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ORIGINAL ARTICLE **Asthma and Rhinitis**

ST13 polymorphisms and their effect on exacerbations in steroid-treated asthmatic children and young adults

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Summary

Background The clinical response to inhaled corticosteroids (ICS) is associated with single nucleotide polymorphisms (SNPs) in various genes. This study aimed to relate variations in genes in the steroid pathway and asthma susceptibility genes to exacerbations in children and young adults treated with ICS.

Methods We performed a meta-analysis of three cohort studies: Pharmacogenetics of Asthma Medication in Children: Medication with Anti-Inflammatory effects (n = 357, age: 4–12 years, the Netherlands), BREATHE (n = 820, age: 3–22 years, UK) and Paediatric Asthma Gene Environment Study (n = 391, age: 2–16 years, UK). Seventeen genes were selected based on a role in the glucocorticoid signalling pathway or a reported association with asthma. Two outcome parameters were used to reflect exacerbations: hospital visits and oral corticosteroid (OCS) use in the previous year. The most significant associations were tested in three independent validation cohorts; the Childhood Asthma Management Programme (clinical trial, n = 172, age: 5–12 years, USA), the Genes- environment and Mixture in Latino Americans II- study (n = 745, age: 8–21, USA) and the Pharmacogenetics of adrenal suppression cohort (n = 391, age: 5–18, UK) to test the robustness of the findings. Finally, all results were meta-analysed.

Results Two SNPs in *ST13* (rs138335 and rs138337), but not in the other genes, were associated at a nominal level with an increased risk of exacerbations in asthmatics using ICS in the three cohorts studied. In a meta-analysis of all six studies, *ST13* rs138335 remained associated with an increased risk of asthma-related hospital visits and OCS use in the previous year; OR = 1.22 (P = 0.013) and OR = 1.22 (P = 0.0017), respectively.

Conclusion and clinical relevance A novel susceptibility gene, *ST13*, coding for a cochaperone of the glucocorticoid receptor, is associated with exacerbations in asthmatic children and young adults despite their ICS use. Genetic variation in the glucocorticoid signalling pathway may contribute to the interindividual variability in clinical response to ICS treatment in children and young adults.

Keywords childhood asthma, corticosteroids, exacerbations, pharmacogenomics, *ST13*
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Introduction

Inhaled corticosteroids (ICS) are considered first line therapy for reducing airway inflammation, improving lung function, and controlling asthma stability in patients with persistent asthma [1, 2]. While most asthmatic patients have a beneficial response to inhaled corticosteroid therapy, approximately 10% of the patients suffer from severe symptoms despite regular use of corticosteroids [3], and almost half of the costs of asthma management arise from unscheduled health care visits due to exacerbations [4]. Heterogeneity in treatment response may partly be due to genetic variation [5]. An example of genetic variation in the *FCER2* gene contributing to exacerbations despite ICS treatment has been published previously [6, 7].

Corticosteroids are thought to exert their anti-inflammatory effects primarily by binding to a ubiquitously expressed glucocorticoid receptor (GR) in the cytoplasm [8]. In the absence of glucocorticoids, the receptor is predominantly sequestered in the cytoplasm in a multiprotein chaperone complex. Various chaperones and cochaperones have been described to be involved in the stabilization and maturation of the receptor [9]. Upon binding of glucocorticoids to receptor, the complex translocates to the nucleus where it can block gene expression of a wide range of pro-inflammatory genes and promote the expression of anti-inflammatory genes. To date, there have been few studies addressing variations in corticosteroid receptor complex genes and steroid treatment response in patients with asthma [10, 11].

We hypothesized that susceptibility genes might also be associated with an increased risk of exacerbations despite steroid treatment, due to a potential link with exacerbation-prone asthma phenotypes. In this study, we aim to relate genetic variations in genes in the steroid pathway and asthma susceptibility genes to asthma exacerbations despite ICS treatment.

Methods

Study population

Tag single nucleotide polymorphisms (SNPs) in 17 candidate genes were studied in three independent north-European cohorts of steroid-treated asthmatic children and adolescents: (1) the Pharmacogenetics of Asthma Medication in Children: Medication with Anti-inflammatory effects (PACMAN) cohort study, (2) the BREATHE study, and (3) the Paediatric Asthma Gene Environment Study (PAGES). For the current analyses, we excluded participants of non-northern European origin.

