**Integration of transcriptomic and genomic data identifies *LTBP1* as a novel locus involved in the response to glucocorticosteroids**

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**ABSTRACT**

**Background.** Inhaled corticosteroids (ICS) are the most commonly prescribed medications to control asthma symptoms. Environmental and genetic factors are involved in the variability in response to ICS, but few genetic markers are described.

**Objective.** To identify novel markers of ICS response by analyzing transcriptomic data and undertaking differential gene expression analysis in patients whose exacerbations were/were not controlled with ICS .

**Methods.** Transcriptome data from ASM cells treated *in vitro* with glucocorticosteroids (GCs) were analysed. Genes with changes in expression levels after GCs exposure were examined in peripheral blood mononuclear cells (PBMCs) from patients whose exacerbations were/were not controlled on ICS. Validation was sought using three publicly available ASM transcriptomic datasets. Genes with evidence of differential expression in response to GCs were tested for association with presence of exacerbations despite ICS in European (n=2,681) and admixed (n=1,347) children and youth.

**Results.** Six genes showed significant changes in expression levels after GCs treatment in ASM cells and PBMCs from patients controlled using ICS. Evidence of overexpression of four of them was validated in independent transcriptomic datasets of ASM exposed to GCs. Variants within *LTBP1* were associated with control using ICS in Europeans (*p* = 3.28x10-6) and in admixed populations (*p* = 6.76x10-5).

**Conclusions.** Overexpression of *LTBP1* was detected in ASM cells after GCs exposure and in PBMCs from patients controlled using ICS. Genetic variants of *LTBP1* were also found to be associated with increased exacerbations despiteICS use in children and young adults from different populations.

**Keywords:** Airway smooth muscle cells, childhood asthma, exacerbations, glucocorticosteroids, peripheral blood mononuclear cells, pharmacogenomics, transcriptome.

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**INTRODUCTION**

Asthma remains the most common childhood chronic disease. While there are increasing therapeutic options, inhaled corticosteroids (ICS) are still the most commonly prescribed, and effective, controller medication for this disease [1, 2]. The majority of asthmatic children treated with ICS show an improvement in symptoms, but30-40% do not respond [3]. Substantial differences in response to asthma treatment with ICS have also been reported among different populations and ethnic groups [3, 4]. While adherence is well recognized as a reason for treatment failure, it can occur despite optimal usage.

Beyond adherence, the variability in ICS response has been suggested to be the result of the interaction of multiple factors including clinical phenotypes, ancestry, environmental, and genetic factors [6, 7]. A high contribution of the individual genetic composition in ICS response has been attributed through heritability estimates of 40-60% [8]. However, to date, the number of genetic markers identified to be involved in ICS response is limited and they are not sufficient to predict if an individual will achieve control of their asthma using ICS [9]. Furthermore, there has been little exploration of potential molecular mechanisms related to variabale asthma control in those using ICS [5].

A broad spectrum of molecular mechanisms of action has been attributed to glucocorticosteroids (GCs), though many of them involve reduction of airway inflammation [2], through regulation of gene transcription [15]. Several cell types have been suggested to be the source of inflammatory mediators in asthma such as structural airways cells, including fibroblasts, epithelial cells, airway smooth muscle (ASM), endothelial, and epithelial cells [15]. Nevertheless, the action of GCs on other important asthma subphenotypes has been described, including airway muscle contractility, hyperresponsiveness, and bronchodilation [16, 17]. Therefore, it has been suggested that epithelial and ASM cells may be the major targets of GCs [18, 19], in addition to inflammatory cells (e.g.: eosinophils, lymphocytes, mast, and dendritic cells), but to a lesser extent [15]. ASM cells are involved in the major asthma-related phenotypes, though the specific molecular mechanisms of action of GCs on ASM cells are not well known [20]. A few studies have recently explored the transcriptome of ASM cells in response to GCs treatment by applying next-generation sequencing techniques to the analysis of the transcriptome (RNA-seq) [20, 21]. In the present study, we aimed to identify novel markers involved in response to GCs by integrating transcriptome data obtained from ASM and peripheral blood mononuclear cells (PBMCs) with genomic data from patients with different patterns of ICS response.

