

Total plasma IgE measures were available for 423 individuals from the discovery study (69 non-food allergic, 106 food sensitised but tolerant and 248 food allergic). We tested log IgE titers as the dependent variable in a regression analysis using genotyped allele frequencies as predictors. The strongest evidence for an association with IgE titres was with the minor allele of rs1295686, which was associated with elevated log transformed total plasma IgE levels (P=0.0003, SE=0.10, Beta=0.38). There was also evidence that three additional variants

(rs3091307, rs1800925 and rs1295683) were associated with elevated IgE levels (**Supplementary table 5 & Supplementary figure 2**).

**Replication confirms *IL13* variant rs1295686 is associated with food allergy**

To support our findings from the discovery phase, we genotyped rs1295686 and rs1295687 in an additional 533 independently selected children. In the replication sample, egg allergy was again the most common food allergy (72.5%), followed by peanut (62.1%). Eczema was more common among food allergic children (41.9%) and there was a higher proportion of Europeans (78.5%) in the control group.

In a logistic regression analysis adjusted for ancestry, we replicated the association between food allergy and rs1295686 in the independent sample group (P=0.03, OR=1.37, CI=1.03-1.82) (**Table 2. A.**). A sensitivity analysis of infants without eczema (96 food allergic cases and 201 non-food allergic controls) again did not suggest eczema was a significant confounder. The rs12954687 SNP did not show evidence of an association with phenotype in this population (**Table 2.A**). To maximize the study power, we performed a meta-analysis of both discovery and replication samples. Genotyped SNP rs1295686 remained the top associated variant (P=0.0006, OR=1.50, Q=0.31, I=3.32) while rs1295687 was not associated (**Table 2. B.**). In this analysis the p-value for Cochrane's Q statistic (Q) and $I^2$ heterogeneity index (I) (0-100) was indicative of low heterogeneity of effect sizes between study populations.

**Discussion**

Genetic variants at *IL13* have been reported to increase the risk of a range of atopic conditions including asthma (3, 6, 7, 9) and eczema (5, 8). To our knowledge this is the first demonstration of an association of *IL13* variants with clinical food allergy in an ethnically diverse population. Our data provide evidence that the minor allele of variant rs1295686 ($r^2$=1 with functional variant rs20541, see **Supplementary figure 1**) predisposes to an increased risk of food sensitisation and food allergy, possibly through enhanced IgE production. These conclusions are based on the following lines of evidence: Association testing of allele frequencies between food allergy cases and controls in two independent samples identified rs1295686 as the strongest association with food allergy phenotype, independently of eczema, exhibiting a consistent direction of effect between discovery and replication analyses. Despite there being noted differences in some clinical characteristics between the discovery and replication populations (eczema rates, ethnicity strata, age of

diagnosis), and criteria used for phenotyping (OFC, 95%PPV) a meta-analysis across both cohorts suggested limited heterogeneity of observed effect sizes between studies, indicative of a robust association. We also found evidence that rs1295686 was associated with elevated total plasma IgE levels supporting a functional role in disease etiology.

The rs1295686 variant is a T -> C polymorphism located in the intronic region of *IL13* that has previously been associated with asthma in a consortium-based meta-analysis (6), with dysregulated total plasma IgE in a US population (4), and with elevated cord blood plasma IgE levels (11, 14). This particular functional variant is in complete linkage disequilibrium with rs20541 (**Supplementary figure 1**), an A -> G missense mutation that has previously been linked to allergic rhinitis in a meta-analysis of eight studies (10), asthma in a meta-analysis of 34 studies (7) and elevated IgE levels (4). In our study we found evidence that rs1295686 was associated with elevated plasma IgE levels in addition to food sensitisation and food allergy, however we are unable to distinguish which variant is driving these associations, and future fine mapping studies would be needed to resolve this.

A strength of this study was the robust phenotyping measures in the discovery cohort determined by open food challenge with predetermined, objective diagnostic criteria, standard for infants as per PRACTALL food challenge guidelines (29). These measures provide the study with the rare opportunity to distinguish between the mechanisms of allergic sensitisation and clinical food allergy. We found only weak evidence ($p = 0.06$) that rs1295686 was associated with food sensitisation although the direction and size of the effect was consistent with that observed for the association between rs1295686 and clinical food allergy. These observations suggest that the lack of association was most likely a function of reduced sample size. There was no evidence of an effect when clinically allergic cases were compared to sensitised tolerant controls suggesting that variant rs1295686 increases the risk of food allergy via sensitisation to dietary antigen.