PACMAN. The PACMAN study is an observational cohort study of children (age: 4–12 years) with a reported (regular) use of asthma medication through community pharmacies in the Netherlands. Details of the study protocol have been described elsewhere [12]. We analysed the PACMAN data obtained between 2009 and 2012. Data were collected with the help of pharmacists belonging to the Utrecht Pharmacy Practice Network for Education and Research (UPPER), and the work was conducted in compliance with the requirements of the IRB of the Department of Pharmacoepidemiology and Clinical Pharmacology, Utrecht University. A detailed history of the subjects is obtained, including information on asthma symptoms, exacerbations and medication use over the preceding 12 months during a study visit in the community pharmacies. Saliva samples are collected for DNA extraction (Oragene DNA Self Collection kit, DNA Genotek, Inc., ON, Canada). The Medical Ethics Committee of the University Medical Centre Utrecht has approved the PACMAN study.

BREATHE. The BREATHE study includes children and young adults (age: 3–22 years) with physician-diagnosed asthma through primary or secondary clinics in either Tayside or Dumfries (Scotland, United Kingdom) [13, 14]. We analysed the BREATHE data obtained between 2004 and 2006. At the asthma clinic, a detailed history was obtained, including information on symptoms, treatment and asthma exacerbations over the preceding 6 months. Mouthwash samples were collected and DNA was isolated using Qiagen DNAeasy 96 kits (Qiagen GmbH, Hilden, Germany. The Tayside Committee on Medical Research Ethics has approved the BREATHE study.

PAGES. The PAGES study recruited children and adolescents (age: 2–16 years) with physician-diagnosed asthma through 15 secondary care asthma clinics across Scotland from 2008 to 2011. Details of the study protocol of the PAGES have been described elsewhere [15]. Briefly, a detailed history was obtained including information on symptoms, treatment and exacerbations over the preceding 6 months. Saliva samples were collected for DNA extraction (Oragene DNA Self Collection kit, DNA Genotek, Inc., Ontaria, Canada). The Plymouth and Cornwall Research Ethics Committee has approved the PAGES study.

Validation cohorts. To test the robustness of our findings, we assessed the identified significant associations of the first meta-analysis in three additional independent populations: Childhood Asthma Management Programme (CAMP), the Pharmacogenetics of adrenal suppression (PASS) cohort and Genes-environments & Admixture in Latino Americans (GALA II) and the meta-analysed results.

CAMP trial. We studied 172 non-Hispanic white corticosteroid-treated children with asthma included in

CAMP (USA). CAMP is a multicentre trial that randomized 1041 children with mild-to-moderate asthma aged 5–12 years to the ICS budesonide, nedocromil or placebo twice daily. The participants were followed for a mean of 4.3 years and follow-up visits took place at 2 and 4 months after randomization and every 4 months thereafter. The design of the study has been described previously [16]. We restricted our analysis to the non-Hispanic white subjects randomized to budesonide with available genotyping data (n = 172).

Pharmacogenetics of adrenal suppression (PASS) cohort. PASS is a multicentre study of children with asthma (age 5–18 years), treated with corticosteroids, who required assessment of adrenal function with a low-dose short synacthen test (LDSST). Participants were recruited from November 2008 to September 2011 from 25 sites in the United Kingdom. Eligibility criteria were as follows: treatment with ICS > 6 months; diagnosis of asthma; under care of a paediatrician experienced in the treatment of asthma; and clinical concern about adrenal suppression sufficient to warrant a LDSST. Study participants were recruited either prospectively (if LDSST not yet undertaken) or retrospectively (if LDSST already undertaken). PASS received full ethical approval from Liverpool Paediatric Research Ethics Committee.

Genes-Environment and Admixture in Latino Americans (GALA II) study. The GALA II study is an ongoing multicentre study of Latino children and young adults with and without asthma, as described elsewhere [17]. Subjects were eligible if they were 8–21 years of age, self-identified all four grandparents as Latino, and had < 10 pack years of smoking history. Asthma was defined based on physician diagnosis and report of symptoms and medication use within the last 2 years. For this study, we only analysed asthmatic children with a reported use of short-acting beta-agonist (SABA) and ICS in the past 12 months. Patients included in this study were recruited from urban study centres across the mainland United States and Puerto Rico from 2008 to 2011. All patients completed a questionnaire with questions regarding their medical, asthma, medication use, allergic, social, environmental, and demographic histories. In addition, all participants provided blood for genetic analysis. Local institutional review boards approved the studies, and all subjects and legal guardians provided written informed assent/consent.

