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

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Complete List of Authors:	Vijverberg, Susanne; Utrecht University, Utrecht Institute for Pharmaceutical Sciences; University Medical Center, Respiratory Medicine Koster, Ellen; Utrecht University, Utrecht Institute for Pharmaceutical Sciences Tavendale, Roger; Dundee University, Population Pharmacogenetics Group Leusink, Maarten; Utrecht University, Utrecht Institute for Pharmaceutical Sciences Koenderman, Leo; University Hospital Utrecht, Respiratory Medicine; Raaijmakers, Jan; Utrecht University, Utrecht Institute for Pharmaceutical Sciences Postma, Dirkje; UMCG, GRIAC Research Institute, University of Groningen, Department of Pulmonary Medicine Koppelman, Gerard; Beatrix Children's Hospital, UMCG, GRIAC Research Institute, University of Groningen, Department of Pediatric Pulmonology and Pediatric Allergology Turner, Steve; University of Aberdeen, Child Health; Mukhopadhyay, Somnath ; Royal Alexandra Children's Hospital, Brighton and Sussex Medical School, Academic Department of Paediatrics Tse, Sze Man; Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School Tantisira, Kelan; Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School Hawcutt, Daniel; University of Liverpool, Women's and Children's Health Francis, Ben; University of Liverpool, Biostatistics Pirmohamed, Munir; University of Liverpool, The Wolfson Centre for Personalised Medicine Institute of Translational Medicine Yanes-Pino, Maria; Instituto de Salud Carlos III, CIBER de Enfermedades Respiratorias; University of California, Medicine Eng, Celeste; University of California, Medicine Burchard, Esteban; University of California, Bioengineering and therapeutic sciences; University of California, Medicine Palmer, Colin; Dundee University, Population Pharmacogenetics Group Maitland-van der Zee, Anke-Hilse; Utrecht University, Utrecht Institute for Pharmaceutical Sciences

ST13 polymorphisms and their effect on ~~increase the risk of~~ exacerbations in steroid-treated asthmatic children and young adults

Susanne J.H. Vijverberg, ~~MSc~~PhD[1,2], Ellen S. Koster, PhD[1], Roger Tavendale, PhD[3], Maarten Leusink, MSc [1], Leo Koenderman, PhD [2], Jan A.M. Raaijmakers, PhD [1], Dirkje S. Postma, MD, PhD[4], Gerard H. Koppelman, MD, PhD [5], Steve W. Turner, MD[6], Somnath Mukhopadhyay, MD, PhD [3,7], Sze Man Tse, MDCM, MPH [8], Kelan G. Tantisira, MD, MPH[8], Daniel B. Hawcutt,PhD[9], Ben Francis[10], PhD Munir Pirmohamed[11], Maria Pino-Yanes, PhD[12,13], Celeste Eng, BS[12], Esteban G. Burchard, MD, MPH[12,14], Colin N.A. Palmer, PhD[3] & Anke H. Maitland-van der Zee, PhD[1]

[1] Division of Pharmacoepidemiology & Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Faculty of Science, Utrecht University, Utrecht, the Netherlands

[2] Department of Respiratory Medicine, University Medical Centre Utrecht, Utrecht, the Netherlands

[3] Population Pharmacogenetics Group, Biomedical Research Institute, University of Dundee, Ninewells Hospital and Medical School, Dundee, United Kingdom

[4] University of Groningen, University Medical Center Groningen, Department of Pulmonology, Groningen Research Institute for Asthma and COPD, Groningen, the Netherlands

[5] University of Groningen, University Medical Center Groningen. Department of Paediatric Pulmonology and Paediatric Allergology, Beatrix Children's Hospital, Groningen Research Institute for Asthma and COPD, Groningen, the Netherlands

[6] Department of Child Health, University of Aberdeen, Aberdeen, United Kingdom

[7] Academic Department of Paediatrics, Royal Alexandra Children's Hospital, Brighton and Sussex Medical School, Brighton, United Kingdom.

[8] Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, United States of America

[9] Department of Women's and Children's Health, Institute of Translational Medicine, University of Liverpool, Liverpool, UK

[10] Department of Biostatistics. University of Liverpool, Liverpool UK

[11] Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, University of Liverpool, UK

[12] Department of Medicine, University of California, San Francisco, CA, USA

[13] CIBER de Enfermedades Respiratorias, Instituto de Salud Carlos III, Madrid, Spain.

[14] Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, CA, USA

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Correspondence:

Dr Anke-Hilse Maitland-van der Zee

Division of Pharmacoepidemiology and Clinical Pharmacology

Utrecht University, Faculty of Science

PO box 80082, 3508 TB Utrecht, the Netherlands

Tel.: 0031-(0)6-22736715

Fax: 0031-302539166

E-mail: a.h.maitland@uu.nl

Abstract

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: PACMAN (n=357, age: 4-12 years, the Netherlands), BREATHE (n=820, age: 3-22 years, UK) and PAGES (n=391, age: 2-16 years, UK). Seventeen ~~G~~genes were selected based on a role in the glucocorticoid signaling 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 CAMP (clinical trial, n=172, age:5-12 years, USA), GALA II (n=745, age:8-21, USA) and PASS cohorts (n=391, age:5-18, UK) ~~A fourth study population (CAMP, clinical trial, n=172, age: 5-12 years, USA) was included~~ to test the robustness of the findings. Finally, all results were meta-analyzed.

