GWIS of inhaled corticosteroids on the severity of bronchial hyperresponsiveness in asthma.
To the editor

Bronchial hyperresponsiveness (BHR) is a key feature of asthma. More severe BHR is associated with more severe asthma symptoms, loss of asthma control and higher frequency of exacerbations. The severity of BHR in asthma is influenced by environmental and genetic factors, and their interaction. Important environmental factors that increase BHR are smoking and allergen exposures.

Inhaled corticosteroids (ICS) are the cornerstone of asthma treatment. Use of ICS is associated with improvement in BHR, yet the size of this effect differs between asthmatic subjects. Most likely, pharmacogenetics, a term used to define inherited genetic differences in response to drugs, may play a role. Most genetic association studies include ICS use as a covariate in the regression analysis of genetic variants and severity of BHR. However, SNPs may be an effect modifier of the association of ICS with BHR severity. In other words, a gene-treatment interaction may exist. One example of such a gene-treatment interaction is provided in a previous study by Tantisira et al. They observed a significant interaction between the H33Q variant located in T-box 21 (TBX21) and ICS on the severity of BHR. The minor allele of the H33Q variant was not associated with the severity of BHR in asthmatics who were not treated with corticosteroids, whereas carriers of the minor allele of H33Q showed a strong improvement in the severity of BHR when using corticosteroids.

The interaction between ICS and SNPs on BHR severity in asthma has never been studied on a genome-wide level. The aim of this study is to identify genes that modify the effect of ICS use on BHR severity in adult asthmatics by performing a genome-wide interaction study (GWIS).

The identification cohort was the Dutch Asthma GWAS (DAG) cohort (table E1). BHR severity was measured with the slope of the dose response curve of either a histamine or methacholine challenge test. SNP association analyses were performed using linear regression models that included the SNP, ICS use, and SNP-by-ICS interaction, as well as smoking. P-values <1.70×10^{-7} on the interaction term were considered genome-wide significant, based on the Bonferroni correction (0.05/294,932). We selected for replication of the SNP-by-ICS interaction results, SNPs with p-values <1×10^{-4} in the interaction term. SNPs with a p-value <1×10^{-3} in the interaction term combined with a p-value <1×10^{-4} for the main SNP effect were used in analyses on ICS and no-ICS users. Replication analyses were performed in the Epidemiological study on the Genetics and Environmental of Asthma (EGEA) and European Community Respiratory Health Survey (ECRHS) study (table E1). Nominal replication of SNP-by-ICS interactions were defined by the same direction of the interaction effect in both replication cohorts as well as a p-value <0.1 in at least one cohort (a p-value <0.1 corresponding to a one-side p-value
<0.05). Subsequently, the functional effect of the replicated SNP-by-ICS interactions on gene expression was investigated in the Groningen and Leiden Universities study of Corticosteroids in Obstructive Lung Disease (GLUCOLD) study⁸ (details in supplemental methods).

In total, 415 and 557 asthmatics were included in the discovery and replication analysis respectively (table E2). Thirty-nine percent of asthmatics in DAG and EGEA used ICS, whereas the prevalence of ICS-use in ECRHS was slightly lower (29%). Overall more females used ICS (table E2). Compared to DAG, asthmatics in ECRHS had more severe BHR and in EGEA less severe BHR.

Based on results of the discovery analysis, 64 SNPs were selected for replication, although none were genome-wide significant. After pruning SNPs based on linkage disequilibrium (r²<0.8), 56 SNPs were analysed for replication (table E3). Of these, 21 SNPs showed an interaction in the same direction in all cohorts, but only 6 SNPs had a one-sided p-value <0.05 in at least one of the replication cohorts. Minor alleles of three SNPs had a negative SNP-by-ICS interaction, i.e. rs1000543 (intronic in LOC339529), rs2454222 (intronic in LOC339529) and rs7816946 (intronic in ZFAND1), and minor alleles of three SNPs had a positive SNP-by-ICS interaction, i.e. rs6429464 (intronic in ADSS), rs13205348 (downstream gene variant of ZDHHC14) and rs8054383 (intergenic between RNU7-24P and RBBP6) (figure 1). Of interest, Rbbp6 (also known as P2P-R) was identified in a steroid-regulated transcription network in a study investigating the top 1000 gene transcripts that are co-expressed with Rbbp6 in fat tissue of rats.⁹ Furthermore, Rbbp6 had an interaction with Ncoa1, a well-known steroid receptor co-regulator.⁹