**METHODS**

**Exploration of changes in the transcriptome of ASM cells in response to GCs**

Differential expression analyses were performed on RNA-seq data obtained from ASM cells isolated from four lung transplant donors of European ancestry publicly available at the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) (accession number SRP033351) [20]. This study performed *in vitro* experiments on cell lines from each donor treating them with control solution (n=4) or dexamethasone for 18 h (n=4) [20] and carried out RNAseq to analyze the effect of treatment in gene expression [20].

RNA-seq raw FASTA files were downloaded from SRA and the subsequent analyses were performed with a custom pipeline in R 3.4.4 [22], including initial quality control (QC) analysis based on parameters obtained with the FastQC 0.11.8 software [23]. Alignment to the GRCh37/hg19 reference genome was performed for paired-end reads using the *Rsubread* package [24, 25]. Mapped sequence reads were assigned to genomic features at gene-level using the *featureCounts* program implemented in *Rsubread* [26]. Counts per million (CPM) were obtained using *edgeR* [27] to filter out very low expressed genes corresponding to those with 10 or fewer read counts (CPM ≤0.3). Retained genes with CPM >0.3 were normalized according to library sizes using the trimmed mean of M values (TMM) method implemented in *edgeR* [28]. Subsequently, mean-variance modeling at the observational level (VOOM) transformation was applied to filter and normalize counts [29]. Evaluation of differential gene expression levels in cells treated with GCs compared to those treated with control solution was performed using linear models with the package LIMMA [30]. Differentially dysregulated genes in response to GCs treatment were identified after multiple comparison adjustment with a false discovery rate (FDR) of 5% (*q*-value≤0.05) [31].

**Analysis of genes dysregulated by GCs treatment in PBMCs from asthma patients treated with ICS**

Genes differentially expressed in ASM cells treated with GCs were followed up to determine if they also showed changes in PBMCs obtained from asthma patients treated with ICS from the SLOVENIA study [32, 33]. For that, RNA was extracted from six children and young adults with asthma with regular use of ICS during the 12 precedent months of the study enrollment and libraries were sequenced using the BGISEQ-500 instrument (BGI Inc.). Based on data on emergency asthma care, hospitalizations, and/or administration of oral corticosteroids because of asthma symptoms in the past 12 months, patients were classified as ICS refractory (n=3), if they had a history of any of such events or non- refractory to ICS (n=3) if they did not experience any of those types of events (**Table S1)**. Further details are described in the Supplementary Material.

RNA-seq results obtained were analyzed following the same methodology as described above for the ASM cells. Genes with CPM >0.7 were included in differential expression analyses. To equate for fold change (FC) direction, differential gene expression was assessed for non-refractory patients relative to ICS refractory patients, assuming that the latter would show the same direction of expression changes as control ASM cells not exposed to GCs, which would imply that ICS treatment would not have an effect on them. FDR was used for multiple comparison adjustment accounting for the genes analyzed in this dataset.

**Validation of transcriptome changes in additional datasets from ASM cells**

Validation of significant differentially expressed genes found in both ASM cells treated with GCs and PBMCs from asthma patients treated with ICS was sought using additional datasets of gene expression profiles of ASM cells after GCs exposure (GSE13168, GSE34313, and SRP098649) (**Table S2**). Differential expression analyses comparing ASM exposed to GCs treatment or control solutions were performed using Reproducible Analysis and Validation of Expression Data (RAVED) (https://github.com/HimesGroup/raved) using the online tool Reducing Associations by Linking Genes And omics Results (REALGAR) (http://realgar.org/) [34, 35]. Differential expression results obtained from these three independent ASM transcriptomic datasets were combined in a meta-analysis by means of a random-effects model accounting for the variance among studies through the *metaVolcanoR* package [36]. Evidence of replication was considered for genes with significantly consistent changes in expression levels in cells treated with GCs relative to the controls (*q*-value≤0.05).