Definition of ICS use

Pharmacological management of asthma was categorized based on the British Thoracic Society (BTS) guidelines [2]: step 0: no use of inhaled albuterol on demand in the past month, step 1: inhaled SABA as needed, step 2: step 1 plus regular ICS, step 3: step 2 plus regular long-acting inhaled beta-2 agonists (LABA), and step 4: step 3 plus oral leukotriene receptor antagonists. For this study, we selected children and young adults on BTS treatment steps 2, 3, and 4.

Single nucleotide polymorphisms selection and genotyping

Ten genes were selected based on their involvement in the glucocorticoid (GC) receptor complex (*NR3C1*, *HSPCA*, *HSPA4*, *FKBP4*, and *ST13*), GC transport (*SERPINA6*) or GC-mediated signalling (*CREBBP*, *TBP*, *NCOA3*, and *SMAD3*). In addition, seven genes were selected based on a previously reported association with asthma susceptibility, severity or asthma medication response (*ARG1*, 17q21 locus, *IL2RB IL18R1*, *PDE4D*, *HLA-DQ*, and *BCL2*) [18–20]. We selected 50 tag SNPs. SNPs were included if the MAF > 0.2. Tag SNPs were selected using Tagger (http://www.broadinstitute.org/mpg/tagger/server.html) with a gene coverage threshold of 90%. Previously described SNPs in the genes of interest were also selected. Genotyping was performed using the Sequenom Mass Array platform (Sequenom, San Diego, California, USA). Genotype calls of all DNA samples and SNPs were examined for quality. Samples that consistently failed genotyping (≥ 20% of the SNPs) were excluded for further analyses. Subsequently, SNPs with a call rate < 95% were excluded, as well as SNPs not in Hardy–Weinberg equilibrium. A total of 38 SNPs (78%) in twelve genes passed this quality control (see Table S1 for selected genes and SNPs). The following genes did not pass quality control and were excluded from further analyses: *HSPCA*, *HSPA4, IL18R1*, *HLA-DQ* and *BCL-2*. Illumina Infinium II 550 K SNP Chips and 610 Quad Chip (Illumina, Inc., San Diego, California) were used for genotyping in the CAMP study. SNPs of interest for replication were imputed based on 1000 Genomes. GALA II subjects were genotyped using the Axiom® LAT1 array (World Array 4, Affymetrix, Santa Clara, CA, USA) as described elsewhere [21]. Imputed data were obtained using the genotyped SNPs, first phasing the data using SHAPE-IT [22] followed by imputation using IMPUTE2 [23] considering all populations from the 1000 Genomes Project Phase I v3 as a reference [24]. The 2 SNPs selected SNPs for the current analyses were accurately imputed (info score of 0.96 and 0.99 for rs138335 and rs138337, respectively). In the PASS cohort, DNA samples of the participants were shipped to ARK Genomics (The Roslin Institute, University of Edinburgh) for genomewide genotyping on the Illumina Human Omni-ExpressExome-8 v1.0 chip (951 117 SNPs). After sample and SNP quality control measures, genotype

data were phased using the software SHAPEIT v2.r644. Imputation of SNPs was then performed using IMPUTE v2.3.0. Statistical analyses were undertaken using PLINK v1.07 and/or SNPTEST v2.4.

Definition of outcome

As indicators for asthma exacerbations, we studied (1) asthma-related hospital visits and (2) course(s) of oral corticosteroid (OCS) use reported by parent or child. The following outcome definitions as a measure for severe exacerbations were used:

(1) asthma-related hospital visits reported by the parent of a child:
   - BREATHE, PAGES, and PASS: asthma-related hospitalization in the past 6 months
   - PACMAN and GALA II: asthma-related ED visits in the past 12 months
   - CAMP: asthma-related ED visits and hospitalizations in the first 12 months of the trial.