Of the six replicated SNP-by-ICS interactions, two SNPs were available in the GLUCOLD gene expression database (rs6429464 and rs8054638) and another SNP had a proxy SNP available (rs2955008 with a LD r²=0.96 with rs7816946). The association of these SNPs with gene expression in airway wall biopsies from 69 patients with COPD was analysed using 11 mRNA expression probes with a probe midpoint located within 250kb of the SNP. There was a significant negative SNP-by-ICS interaction between the minor allele of rs6429464 and ICS use on the expression of Chromosome 1 open reading frame 100 (C1orf100), a protein coding gene (Figure 2). The minor allele was associated with decreased expression of C1orf100 in asthmatics using ICS (Interaction beta=-0.41 p=0.02; ICS use beta=-0.04 p=0.53; non-ICS use beta=0.37 p=0.03). Recently, the expression of C1orf100 in CD4+ T-cells was associated with a lower FEV₁ in asthmatic children from the CAMP study¹⁰. In this study 4-year treatment with ICS (i.e. budesonide) significantly attenuated the negative association between C1orf100-expression and FEV₁ compared to treatment with nedocromil or placebo. No interacting SNP effect was reported. Combined with our results indicating a SNP-by-ICS interaction on both BHR severity and gene expression, this indicates the potential importance of C1orf100 in ICS-response in asthmatics.
Figure 1: SNP effects stratified by ICS-use in the discovery (DAG) and replication (EGEA and ECRHS) cohorts of the 6 replicated interactions on the severity of BHR. A: SNPs
Even though we have a well phenotyped cohort, our study is limited by the small number of asthmatics studied in the GWIS, leading to a low statistical power to find a significant interaction. However, we were able to provide suggestive evidence for interaction between ICS and six SNPs in two independent cohorts and to associate one of these interactions with gene expression. Further, our analyses were cross-sectional in design and future studies should investigate gene-ICS interaction on BHR severity in a longitudinal way.

In summary, we identified several loci that interact with ICS on BHR severity in asthma, one of these (i.e. RBBP6) is already known to be involved in steroid regulated transcription. Gene expression analysis identified C1orf100 as an important gene that interacts with ICS in modifying the severity of BHR. Further studies should investigate the functional implication of this finding.

Figure 2: rs6429464A SNP effect stratified by ICS-use in the GLUCOLD study on the expression of C1orf100.
References


Supplement

**DAG cohort**

*Study population and phenotype definition*

Asthma patients were recruited from the Dutch Asthma GWAS (DAG)\(^1\) cohort. All included cases were of Caucasian descent and had a doctor diagnosis of asthma combined with a positive BHR challenge test. Only participants with data on smoking and ICS use at the time of testing were included. Patients labelled ICS were either on ICS during the challenge test or stopped using ICS between 0-2 weeks before the challenge test, patients labelled as no ICS did not use ICS >4 weeks before the challenge test. Patient who stopped using ICS between 2-4 weeks before the challenge test were excluded from the analysis due to possible effect of ICS on the severity of BHR.

*Calculation of the severity of BHR*

All subjects performed a challenge test using either histamine or methacholine according to standardized protocols\(^2\). The BHR slope was calculated by dividing the difference between FEV\(_1\) at baseline and at the dose step at which a ≥20% fall in FEV\(_1\) was reached or the highest dose inhaled, by the dose that was taken at this last step. The following formula was used to calculate the slope: Ln ((FEV\(_1\) at baseline – FEV\(_1\) at the dose step at which a ≥20% fall in or the highest dose inhaled)\(^*\)100/ the dose that was taken at this last step). The FEV\(_1\) used in this formula is the absolute FEV\(_1\) in litres. We divided the BHR slopes of the 30-second tidal breathing method by 4 in order to compare the slope of the 30-second and the 2-minute tidal breathing methods\(^3\). Values were log transformed (Ln) to reach normal distribution.

*Quality control of GWAS*

DNA of subjects was genotyped with the Illumina 317k Chip or with the Illumina 370k Duo Chip (Illumina Inc, San Diego, CA). Quality control was applied; subjects were removed from analysis if they were not of Caucasian descent based on principal component analysis, had a low genotyping call rate (<95%) or were discrepant or ambiguous for genetic sex. SNPs were deleted if the call rates were low (<95%), not in Hardy-Weinberg Equilibrium (p<1*10\(^{-4}\)), or if the minor allele frequency was <0.05. After quality control 294,932 SNPs were selected for analysis.

*Statistical analysis*

All statistical analyses were performed using PLINK v1.07. Linear regression analyses, adjusted for smoking, included the SNP, ICS use and the SNP-by-ICS interaction were performed on the Ln-transformed slope of the BHR challenge test. Smoking was categorised as never (reference), current or ex-smokers.
GLUCOLD study

Study population

Patients with Chronic Obstructive Pulmonary Disease (COPD) were included in a randomized placebo-controlled trial\(^4\). In this trial long-term ICS therapy with and without LABAs was compared to placebo in order to investigate inflammation and pulmonary function in COPD. Patients were randomized into four groups: 1) treated with ICS without LABA for six months followed by placebo for 24 months, 2) treated with ICS without LABA for 30 months, 3) treated with ICS with LABA for 30 months, or 4) treated with placebo for 30 months. Patients underwent a Methacholine challenge test at baseline (before start of the treatment), at six months and at 30 months. Data from the six months visit was used for the gene expression analysis in this study, to include the largest group of patients. ICS was categorised as ICS use when ICS were prescribed with or without LABA, patients included in the placebo group were categorised as no ICS.