**Association of genetic variants within identified genes with ICS refractoriness in children and young adults with asthma**

The association of genetic variants located within 100 kilobases (kb) upstream or downstream from the genes with evidence of differential expression in all the transcriptomic datasets with ICS refractorinesswas explored. For that, ten studies participating in the Pharmacogenomics in Childhood of Asthma (PiCA) consortium [37] were analyzed. A total of eight European descent populations were included: the Pharmacogenetics of Asthma Medication in Children: Medication with Anti-inflammatory effects (PACMAN); the Paediatric Asthma Gene-Environment Study (PAGES); BREATHE; Genetics of the Scottish Health Research Register (GoSHARE); the Pharmacogenetics of Adrenal Suppression study (PASS); SLOVENIA; the follow-up stage of the Multicenter Asthma Genetics in Childhood Study (followMAGICS); Effectiveness and Safety of Treatment with Asthma Therapy in Children (ESTATe). Additionally, two admixed populations were also analyzed: Latinos/Hispanics from the Genes-Environment and Admixture in Latino Americans (GALA II) study, and African Americans included in the Study of African Americans, Asthma, Genes and Environments (SAGE) [33].

Children and young adults with asthma (2-25 years old) with available genome-wide genotype data, reported use of ICS, and data related to the ICS refractoriness based on the presence or absence of asthma exacerbations during the 6 or 12 months preceding the study enrollment were included (Supplementary Material). Further details have been described elsewhere [33]. Asthma exacerbations were defined by the need for emergency care, hospitalizations or administration of systemic corticosteroids because of asthma (**Table S3** and **Table S4**). Alternative definitions were used for those studies without available information regarding any of these events so that, such as unscheduled general practitioner or respiratory system specialist visits and school absences (**Table S3**). Individuals with asthma exacerbations despite ICS treatment were considered as ICS refractory and those without asthma exacerbations as non-refractory.

Association between imputed genetic variants and ICS refractoriness was tested for each study through Wald logistic regressions using EPACTS 3.2.6 [38]. Age, gender and principal components were included as covariates in the regression models. Two meta-analyses were performed based on the ancestry of the studies included: European and non-Europeans. Single nucleotide polymorphisms (SNPs) with a minor allele frequency (MAF)≥1% and imputation quality (Rsq)≥0.3 shared among the studies included in each group were meta-analyzed applying fixed-effects or random-effects models through the Cochran Q-test implemented on METASOFT [39]. A Bonferroni-like correction was applied accounting for the total number of independent variants tested within the gene regions evaluated. Evidence of replication was considered for those variants reaching the threshold established as α=0.05/total number of independent variants. For that, empirical autocorrelations based on the -log10 *p*-value of each SNP analyzed were estimated using *coda* R package [40].

**RESULTS**

**Differential gene expression analyses in airway smooth muscle cells in response to GCs**

An average of 48.8 million total reads was obtained per sample and an average of 81.9% of the reads was successfully mapped to the GRCh37/hg19 build of the reference genome (**Table S5**). A total of 14,707 genes were analyzed and 1,865 of them were found to be differentially expressed in cells treated with GCs compared to those exposed to control solution (*q-*value≤0.05) (**Table S6**).

**Analysis of changes in gene expression driven by GCs exposure in peripheral blood mononuclear cells from asthma patients treated with ICS**

Genes with evidence of altered expression levels after treatment with GCs were followed up for replication using PBMCs transcriptome profiles obtained from six patients treated with ICS from the SLOVENIA study (**Table S1**). From the 1,865 genes found to be differentially expressed in ASM cells after *in vitro* GCs exposure, a total of nine genes showed significant changes in expression levels depending on the ICS refractoriness status (*q*-value≤0.05) (**Table S7**). Of those, six genes showed consistent alteration in expression levels compared to samples taken as controls in both datasets based on log2 FC values (**Figure 1**, **Table S8**).

**Validation of transcriptome changes in response to GCs in additional datasets from ASM cells**

From the six genes with consistent changes in expression levels in response to GCs stimulation and related to ICS refractoriness, four genes were found to be overexpressed in cells experimentally treated with different GCs compared to those exposed to control solutions after combining the publicly available ASM transcriptomic dataset: *LTBP1* (*q*-value=7.45x10-4), *CALD1* (*q*-value=5.21x10-5), *WASF3* (*q*-value=4.02x10-7), and *TGFB1I1* (*q*-value=0.025) (**Table S9**, **Figure S1**).