(2) burst(s) of OCS reported by the parent or child:
   - BREATHE, PAGES, and PASS: in the past 6 months
   - PACMAN and GALA II: in the past 12 months
   - CAMP: in the first 12 months of the trial

Statistical analysis

Logistic regression analysis was used to study the association between the SNPs and risk of exacerbations (OCS use or asthma-related hospital visits). Odds ratios (OR), 95% confidence intervals (CI) and *P*-values were calculated per study. The model was adjusted for age, gender and BTS treatment step. An additive genetic model was assumed. ORs were meta-analysed assuming random effects with the inverse variance weighing method. I^2 was used to quantify between-study heterogeneity [25]. The Bonferroni-corrected *P*-value was set at *P*: 0.0007 (0.05/76). Statistical analysis was carried out using IBM SPSS 19.0 for Windows (SPSS, Inc., Chicago, IL, USA) and PLINK [26]. Forest plots were made with R and the 'meta' package [27].

Functional annotation of associated SNPs

Functional annotation of associated SNPs was carried out querying the Encyclopaedia of DNA Elements (ENCODE) data with the online software HAPLOREG (http://www.broadinstitute.org/mammals/haploreg/haploreg.php) [28]. Additional search for evidence of associated loci being expression quantitative trait loci (eQTLs) was performed with using the Geuvadis Data Browser (http://www.ebi.ac.uk/Tools/geuvadis-das) [29].

Results

Characteristics of the study populations

Data were available for 820 children and young adults of the BREATHE cohort, 391 children and adolescents of PAGES and 357 children of the PACMAN cohort (Table 1). Most patients were on BTS treatment step 2 (as-needed SABA use combined with regular low-dose ICS). Compared with the other studies, the participants in the PACMAN cohort reported the lowest rates of asthma-related hospital visits (6.2%) and OCS usage (6.2%) in the past year.

Furthermore, data from three additional studies were available for the replication phase; 172 non-Hispanic white children of the CAMP trial, 391 children of the PASS cohort and 745 Latino children and young adults of the GALA II study (Table 2).

Discovery phase: associations with severe exacerbations in BREATHE, PAGES and PACMAN

In a meta-analysis of the three north-European cohorts BREATHE, PAGES and PACMAN, we found two of the 38 SNPs to be associated with an altered risk of severe exacerbations as defined by asthma-related hospital visits.

Table 1. Baseline characteristics study population in the discovery phase

	BREATHE (n = 820)	PAGES (n = 391)	PACMAN (n = 357)
Child characteristics			
Age, mean (range) years	9.8 (2–22)	9.0 (2–16)	8.7 (4–13)*
Male gender, %	61.2	55.8	61.1
Asthma exacerbations in preceding 12 months/6 months			
Asthma-related ED visit/hospital admission†, %	19.0 (156/819)‡	15.5	6.2 (22/356)‡
Oral steroid use†, %	31.6 (259/819)‡	43.2	6.2
BTS treatment step			
2, %	65.9	48.8	71.7
3, %	18.3	42.2	23.0
4, %	15.9	9.0	5.3

BTS, British Thoracic Society; ED, emergency department; PACMAN, Pharmacogenetics of Asthma Medication in Children; PAGES, Paediatric Asthma Gene Environment Study.

*Children within the PACMAN cohort were selected between the age of 4 and 12. However, the child might have been 13 at the moment of the study visit.

†PACMAN cohort: preceding 12 months, BREATHE/PAGES: preceding 6 months.

‡Data not available for all individuals; (number of individuals / number of individuals with data available). For BREATHE, the individual with missing hospital data is different from the individual with missing OCS data.

Table 2. Baseline characteristics study population in the replication phase

	CAMP (n = 172)	PASS (n = 391)	GALA II (n = 745)
Child characteristics			
Age, mean (range) years	8.8 (5–13)*	11.1 (5–18)	12.1 (8–21)
Male gender, %	55.2	55.8	56.8
Asthma exacerbations in preceding 12 months/6 months			
Asthma-related ED visit/hospital admission[#], %	13.4	75.4	42.4 (313/739)
Oral steroid use[#], %	47.1	51.9	41.6 (310/745)
BTS treatment step			
2, %	†	7.7	41.1
3, %	–	33.0	43.6
4, %	–	58.8	15.3

BTS, British Thoracic Society; CAMP, Childhood Asthma Management Programme; ED, emergency department; GALA II, Genes-environments & Admixture in Latino Americans; PASS, Pharmacogenetics of adrenal suppression.