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 ~~despite corticosteroid treatment~~using ICS in the three cohorts studies~~d~~. ~~When CAMP was included in the meta-analysis the two SNPs remained associated with exacerbations.~~ In a meta-analysis of ~~the four~~all six studies, *ST13* rs138335 remained~~was~~ associated with an increased risk of asthma-related hospital visits and OCS use in the previous year.; OR=1.2~~8~~2 ~~per G allele for rs138335~~ (p=0.0~~2~~13) and OR=1.22~~31 per G allele for rs138337~~ (p=0.0017) respectively. ~~and OCS usage in the previous year, OR= 1.30 per G allele for rs138335 (p=0.003) and OR=1.18 per G allele for rs138337 (p=0.03).~~

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

Key words

Childhood asthma, corticosteroids, exacerbations, pharmacogenomics, *ST13*

Abbreviations used

BTS, British Thoracic Society; CAMP, Childhood Asthma Management Program; ED, Emergency Department; GALA II, Genes-environments & Admixture in Latino Americans; GC, glucocorticoid; GR, glucocorticoid receptor; ICS, Inhaled Corticosteroids; LABA, long-acting beta-2 agonist; LD, linkage disequilibrium; LTRA, leukotriene receptor antagonist; OCS, Oral Corticosteroids; PACMAN, Pharmacogenetics of Asthma Medication in Children: Medication with Anti-inflammatory effects; PAGES, Paediatric Asthma Gene Environment Study; SABA, short-acting beta agonist; SNP, Single Nucleotide Polymorphism; UPPER, Utrecht Pharmacy Practice Network for Education and Research.

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 arises 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 multi-protein chaperone complex. Various chaperones and co-chaperones 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 the present 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 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 analyzed 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., Ontaria, 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 analyzed 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

~~First and second meta-analysis~~

~~We performed two meta-analyses; in the first we included PACMAN, BREATHE and PAGES, according to the similarity in design (cohort) and uniformity in genotyping strategy (Sequenom platform, performed at Dundee University).~~ In order to test the robustness of our findings, we assessed the identified significant associations of the first meta-analysis in three additional independent populations: CAMP, the PASS cohort and GALA II and meta-analyzed the results. ~~a fourth population, the CAMP trial. This study differed in design (clinical trial) and genotyping strategy (imputation of GWAS data), and therefore was included in a second meta-analysis.~~

CAMP trial

We studied 172 non-Hispanic white corticosteroid-treated children with asthma included in CAMP (USA). CAMP is a multi-center trial that randomized 1,041 children with mild-to-moderate asthma aged 5 to 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 multicenter 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 UK. Eligibility criteria were as follows: treatment with ICS >6 months; diagnosis of asthma; under care of a pediatrician experienced in the treatment of asthma; 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 multi-center 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 analyzed asthmatic children with a reported use of SABA and ICS in the past 12 months. Patients included in this study were recruited from urban study centers 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 short-acting beta-2 agonists (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 the present study we selected children and young adults on BTS treatment steps 2, 3 and 4.

SNP selection and genotyping

Ten genes were selected based on their involvement in the glucocorticoid (GC) receptor complex (*NR3C1, HSPCA, HSPA4, FKBP4, ST13*), GC transport (*SERPINA6*) or GC-mediated signalling (*CREBBP, TBP, NCOA3, 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, BCL2*) [178-1920]. 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 Supplementary Table 1 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. ~~Selected genes and corresponding SNPs that passed quality control are listed in Supplementary Table 1~~. 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) as described elsewhere [21]. Imputed data was 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 genome-wide genotyping on the Illumina Human OmniExpressExome-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, ~~and~~ PAGES, PASS: asthma-related hospitalization in the past 6 months
   - PACMAN, 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, ~~and~~ PAGES, PASS: in the past 6 months
- PACMAN, 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-analyzed assuming random effects with the inverse variance weighing method. I^2 was used to quantify between-study heterogeneity [2~~0~~5]. The Bonferroni-corrected p-value was set at p: 0.0007 (0.05/76) ~~False Discovery Rates (FDR) were calculated to estimate the proportion of false positives due to multiple testing [21]. In addition, the risk estimates per genotype were calculated.~~ Statistical analysis was carried out using IBM SPSS 19.0 for Windows (SPSS, Inc, Chicago, Ill, USA) and PLINK [2~~2~~6]. ~~Haplotype frequencies of the two *ST13* SNPs were estimated using the EM algorithm implemented in the 'haplo.stats' package in R,~~ F~~f~~orest plots were made with R and the 'meta' package [2~~3~~7].

Functional annotation of associated SNPs

Functional annotation of associated SNPs was carried out querying the Encyclopedia 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 and 172 children of CAMP (Table 1). Most patients were on BTS treatment step 2 (as-needed short-acting beta-agonist use combined with regular low dose ICS). Compared to 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 out of the 38 SNPs to be associated with an altered risk of severe exacerbations as defined by asthma-related hospital visits. *ST13* SNP rs138335 increased the risk of asthma-related hospital visits (OR=1.35 per G allele; 95%CI: 1.07-1.7069, p=0.01, FDR: 25%) (Figure 1). Rs138337 in the same gene, had a similar comparable effect on the risk of asthma-related hospital visits (OR: 1.36 per G allele, 95%CI: 1.11-1.676, p=0.003, FDR: 11%) (Figure 2Table 2). In addition, rs138335 was also associated with an increased risk of OCS use (OR: 1.33 per G allele; 95%CI: 1.11-1.5960, p=0.002, FDR: 11%) (Figure 3). In Supplementary tables 2 and 3 show the summary effect estimates of all investigated SNPs are shown.

Replication phase: ST13 in a fourth independent asthma populationthe CAMP, GALA II and PASS cohorts

In order to assess the robustness of findings we studied rs138337 and rs138335 in a fourth three additional independent study populations; the North-American 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 (Figure 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; Figure 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=5.8x10^{-70} and p=1.9x10^{-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.

~~Genotyping data for *ST13* were available for 172 asthmatic steroid-treated non-Hispanic white children. In this clinical trial population, the two SNPs in *ST13* were not significantly associated with risk of severe exacerbation, but this might be due to a lack of power considering the small study population. In a second meta-analysis including all four studies (Table 2), both SNPS increased the risk of asthma related hospital~~

visits. For rs138337 the OR per G-allele for asthma-related hospital visits was 1.31 (95%CI: 1.08-1.59, p=0.007, FDR: 19%) and for rs138335 the OR per G-allele was 1.28 (95%CI: 1.04-1.59, p=0.02, FDR: 36%). Furthermore, both SNPs were associated with an increased risk of OCS usage; the OR per G-allele was 1.18 for rs138337 (95%CI: 1.01-1.38, p=0.03, FDR: 41%) and 1.30 per G-allele for rs138335 (95%CI: 1.09-1.54, p=0.003, FDR: 10%). Forest plots are shown in Figure 1.