**Association of genetic variants within identified genes with ICS refractoriness in children and young adults with asthma**

Association of 5,680 SNPs within 100 kb upstream and downstream from the genes *LTBP1*, *CALD1*, *WASF3*, and *TGFB1I1* with ICS refractoriness was evaluated in European children and young adults with asthma. After applying a Bonferroni-like correction considering the total number of independent variants tested across all the gene regions (*p*≤6.59x10-5 for 759 independent variants tested), the SNP rs11681246 located within *LTBP1* was significantly associated with ICS refractoriness in Europeans (OR for G allele: 0.72, 95% CI: 0.63-0.83, *p* = 3.28x10-6) (**Figure 2**). However, this SNP was not replicated in admixed populations. Nonetheless, we assessed the association of alternative polymorphisms in this genomic region in admixed populations, revealing the association of an intronic *LTBP1* variant, rs76390075 (OR for C allele: 0.40, 95% CI: 0.26-0.63, *p* = 6.76x10-5), after adjustment for 676 independent variants tested (*p*≤7.40x10-5) (**Figure 3**).

**DISCUSSION**

This study describes the results of transcriptomic analyses of several datasets to reveal candidate genes related to ICS refractoriness among asthma patients. After combining RNA-seq data from ASM cells treated with GCs with data from PBMCs from asthma patients with different patterns of ICS refractoriness, six genes showed consistent changes in expression levels. From these, overexpression of four genes after GCs exposure was validated in three independent ASM transcriptomic datasets. Furthermore, the implication of *LTBP1* in the response to GCs was validated by revealing the association of seven variants within this gene with ICS refractoriness among Europeans, Latinos/Hispanics, and African Americans. GCs were found to increase *LTBP1* expression levels in ASM cells experimentally exposed independently of the type of GCs. Similar effects were detected in PBMCs obtained from asthma patients non-refractory to ICS treatment, but not in ICS refractory patients. This suggests that ICS treatment could not have an effect on *LTBP1* expression in PBMCs from ICS refractory patients.

*LTBP1* encodes a member of the family of latent-transforming growth factor-beta (TGF-β) binding proteins. LTBP1 is involved in the regulation of the TGF-β1 activity [41], including its activation from a precursor form [42], folding, secretion out from the cell [43] and deposition at the extracellular matrix [44] through interactions with fibrillin molecules [45]. Interestingly, TGF-β1 has been proposed to play a key role in cell growth and differentiation, immune response, and airway remodeling [46]. Specifically, increased levels of the active form of TGF-β1 have been detected in asthma patients, which has been suggested to recruit myofibroblasts triggering an increased collagen deposition and the development of subepithelial fibrosis in asthma [47]. *LTBP1* has been also proposed to be involved in allergic diseases and idiopathic pulmonary fibrosis (IPF) [48], where LTBP1 has been found to interact with fibulin 1c (FBLN1), modulating lung remodeling and fibrosis through the regulation of TGF-β1 activation [48]. Additionally, TGF-β1 induces the conversion of fibroblasts to myofibroblasts, which is significantly reduced by means of inhibition of FBLN1. For this reason, the inhibition of FBLN1 binding to LTBP1 has been proposed as a therapeutic strategy to reduce fibrotic processes [48]. Additionally, *LTBP1* has been associated with lung function measurements among participants from the UKBiobank (http://www.nealelab.is/uk-biobank/) [49]. These findings support that *LTBP1* could play an important role in asthma-related phenotypes.

Interestingly, the variants rs11681246 and rs76390075 found to be associated with asthma exacerbations in patients treated with ICS from European and admixed populations, respectively, have been suggested to be involved in the regulation of gene expression in pulmonary cells, according to the Encyclopedia of DNA Elements (ENCODE) [50, 51]. The SNP rs11681246 has been related to histone marks such as the monomethylation of histone H3 at lysine 4 (H3K4me1) and acetylation of histone H3 at lysine 27 (H3K27ac) in several cell lines, including fetal fibroblasts and adult lung fibroblast primary cells. Moreover, this has been proposed to be a DNAse hypersensitivity site in pulmonary fibroblast primary cells [50, 51]. On the other hand, the SNP rs76390075 is in linkage disequilibrium (r2 = 1) with several variants with a potential functional role in pulmonary tissues, including epigenetic modifications such as the acetylation of histone H3 at lysine 9 and, H3K4me1 and H3K27ac marks (rs149277643, rs3769528, rs75486357, rs3820912 and, rs182443958), and the location at DNAse hypersensitivity sites in fetal fibroblasts and pulmonary fibroblast primary cells [50, 51].