*Prospective trial; children were 5–13 years at the start of the trial.

†CAMP is randomized clinical Trial of mild-to-moderate asthmatics. All children were on 200 μg of budesonide (ICS) plus SABA as needed.

#CAMP: first 12 months of the trial, PASS: preceding 6 months, GALA II: preceding 12 months.

ST13 SNP rs138335 increased the risk of asthma-related hospital visits (OR = 1.35 per G allele; 95% CI: 1.07–1.69, P = 0.01) (Fig. 1). Rs138337 in the same gene had a comparable effect on the risk of asthma-related hospital visits (OR: 1.36 per G allele, 95% CI: 1.11–1.66, P = 0.003) (Fig. 2). In addition, rs138335 was also associated with an increased risk of OCS use (OR: 1.33 per G allele; 95% CI: 1.11–1.60, P = 0.002) (Fig. 3). Tables S2 and S3 show the summary effect estimates of all investigated SNPs.

Replication phase: ST13 in the CAMP, GALA II and PASS cohorts

To assess the robustness of findings, we studied rs138337 and rs138335 in three additional independent study populations; the CAMP study (n = 172 non-Hispanic white asthmatic children), the PASS cohort (n = 391 north-European asthmatic children) and the GALA II study (n = 745 Latino asthmatic children). In a meta-analysis of the three cohorts, none of the *ST13* SNPs was significantly associated with severe exacerbations (Figs 1–3). When investigating the study populations independently, we observed a trend (P = 0.06) in the north-European PASS cohort suggesting that carrying a G allele at rs138335 increased the risk of asthma-related hospital visits in this study population (OR: 1.42, 95% CI: 0.97–2.07; Fig. 1), whereas the other 2 cohorts did not significantly contribute.

Meta-analysis of the six study populations

In the meta-analysis of all six study populations, *ST13* rs138335 remains associated with asthma-related hospital visits (OR per increase in G allele: 1.22, 95% CI: 1.04–1.43, P = 0.013) and OCS usage (OR per increase in G allele: 1.22, 95% CI: 1.08–1.39, P = 0.0017). The effect estimates in the different cohorts largely pointed in the same direction for both outcomes, yet these associations did not pass the Bonferroni-corrected significance threshold of 0.0007.

Functional annotation of associated SNPs

The associated SNPs rs138337 and rs138335 are eQTLs in lymphoblastoid cell lines from Europeans ($P = 5.8 \times 10^{-70}$ and $P = 1.9 \times 10^{-36}$, respectively). In addition, the SNP rs138335 is in strong linkage disequilibrium (LD, r^2 = 0.95) with another SNP (rs138349) that is located in a promoter histone mark, an enhancer histone mark, a DNase I hypersensitive site, and acts as a binding site for an enhancer binding protein and transcription factors. Furthermore, the SNP rs138335 is in high LD (r^2 = 0.86) with the SNP rs2899341, which is located in an enhancer histone mark and in a DNase I hypersensitive site.

Discussion

In a meta-analysis of three independent north-European studies, we identified *ST13* as a novel risk gene for the occurrence of asthma exacerbations despite inhaled corticosteroid treatment in asthmatic children and young adults. For rs138335, the risk of exacerbations was increased with each substitution of the minor allele for the major allele variant. For rs138337, oppositely, the minor allele variant was found to be associated with an increased risk of exacerbations. The two SNPs were in moderate LD (r^2 = 0.47) in our study. None of the other investigated genes could be linked to an increased risk of severe exacerbations.