To assess the risk stratified per genotype we additionally performed a genotypic analysis whereby rs138335 CC and rs138337 AA were used as reference groups (Table 3). In a meta-analysis the highest risk estimates were found for carriers of rs138335 GG and risk of OCS usage (OR rs138335 GG compared to CC: 1.98, p=0.002) and carriers of rs138337 GG and risk of asthma-related hospital visits (OR rs138337 GG compared to rs138337 AA: 1.75, p=0.007).

ST13 haplotypes are associated with asthma-related hospital visits

Based on the available genotype information, three haplotypes were estimated to have a frequency >2% (Table 4). The most common haplotype was rs138335-G/rs138337-G. There was a significant association between *ST13* haplotype and asthma-related hospital visits, whereby compared to the most common haplotype, the haplotype without risk alleles (rs138335-C/rs138337-A) conferred the most protection (OR: 0.96, 95%CI: 0.94-0.99) (Table 5).

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.

SNPs rs138335 and rs138337 both lie in the non-coding intronic regions of the *ST13* gene, but ~~may still affect gene expression, splicing or be in high LD with a variant that has functional consequences. However, there were no expression quantitative trait loci (eQTL) data available for both SNPs (http://www.ncbi.nlm.nih.gov/gap/PheGenI), nor could we identify a coding SNP in high LD with rs138335 and/or rs138337 (www.ensembl.org)~~ 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 co-chaperone 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 [~~24~~30]. *STIP1* (coding for another co-chaperone 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 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~~24~~], building evidence that alterations in the expression or folding of these co-chaperones may influence the binding of corticosteroids to the receptor or downstream signaling and therefore, ICS responsiveness. Functional studies are necessary to support our hypothesis.

A number of limitations need to be noted regarding the present study. Two SNPs in *ST13* were associated with both outcomes of exacerbations in the meta-analysis of all ~~four~~ 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 analyze a large study population (including 2876 asthmatic children and young adults), a post-hoc power analysis showed 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 expected proportions of false discoveries (FDR rates) due to multiple testing ranged for both SNPs and the two outcomes in the second meta-analysis between 10-41%. We used FDR rates as a measure to correct for multiple testing, Bonferroni corrected p-values might be too conservative in candidate-gene approaches were SNP are in background LD. Although, the FDR rates are > 5% (which is often used as cut-of value), the biological function of *ST13* and the previous identified association of *STIP1* with ICS response in asthmatic patients [10], strongly suggest that the identified association is not a false discovery. Furthermore, the SNPs were tested in 4 distinct study populations, and the effect estimates for both measures of severe exacerbations pointed in the same direction in the three largest study populations included in our study.~~

The ~~study~~ populations we studied varied in age and severity of asthma symptoms, ~~.~~ ~~This~~ probably due to the design of the studies ~~; t~~The PACMAN population is recruited in community pharmacies, whereby most participants had well-controlled symptoms [32~~25~~], while patients in PAGES, BREATHE and CAMP were recruited 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 behavior between the ~~two~~ different countries might also play a role [33~~26~~]. 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 ≥ 0.20. Due to the sample size, we ~~We~~ could not investigate~~study~~ rare variants, which might have had larger effects. ~~However, our study population was too small to study rare variants.~~ 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* ~~are~~ seem to be associated with an increased risk of severe exacerbations in children and young adults despite their use of ICS ~~treatment~~. 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 non-genetic risk factors (e.g: exposure to tobacco smoke [34~~27~~] or vitamin D levels [35~~28~~]) ~~might explain a larger part of the observed variability in~~may reveal more precisely interindividual ICS ~~treatment~~ responses.

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Table 1. Baseline characteristics study population in the discovery phase

	BREATHE (n=820)	PAGES (n=391)	PACMAN (n=357)
Child characteristics			
Age, ~~mean (SD)~~range (yrs)	9.8 (~~4.0~~2-22)	9.0 (2-16~~3.8~~)	8.7 (4-13~~2.3~~)±
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, * 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.

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

~~¶ CAMP is Randomized Clinical Trial of mild-to-moderate asthmatics. All children were on 200 µg of budesonide (ICS) plus SABA as needed.~~

Table 2. Baseline characteristics study population in the replication phase

	CAMP (n=172)	PASS (n=391)	GALA II (n=745)
Child characteristics			
Age, range (yrs)	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

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

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

Figure 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.