Quality control of genotyping

DNA of subjects was genotyped with the CytoSNP 12v2 array (Illumina Inc, San Diego, CA). Quality control applied was the same as used in the DAG cohort; subjects were removed from the analysis if they were not of Caucasian descent based on principal component analysis, had a low genotyping call rate (<95%) or were discrepant or ambiguous for genetic sex. SNPs were deleted if the call rates were low (<95%), not in Hardy-Weinberg Equilibrium \(p<1\times10^{-4}\), or if the minor allele frequency was <0.05.

Expression analysis in airway wall biopsies at six months

Quality control of mRNA expression data of the GLUCOLD has been previously described\(^5\). All microarray data from samples in this study have been deposited in gene expression omnibus under accession #36221. A linear regression analysis, adjusted for smoking, including the SNP (in an additive model), ICS use and the SNP-by-ICS interaction, was performed on expression levels of selected probes. Only gene expression probes with a probe midpoint located within 250kb of a replicated SNP were selected for the analysis. A total number of 11 probes was selected for the analysis based on availability of SNPs and probes.
Table E1: Overview of the inclusion characteristics, ethnicity and study methods of the discovery (DAG) and replication (EGEA, ECRHS) cohorts.

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<th>DAG cohort</th>
<th>EGEA</th>
<th>ECRHS</th>
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<tr>
<td>Number of participants</td>
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<td>318</td>
<td>239</td>
</tr>
<tr>
<td>Study design</td>
<td>Case-control</td>
<td>Longitudinal case-control and family study</td>
<td>Case-control</td>
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<tr>
<td>Ethnicity</td>
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<td>Caucasian</td>
<td>Caucasian</td>
</tr>
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<td>Population included</td>
<td>93% Adult asthmatics + BHR</td>
<td>Adult asthmatics + BHR</td>
<td>Adult asthmatics + BHR</td>
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<td>Definition of lung disease</td>
<td>Asthma doctor diagnosis + BHR</td>
<td>Questionnaire; Symptoms</td>
<td>Questionnaire; Symptoms or asthma medication</td>
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<td>Agent used for BHR</td>
<td>Methacholine + histamine</td>
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<td>Severity phenotype</td>
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<td>Slope</td>
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<td>Covariates used</td>
<td>Smoking + ICS</td>
<td>Smoking + ICS</td>
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<td>CHIP</td>
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BHR: Bronchial Hyperresponsiveness; ICS: Inhaled corticosteroids
Table E2: Patient characteristics of the discovery cohort (DAG) and replication cohorts (ECRHS and EGEA)

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<td>65 (41)</td>
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<td>Age, years</td>
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<tr>
<td>Height, m</td>
<td>1.73 ± 0.10</td>
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<td>Weight, kg</td>
<td>78 ± 17</td>
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<tr>
<td>Smoking</td>
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<td>Current</td>
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<td>Ex</td>
<td>89 (56)</td>
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<td>Packyears, years</td>
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<tr>
<td>FEV₁/FVC</td>
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<td>FEV₁,% predicted</td>
<td>78 ± 22</td>
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<td>FVC% predicted</td>
<td>108 ± 5</td>
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<tr>
<td>Slope (ln)</td>
<td>3.2 ± 1.7</td>
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<td>Allergy, % of total</td>
<td>85</td>
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n (%), mean ± standard deviation, median [range]

Allergy was based on positive skin prick test
Table E3: All replication results with data from the discovery cohort. A: Effected allele frequency of all cohorts and location on the genome. B: Data shows SNP main effect on BHR severity in asthmatics not using ICS, asthmatics using ICS and interaction term ICS x SNP.

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<th>CHR</th>
<th>Position (bp)</th>
<th>SNP</th>
<th>Effect allele</th>
<th>DAG Effect allele frequency</th>
<th>EGEA Allele frequency</th>
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Table E3: All replication results with data from the discovery cohort. A: Effected allele frequency of all cohorts and location on the genome. B: Data shows SNP main effect on BHR severity in asthmatics not using ICS, asthmatics using ICS and interaction term ICS x SNP.

### Nominal replicated results

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<th>rs6429464</th>
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<tr>
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### Results in the same direction as the DAG cohort but not significant

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<table>
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<th>SNP</th>
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<td>SNP effect no ICS</td>
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<tr>
<td>Interaction effect*</td>
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<td>0.04</td>
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</table>
Results in the opposite direction as the DAG cohort in one or both of the replication cohorts

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<th>Beta (SE) no ICS</th>
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<th>Interaction effect*</th>
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