Single nucleotide polymorphisms rs138335 and rs138337 both lie in the noncoding intronic regions of the *ST13* gene, but our *in silico* functional evaluation revealed a functional role for these two SNPs as eQTLs and also for SNPs in high LD with them. *ST13* encodes a cochaperone protein (Hsp70 interacting protein; hip) of the steroid–receptor complex and is involved in the functional maturation of the corticosteroid receptor, but the mechanism by which it does so remains to be elucidated [30]. *STIP1* (coding for another cochaperone protein in the GR receptor complex, namely Hsp70/Hsp90 organizing protein: hop) has previously been associated with lung function and lung function improvement in 382 asthmatic patients treated with ICS [10]. At the

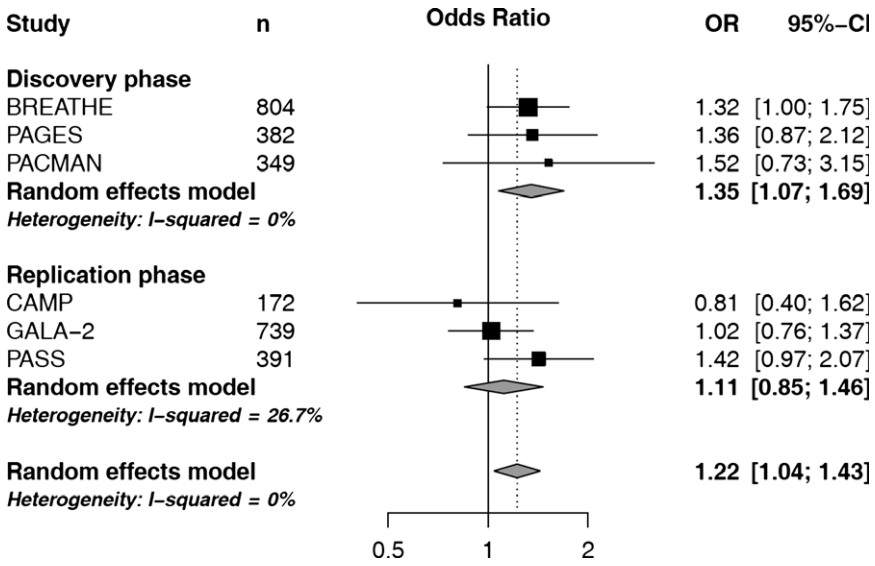

Fig. 1. Forest plot for the association between *ST13* rs138335 and asthma-related hospital visits. Odds ratios (OR) and corresponding 95%CI per increase in G-allele, controlling for age, sex and treatment step.

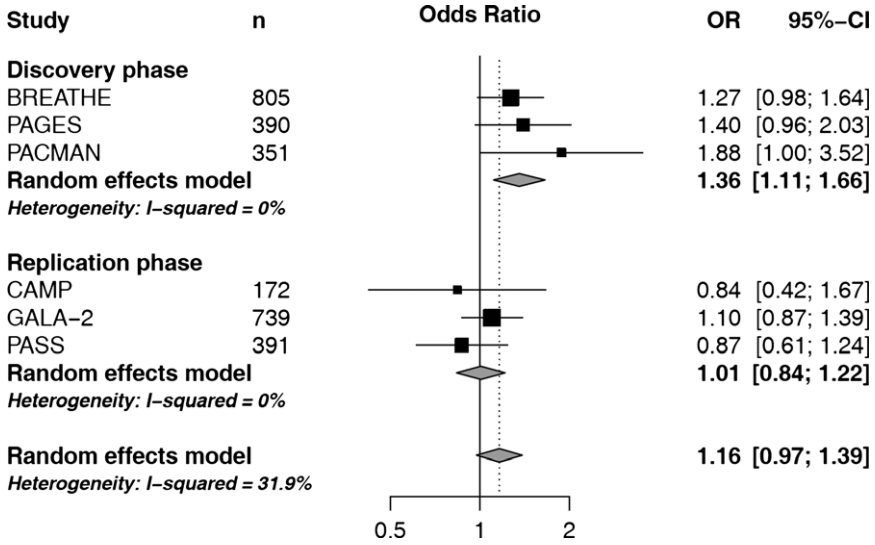

Fig. 2. Forest plot for the association between *ST13* rs138337 and asthma-related hospital visits. Odds ratios (OR) and corresponding 95%CI per increase in G-allele, controlling for age, sex and treatment step.

time of SNP selection, *STIP1* was not included in our study. Hip (encoded by *ST13*) and hop (encoded by *STIP1*) are thought to function in a cooperative manner in GR maturation [30], building evidence that alterations in the expression or folding of these cochaperones may influence the binding of corticosteroids to the receptor or downstream signalling and therefore, ICS responsiveness. Functional studies are necessary to support our hypothesis.