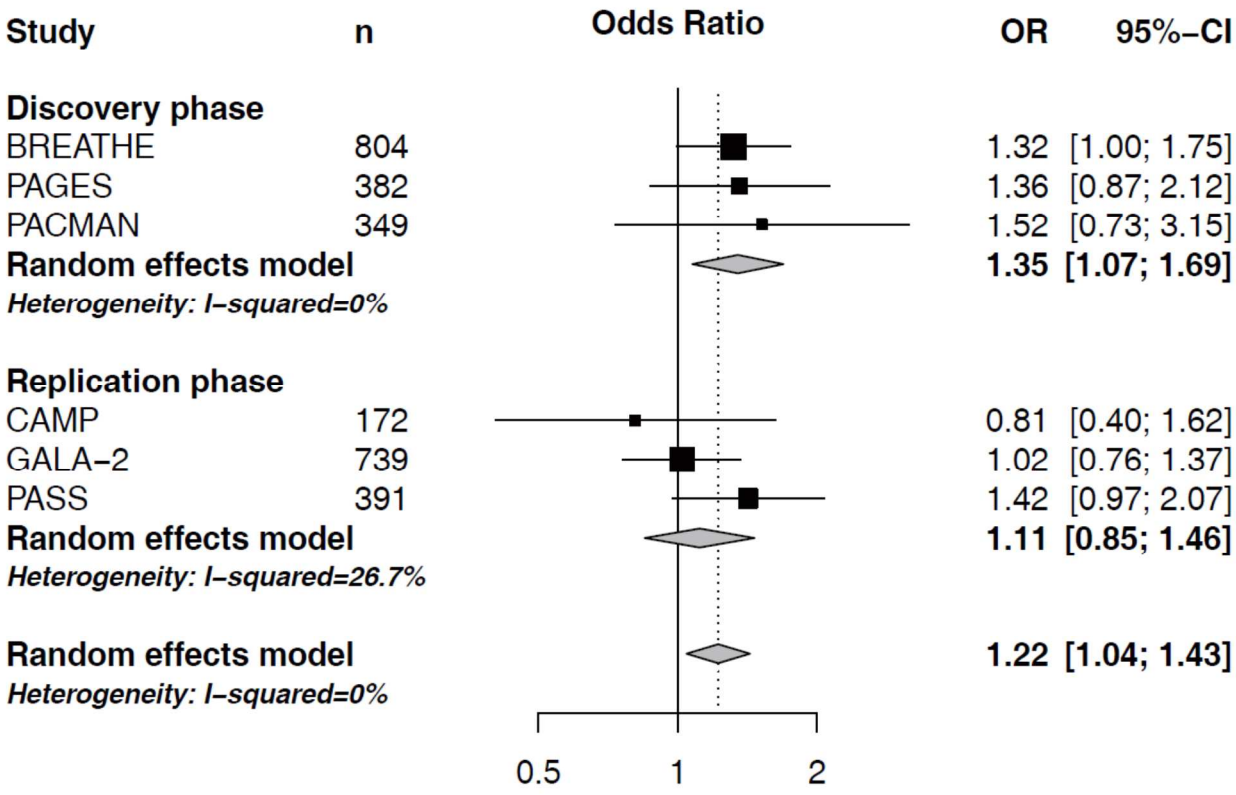


Figure 2. Forest plot for the association between *ST13* rs138337 and asthma-related hospital visits. Odds ratios (OR) and corresponding 95%CI per increase in G-allele, controlling for age, sex and treatment step.

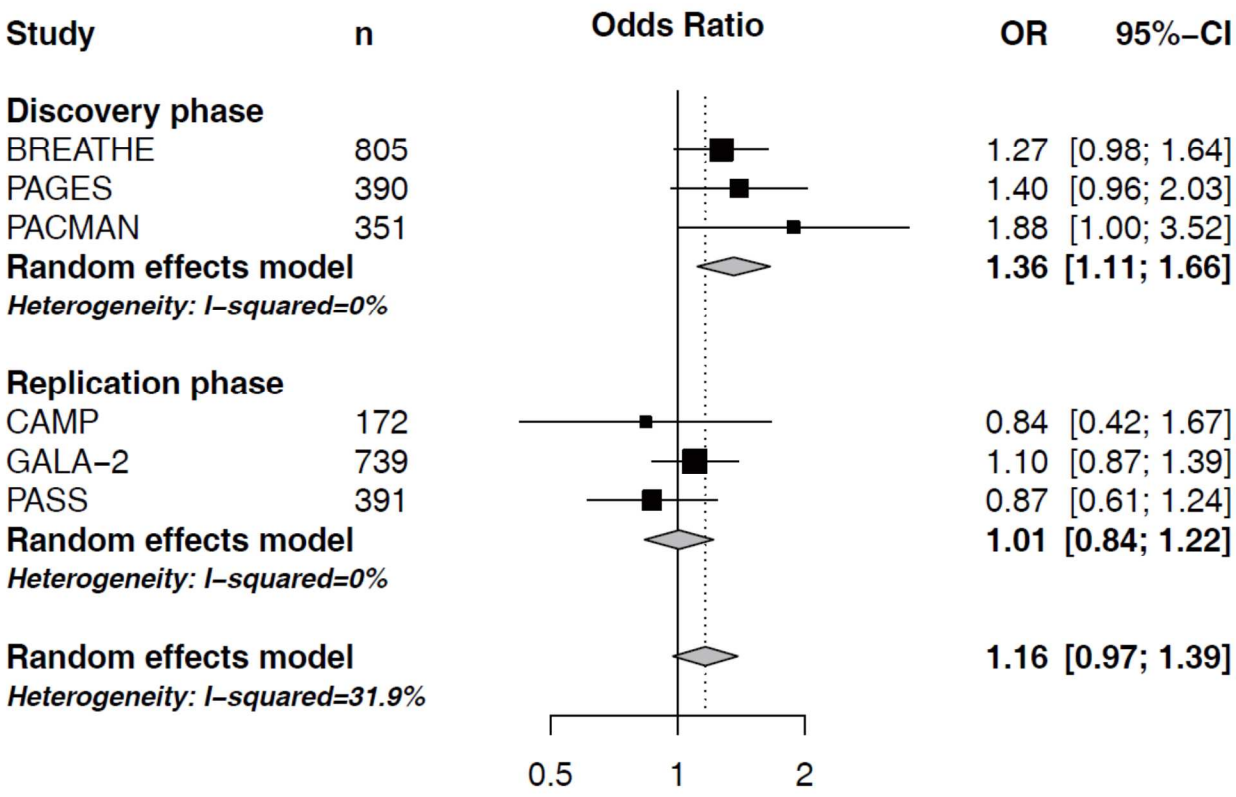
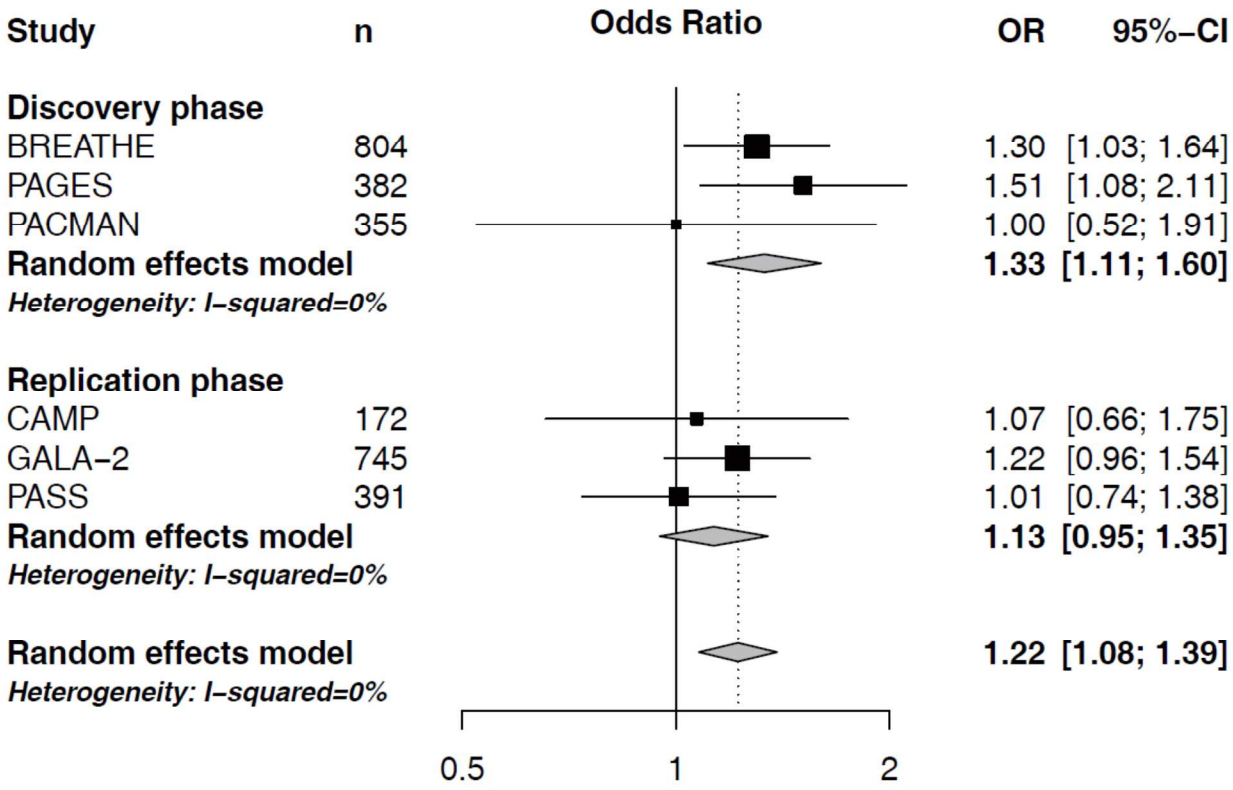