To the best of our knowledge, our study is one of the few integrating different omics layers to identify genetic markers of ICS response [12, 52, 53]. The strengths of our study include the assessment of several different transcriptomic datasets, the fact that association analyses were carried out using data from the largest consortium studying the pharmacogenetic factors involved in asthma treatment response in children and youth, and the analysis of populations with different ancestries. This contrasts with previous studies, which have explored the association of a low number of variants in reduced sample sizes [9, 33, 54].

Nonetheless, some limitations need to be acknowledged. First, gene expression levels in response to GCs or control exposure were compared between reduced groups of ASM cells in the discovery phase of this study. Moreover, these cells were obtained from non-asthmatic individuals belonging to European-descent males, not representing the two genders and different ethnic groups [20]. Additionally, the sample size analyzed in the transcriptomic set of PBMCs was also reduced. Second, gene expression profiles obtained using microarrays and RNA-seq assays were compared, even though many discrepancies have been attributed between these approaches [55]. We attempted to avoid biases by analyzing the two sources of data in different stages of the analyses. Third, transcriptome data from different cell types were compared, although cell specificity has been attributed to the effects of GCs [56]. Fourth, transcriptomic datasets obtained by means of different study designs were analyzed, including cells experimentally exposed to GCs and those extracted from patients under ICS therapy. Despite all these limitations, overall consistencies in changes in gene expression levels in response to GCs were found among ASM cells and PBMCs.

In summary, our study revealed *LTBP1* as a novel locus for ICS refractoriness in asthma patients. These results indicate that integrating publicly available data from different omic sources could be a powerful approach to provide novel insights about the mechanisms involved in the response to ICS treatment and thus, to develop alternative diagnosis and therapeutic strategies that could improve asthma management strategies in clinical practice.

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**FIGURE LEGENDS**

**Figure 1. Dot plots of differential expression for genes with evidence of changes in expression levels in response to GCs in ASM cells and PBMCs.** (A) *LTBP1*; (B) *CALD1*; (C) *KCNMA1*; (D) *CPT1A*; (E) *WASF3*; (F) *TGFB1I1*. Gene expression levels are represented in terms of the logarithm base 2 of counts per million (CPM) (log2 CPM) in the *y*-axis as dots for cases (red) and control (blue) samples. ASM cells treated with glucocorticosteroids (GCs) were considered were compared to cells exposed to control solutions. Gene expression levels in PBMCs from ICS non-refractory patients were compared to cells from ICS refractory patients. The median of expression levels is represented for each sample group by a black horizontal line. *P*-values adjusted by false discovery rate are shown (*q*-value).

**Figure 2. Association results with ICS refractoriness for *LTBP1* in European populations.** A) Forest plot of association effects SNP rs11681246 across the European studies included in the association analyses: association effects are shown in terms of odds ratio (OR) for the effect allele (G) for each study and after performing a meta-analysis of the results by black boxes and a blue diamond, respectively; the 95% Confidence Intervals (95% CI) are represented by black dash lines; results are not provided for BREATHE since rs11681246 did not pass quality control checks in this study. B) Regional plot of association results with ICS refractoriness: the logarithmic transformation of the association results (-log10 *p*-value) is represented in the *y*-axis by chromosome position (*x*-axis) for each SNP as a dot; the most significant variant after Bonferroni-like correction is represented by a diamond; the remaining SNPs are color-coded based on pairwise linkage disequilibrium (*r*2 values) with that SNP for European populations from 1KGP (GRCh37/hg19 build).

**Figure 3. Association results with ICS refractoriness for *LTBP1* in Latinos/Hispanics and African Americans.** A) Forest plot of association effects of rs76390075 with ICS refractoriness in admixed populations; association effects are shown in terms of odds ratio (OR) for the effect allele (G) for each study and after performing a meta-analysis of the results for admixed populations by black boxes and a blue diamond, respectively; the 95% Confidence Intervals (95% CI) are represented by black dash lines. B) Regional plot of association results of *LTBP1* variants with ICS refractoriness in admixed populations: the *y*-axis represents the logarithmic transformation of the association results (-log10 *p*-value) by chromosome position (*x*-axis) for each SNP as a dot; the diamond corresponds to the most significant variant after Bonferroni-like correction; the remaining SNPs are color-coded based on pairwise linkage disequilibrium (*r*2 values) with that SNP for Admixed American populations from 1KGP (GRCh37/hg19 build).