A number of limitations need to be noted regarding this study. Two SNPs in *ST13* were associated with both outcomes of exacerbations in the meta-analysis of all six cohorts, but did not pass the Bonferroni-corrected significance threshold. Therefore, we cannot exclude that our findings are false positives. Even though we were able to analyse a large study population (including 2876 asthmatic children and young adults), a *post hoc* power analysis showed that we were underpowered to identify a significant association with an OR < 1.5 for asthma-related hospital visits and OR < 1.4 for OCS use. This underlines the need for large-scale international collaboration in this field [31].

The populations that we studied varied in age and severity of asthma symptoms, probably due to the design of the studies. The PACMAN population is recruited in community pharmacies, whereby most participants had well-controlled symptoms [32], while patients in PAGES, BREATHE and CAMP were recruited

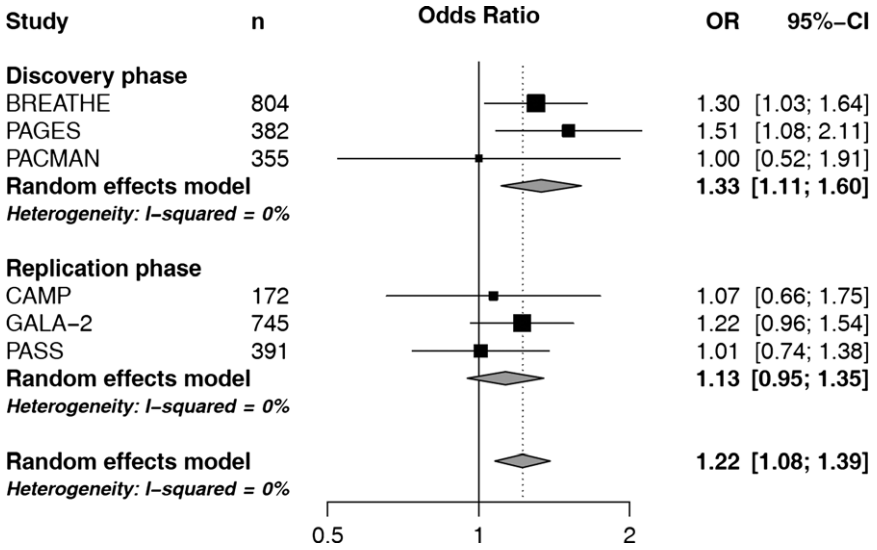

Fig. 3. Forest plot for the association between *ST13* rs138335 and oral corticosteroid (OCS) usage. Odds ratios (OR) and corresponding 95%CI per increase in G-allele, controlling for age, sex and treatment step.

through primary and secondary care. PASS participants were recruited through secondary care based on clinical concern about adrenal suppression, while participants in GALA II were recruited using a combination of community and clinic-based recruitment. In addition, differences in health system and prescription behaviour between the different countries might also play a role [33]. Notwithstanding these differences, statistical heterogeneity (I^2) was limited for *ST13* in the meta-analysis.

Our study was also limited due to the selection of tagging SNPs with a MAF $\geq$ 0.20. Due to the sample size, we could not investigate rare variants, which might have had larger effects. Furthermore, the incorporation of common variants with smaller effects in clinical risk models might be valuable for a larger group of the asthma patient population.

In summary, variations in a novel risk gene *ST13* seem to be associated with an increased risk of severe exacerbations in children and young adults despite their use of ICS. Although the effect sizes are modest, these results may provide insights into the biological mechanisms that underlie severe exacerbations in asthmatic patients treated with steroids. Heterogeneity in corticosteroid response is probably caused by a complex interaction of genetic and environmental factors. Including *ST13* risk status in a multidimensional model with other genetic and nongenetic risk factors (e.g.: exposure to tobacco smoke [34] or vitamin D levels [35]) may reveal more precisely interindividual ICS responses.

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Conflict of interest

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Supporting Information

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

Table S1. Selected genes with corresponding SNPs that passed quality control.

Table S2. Summary effect estimates of SNPs in candidate genes and asthma-related hospital visits in a meta-analysis of BREATHE, PAGES & PACMAN.

Table S3. Summary effect estimates of SNPs in candidate genes and oral corticosteroids use in the previous year in a meta-analysis of BREATHE, PAGES & PACMAN.