Figure 3. Forest plot for the association between *ST13* rs138335 and OCS usage. Odds ratios (OR) and corresponding 95%CI per increase in G-allele, controlling for age, sex and treatment step.



Supplementary Table 1. Selected genes with corresponding SNPs that passed quality control

Gene	SNPs	Description	Function
NR3C1	*rs4607376* *rs4912912* *rs7701443* *rs9324924* *rs2963155* *rs4912905* *rs6865292* *rs17209258* *rs6196*	Nuclear receptor subfamily 3 group C member 1	Glucocorticosteroid receptor (GR) [1]
KFBP4	*rs1981655* *rs11833878*	FK506 binding protein 4	Chaperone, binds dynein upon ligand binding of GR [2]
ST13	*rs138335* *rs138337*	suppression of tumorigenicity 13	Co-chaperone, mediates assembly chaperone GR complex [3]
CREBBP	*rs130021* *rs11076787* *rs886528* *rs2526689*	cAMP-response element binding protein	Transcriptional co-activator [4]
TBP	*rs2235506* *rs3800235*	TATA box binding protein	Transcriptional co-activator [5]
NCOA3	*rs2425941* *rs6066394* *rs2143491* *rs6018600* *rs11700063*	nuclear receptor coactivator 3	Transcriptional co-activator, acylates histones. [6]
SMAD3	*rs744910*	Mothers against decapentaplegic homolog 3	Transcription factor, regulated by GR. Associated with asthma susceptibility [7]

SERPINA6	*rs1956179* *rs7158343* *rs10498639* *rs1998056* *rs2281518* *rs2281519* *rs2281520* *rs11629171*	Corticosteroid-binding globulin	Protein involved in plasma corticosteroid-binding globulin activity [8]
ARG1	*rs2781667*	Arginase, liver	Arginase (enzyme), thought to be involved in asthma pathogenesis through effects on nitrosative stress. Associated with bronchodilator response [9]
17q21 locus	*rs7216389*	Involved in expression of orosomucoid - like protein 3	Locus thought to be associated with asthma susceptibility and therapy response [10-13]
PDE4D	*rs1544791* *rs1588265*	phosphodiesterase 4D	Degrades cAMP. Thought to be associated with asthma susceptibility [14]
IL2RB	*rs2284033*	Interleukin 2 receptor, beta	Binds interleukin 2, involved in T cell mediated immune responses. Associated with asthma susceptibility [7]

Supplementary Table 2. Summary effect estimates of SNPs in candidate genes and asthma-related hospital visits in a meta-analysis of BREATHE, PAGES & PACMAN[1]