We carefully addressed the issue of population structure in the discovery phase by CMH analyses with clusters defined by parental country of birth and validated our categories using genome-wide SNP data. In the replication sample population structure was adjusted for with principal components. One limitation of the study was the absence of challenge-proven food allergy phenotyping measures in some of the studies utilised for replication, instead relying upon 95% PPV definitions. This may have led to inclusion of some food sensitised but tolerant individuals in the groups and/or the exclusion of some true food allergy cases. We addressed this by comparing effect sizes across the different study populations within the

replication sample, which did not suggest phenotype definition or age at diagnosis between studies as a significant source of heterogeneity.

Our study establishes association between food allergy and *IL13* genetic variation in two Australian food allergy samples. Further independent studies to confirm and extend these findings are now warranted. Understanding the genetic factors and molecular pathways that contribute to perturbation of Th2 immune responses will assist in the development of novel therapeutic approaches to immune-mediated diseases.

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**Table 1:** Cochran-Mantel-Haenszel 2x2xK associations between IL13 SNPs and food allergy phenotypes in the discovery sample, with strata defined by reported parental country of birth A) 367 food allergic cases and 156 non-allergic controls only B) 199 food sensitised, tolerant and 156 non-allergic controls C) 367 food allergic cases and 199 food sensitised-tolerant cases

| | | A. Food allergic cases vs non-food allergic controls | | | | B. Food sensitised-tolerant vs non-food allergic controls | | | | C. Food allergic cases vs sensitised-tolerant cases | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SNP | A1 | P | OR | L95 | U95 | P | OR | L95 | U95 | P | OR | L95 | U95 |
| rs1295686 | A | **0.003** | 1.75 | 1.20 | 2.53 | 0.06 | 1.48 | 0.98 | 2.23 | 0.23 | 1.18 | 0.90 | 1.55 |
| rs2243297 | A | 0.13 | 2.10 | 0.80 | 5.51 | 0.23 | 1.91 | 0.67 | 5.42 | 0.72 | 1.12 | 0.61 | 2.04 |
| rs1295687 | G | 0.19 | 1.63 | 0.78 | 3.41 | 0.05 | 2.19 | 1.01 | 4.76 | 0.25 | 0.77 | 0.49 | 1.21 |
| rs2243211 | A | 0.22 | 1.45 | 0.80 | 2.64 | 0.87 | 0.94 | 0.48 | 1.87 | 0.08 | 1.52 | 0.95 | 2.44 |
| rs1295683 | T | 0.25 | 1.32 | 0.82 | 2.13 | 0.32 | 1.30 | 0.78 | 2.19 | 0.88 | 1.03 | 0.72 | 1.46 |
| rs2243248 | G | 0.51 | 1.21 | 0.69 | 2.11 | 0.37 | 0.74 | 0.38 | 1.42 | 0.03 | 1.68 | 1.05 | 2.69 |
| rs2243300 | T | 0.51 | 1.19 | 0.70 | 2.02 | 0.43 | 0.78 | 0.42 | 1.45 | 0.11 | 1.43 | 0.92 | 2.23 |
| rs1800925 | T | 0.60 | 1.11 | 0.75 | 1.63 | 0.84 | 1.05 | 0.68 | 1.60 | 0.74 | 1.05 | 0.78 | 1.43 |
| rs3091307 | G | 0.62 | 1.10 | 0.75 | 1.60 | 0.67 | 1.10 | 0.72 | 1.66 | 0.90 | 1.02 | 0.76 | 1.37 |

*P is the p-value determined by CMH testing with clusters defined by parental country of birth, A1 is the effect allele, OR is the odds ratio and L95 and U95 the lower and upper 95% confidence intervals.

**Table 2 A.** Logistic regression analysis of SNP-food allergy associations in the replication sample, adjusted for ancestry by principal components. 203 food allergic cases vs 330 non-food allergic controls. **B.** Meta-analysis of discovery and replication samples modelled with random and fixed effects. 570 food allergic cases vs 486 non-food allergic controls

| | A. Replication analysis: food allergic cases vs non-food allergic controls | | | | B. Meta-analysis – Discovery and Replication | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SNP | A1 | OR | L95 | U95 | P | P | P(R) | OR | OR(R) | Q | I |
| rs1295686 | A | 1.37 | 1.03 | 1.82 | 0.03 | 0.0005 | 0.0006 | 1.50 | 1.50 | 0.31 | 3.32 |
| rs1295687 | C | 1.10 | 0.68 | 1.78 | 0.70 | 0.30 | 0.30 | 1.24 | 1.24 | 0.38 | 0.00 |

*__A)__ A1 is the reference allele; OR is the odds ratio; L95 and U95 are the lower and upper confidence intervals; P is the p-value obtained from logistic regression __B)__ P is the P-value calculated by fixed-effects meta-analysis model; P(R) is the P-value determined from random-effects meta-analysis model. OR is the odds ratio determined from fixed-effects; OR(R) is the odds ratio determined from random-effects. The Q value is Cochrane's P-value, a measure of effect size heterogeneity between studies. I is the I-index (0-100), another measure of effect size heterogeneity between studies.