SNP	CHR	BP	Gene	Effect allele	p-value	OR	I (%)	q-value
rs138337	22	39560999	ST13	G	0.003	1.36	0	0.11
rs138335	22	39557032	ST13	C	0.010	0.74	0	0.25
rs6066394	20	45643560	*NCOA3*	T	0.099	1.21	0	0.92
rs6018600	20	45691984	*NCOA3*	A	0.116	1.18	0	0.92
rs1956179	14	93855495	*SERPINA6*	G	0.129	1.18	0	0.92
rs2963155	5	142736197	*NR3C1*	G	0.207	0.86	0	0.92
rs4607376	5	142776725	*NR3C1*	A	0.219	1.13	0	0.92
rs7216389	17	35323475	*17q21locus§*	C	0.266	0.81	59	0.92
rs2425941	20	45590796	*NCOA3*	T	0.269	0.89	0	0.92
rs17209258	5	142653590	*NR3C1*	G	0.273	1.25	55	0.92
rs2526689	16	3857884	*CREBBP*	G	0.337	0.90	0	0.92
rs2281518	14	93858870	*SERPINA6*	C	0.342	1.13	0	0.92
rs2281520	14	93846140	*SERPINA6*	C	0.358	0.88	10	0.92
rs10498639	14	93845279	*SERPINA6*	A	0.400	1.09	0	0.92
rs3800235	6	170718978	*TBP*	C	0.410	1.10	0	0.92
rs11700063	20	45586555	*NCOA3*	A	0.427	0.86	44	0.92
rs1588265	5	59405551	*PDE4D*	G	0.438	0.88	44	0.92
rs1998056	14	93859248	*SERPINA6*	G	0.448	0.92	0	0.92
rs2281519	14	93846385	*SERPINA6*	T	0.484	1.08	0	0.92
rs4912912	5	142787343	*NR3C1*	C	0.516	0.93	0	0.92
rs1544791	5	59474839	*PDE4D*	A	0.531	0.90	44	0.92
rs886528	16	3751557	*CREBBP*	C	0.566	1.14	70	0.92
rs130021	16	3772472	*CREBBP*	G	0.606	0.89	67	0.92
rs4912905	5	142710569	*NR3C1*	C	0.625	0.94	0	0.92
rs6865292	5	142773183	*NR3C1*	C	0.662	1.05	0	0.92
rs7701443	5	142772843	*NR3C1*	G	0.699	0.96	0	0.92
rs2235506	6	170720811	*TBP*	C	0.721	0.96	0	0.92
rs11833878	12	2780498	*KFBP4*	G	0.738	0.95	0	0.92
rs744910	15	65233839	*SMAD3*	A	0.741	0.96	28	0.92
rs2143491	20	45662074	*NCOA3*	A	0.747	0.97	0	0.92
rs6196	5	142641683	*NR3C1*	G	0.748	0.96	0	0.92
rs9324924	5	142772677	*NR3C1*	T	0.762	0.94	55	0.92
rs11629171	14	93843203	*SERPINA6*	T	0.867	1.02	21	0.96
rs2284033	22	35863980	*IL2RB*	A	0.875	1.02	0	0.96
rs11076787	16	3792777	*CREBBP*	T	0.901	0.98	0	0.96

rs1981655	12	2777987	*KFBP4*	A	0.926	1.03	0	0.96
rs2781667	6	131936837	*ARG1*	T	0.950	1.01	0	0.96
rs7158343	14	93852906	*SERPINA6*	G	0.963	1.01	0	0.96

* OR adjusted for age, gender and BTS treatment step, [1]PACMAN: asthma-related ER visits in the preceding 12 months, BREATHE/PAGES: asthma-related hospitalization in the preceding 6 months. § locus involved in the regulation of several genes. OR= Odds Ratio assuming random effect model, I= heterogeneity (%).BP: base pair. Positions were based on NCBI Build 36.

Supplementary Table 3. Summary effect estimates of SNPs in candidate genes and oral corticosteroids use in the previous year in a meta-analysis of BREATHE, PAGES & PACMAN[1]

SNP	CHR	BP	Gene	Effect allele	p-value	OR	I (%)	q-value
rs138335	22	39557032	*ST13*	C	0.002	0.75	0	0.11
rs138337	22	39560999	*ST13*	G	0.099	1.18	20	0.92
rs7216389	17	35323475	*17q21 locus§*	C	0.102	0.80	49	0.92
rs6196	5	142641683	*NR3C1*	G	0.169	1.24	40	0.92
rs2963155	5	142736197	*NR3C1*	G	0.179	1.20	39	0.92
rs744910	15	65233839	*SMAD3*	A	0.188	0.89	6	0.92
rs6018600	20	45691984	*NCOA3*	A	0.215	1.11	0	0.92
rs4912912	5	142787343	*NR3C1*	C	0.224	0.90	0	0.92
rs7158343	14	93852906	*SERPINA6*	G	0.262	1.12	5	0.92
rs2284033	22	35863980	*IL2RB*	A	0.292	1.15	47	0.92
rs11629171	14	93843203	*SERPINA6*	T	0.308	0.89	17	0.92
rs1998056	14	93859248	*SERPINA6*	G	0.317	0.92	0	0.92
rs11833878	12	2780498	*KFBP4*	G	0.326	1.19	42	0.92
rs6865292	5	142773183	*NR3C1*	C	0.348	1.11	25	0.92
rs1981655	12	2777987	*KFBP4*	A	0.357	1.23	0	0.92
rs10498639	14	93845279	*SERPINA6*	A	0.410	0.89	56	0.92
rs2235506	6	170720811	*TBP*	C	0.458	1.07	0	0.92
rs9324924	5	142772677	*NR3C1*	T	0.463	1.07	0	0.92
rs7701443	5	142772843	*NR3C1*	G	0.466	0.94	0	0.92
rs4607376	5	142776725	*NR3C1*	A	0.543	1.05	0	0.92
rs130021	16	3772472	*CREBBP*	G	0.554	0.95	0	0.92
rs11700063	20	45586555	*NCOA3*	A	0.620	1.05	0	0.92
rs6066394	20	45643560	*NCOA3*	T	0.642	1.06	30	0.92
rs1544791	5	59474839	*PDE4D*	A	0.646	0.94	48	0.92
rs2143491	20	45662074	*NCOA3*	A	0.654	1.04	0	0.92
rs2281519	14	93846385	*SERPINA6*	T	0.686	0.96	0	0.92
rs2281520	14	93846140	*SERPINA6*	C	0.693	1.06	43	0.92
rs2526689	16	3857884	*CREBBP*	G	0.710	0.97	0	0.92
rs17209258	5	142653590	*NR3C1*	G	0.714	0.96	0	0.92
rs1588265	5	59405551	*PDE4D*	G	0.749	0.95	64	0.92
rs2281518	14	93858870	*SERPINA6*	C	0.750	1.04	23	0.92
rs11076787	16	3792777	*CREBBP*	T	0.809	1.03	0	0.96
rs2425941	20	45590796	*NCOA3*	T	0.823	0.98	0	0.96
rs2781667	6	131936837	*ARG1*	T	0.864	1.02	15	0.96
rs1956179	14	93855495	*SERPINA6*	G	0.872	0.98	31	0.96
rs3800235	6	170718978	*TBP*	C	0.912	1.01	0	0.96

rs886528	16	3751557	*CREBBP*	C	0.931	1.01	0	0.96
rs4912905	5	142710569	*NR3C1*	C	0.950	0.99	12	0.96

OR adjusted for age, gender and BTS treatment step, [1]PACMAN: OCS use in the past 12 months, BREATHE/PAGES: OCS use in the past 6 months. § locus involved in the regulation of several genes. OR= Odds Ratio assuming random effect model, I= heterogeneity (%). BP: base pair. Positions were based on NCBI Build 36.

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Dear Prof. dr. Wardlaw

We would like to thank you and the expert reviewer for the comments and suggestions regarding our manuscript. We have rigorously revised the manuscript, added new figures and tables, and have marked all changes by underlining in the revised manuscript.

In the revised manuscript we have added two new cohorts (PASS and GALA II), thereby almost doubling our study population (from 1740 to 2876 study subjects). The final meta-analysis includes 6 cohorts and indicates a strong collaborative effort in the field of pharmacogenetics of pediatric asthma. Furthermore, we have put more focus on the discovery and the replication phase in order to increase the readability of the paper.

In this letter we would like to respond to the remarks and suggestions of the reviewer point by point:

Major comments:

Power – The power of the study was not stated, although FDR rates were analysed. The 95% CIs show a wide range of effect, with some ORs close to 1, so limited power is certainly a consideration in this study. It is recognized that there are few cohorts available like these, for ICS pharmacogenomics studies, so the authors have done well to include all of these studies.

We agree with the reviewer that limited power is an issue in this study, and in the revised manuscript we have put a lot of effort in including additional cohorts to increase the study sample size. We have added two new cohorts to our study, increasing our study population from 1740 to 2876 individuals. Nevertheless, the power in the discovery phase remains limited. We have now addressed this in our discussion at p.18:

"Even though we were able to analyze a large study population (including 2876 asthmatic children and young adults), a post-hoc power analysis showed 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 underlies the need for large-scale international collaboration in this field.[31]"

2 meta-analyses – The authors have employed the technique of 2 meta-analyses, to provide an approach to replication of genetic associations. Whilst the CAMP cohort is well-characterised and important to include, the additional of approximately another 10% of participants to the total number does not seem to add substantially to the replication. i.e. a small addition of participants may not significantly alter the effect sizes of the first meta-analysis. It may be better just to include all 4 studies in one combined analysis.

In the revised manuscript we have added two additional replication cohorts (PASS and GALA II). We have stratified our analyses in a discovery phase (analyzing PACMAN, BREATHE and PAGES) and a replication phase, in which we have analyzed the most significant SNPs from the discovery phase in CAMP, PASS and GALA II. Lastly, we have performed a meta-analysis of all six studies. Rs138335 remains associated with an increased risk of exacerbations (at nominal level) in this final meta-analyses. This is now depicted in figures 1 and 3:

Figure 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.

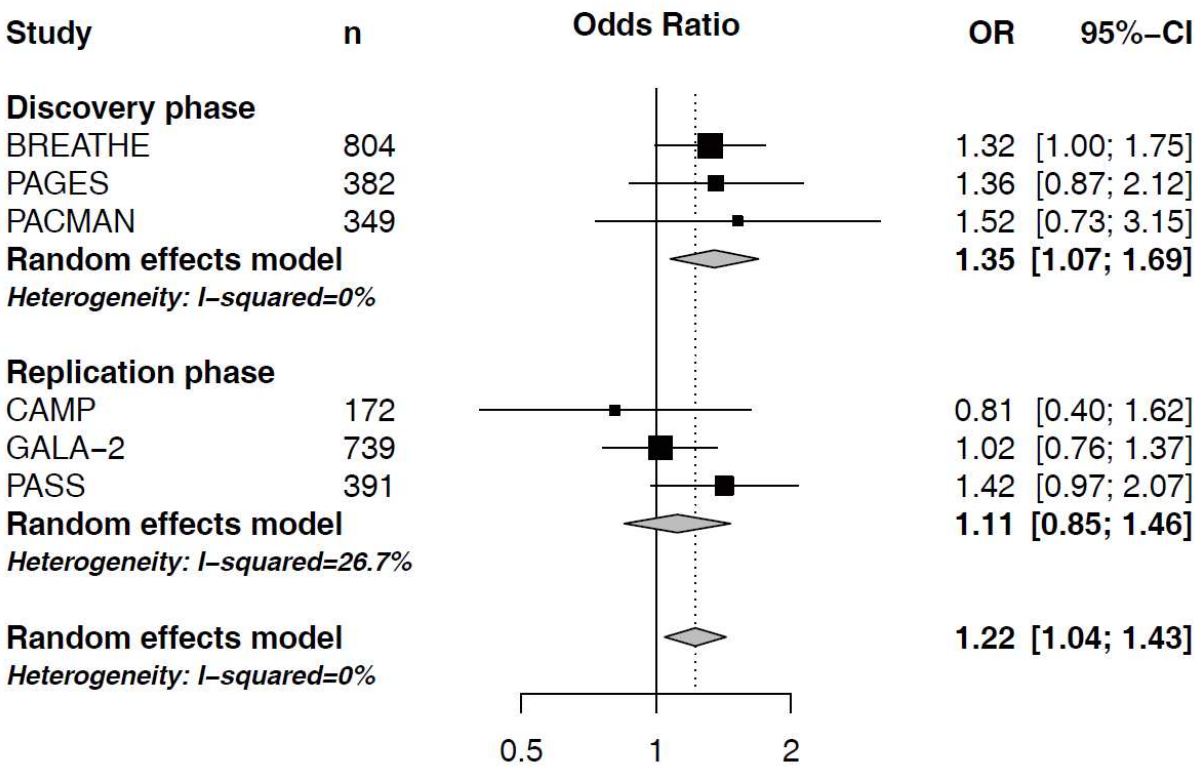
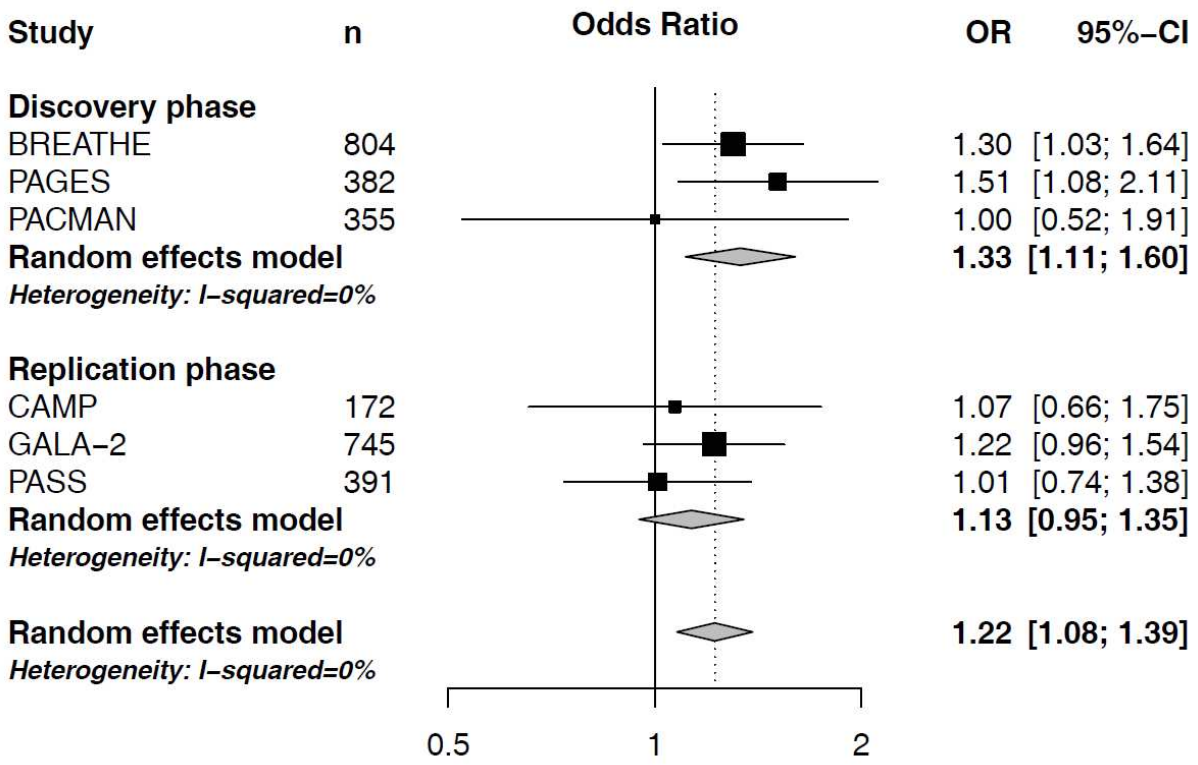


Figure 3. Forest plot for the association between *ST13* rs138335 and OCS usage. Odds ratios (OR) and corresponding 95%CI per increase in G-allele, controlling for age, sex and treatment step.



Other SNPs – In the list of candidate SNPs, GLCCI1 SNPs (from a previous large study of ICS respons) was not included. I presume this is because the GLCCI1 SNPs had already been studied in the CAMP cohort, and are not a direct member of the GR complex.

GLCCI1 was not included in this manuscript. At the time of the discovery phase the relation between treatment response and *GLCCI1* SNPs (rs37972/rs37972) had not been published and was not included in the study protocol. However, since then *GLCCI1* rs37972 has been analyzed separately in BREATHE, PAGES and PACMAN, as a replication study of the CAMP results. That paper has been published recently in Pharmacogenomics:

Vijverberg et al. Pharmacogenetic analysis of GLCCI1 in three north European pediatric asthma populations with a reported use of inhaled corticosteroids. Pharmacogenomics 2014 15(6): 799-806 doi 10.2217/pgs.14.37.

Dose-response – Even though this study is relatively underpowered, and involves a range of ages of children and young adults,

Unfortunately, dosages of the inhaled and oral corticosteroids have not been reported in all studies. Furthermore, the children included in CAMP have all been treated with the same amount of ICS due to the clinical trial set up. Therefore, we were unable to perform a dose-response analysis.

Variability – Can the authors estimate how much of the steroid-response variability is contributed to by these SNPs?

The estimation of the *ST13* SNPs on the steroid-response variability is very complicated. Especially since we were unable to perform a dose-response analysis, it is difficult to quantify the contribution of the SNPs on the variability in response. Based on the limited effect sizes of the SNPs we assume that they will have a small effect on the variability of response. Nevertheless, this effect might still be valuable to understand the pathobiological background of ICS response in asthmatic children and young adults.

Minor comments:

Discussion paragraph 3 –
'We used FDR rates as a measure to correct for multiple testing, Bonferroni corrected p-values might be too conservative in candidate-gene approaches were SNP are in background LD'
Should this read 'where SNPs are in….'

In the revised manuscript this sentence has been removed.

Table 1 – The actual age range of the participants would be useful to include in this demographics table.

We have added the age range in table 1 (baseline characteristics of the studies included in the discovery phase), and included this in our newly added table 2 (baseline characteristics of the studies included in the replication phase):

Table 1. Baseline characteristics of the study population in the discovery phase

	BREATHE (n=820)	PAGES (n=391)	PACMAN (n=357)
Child characteristics			
Age, range (yrs)	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, * 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)

± children within the PACMAN cohort were selected between the age of 4-12. However, the child might have been 13 at the moment of the study visit.

Table 2. Baseline characteristics study population in the *replication phase*

	CAMP (n=172)	PASS (n=391)	GALA II (n=745)
Child characteristics			
Age, range (yrs)	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

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

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