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Elena Goleva, PhD, Denise C. Babineau, PhD, Michelle A. Gill, MD, PhD, Leisa P. Jackson, BS, Baomei Shao, BS, Zheng Hu, BS, Andrew H. Liu, MD, Cynthia M. Visness, PhD, MPH, Christine A. Sorkness, PharmD, Donald Y.M. Leung, MD, PhD, Alkis Togias, MD, William W. Busse, MD



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**Expression of Corticosteroid Regulated Genes By Peripheral Blood Mononuclear
Cells in Children with Asthma**

Elena Goleva, PhD¹, Denise C. Babineau, PhD², Michelle A. Gill, MD, PhD³, Leisa P.
Jackson, BS¹, Baomei Shao, BS³, Zheng Hu, BS³, Andrew H. Liu, MD¹, Cynthia M.
Visness, PhD, MPH², Christine A. Sorkness, PharmD⁴, Donald Y.M. Leung, MD, PhD¹,
Alkis Togias, MD⁵, William W. Busse, MD⁴

¹National Jewish Health, Denver, CO

²Rho Federal Systems Division, Chapel Hill, NC

³University of Texas Southwestern Medical Center, Dallas, TX

⁴University Wisconsin School of Medicine and Public Health, Madison, WI

⁵National Institute of Allergy and Infectious Diseases, Bethesda, MD

Corresponding Author:

Elena Goleva, PhD

National Jewish Health

1400 Jackson St., K1016A

Denver, CO 80206, USA

Tel: (303) 398-1258

Fax: (303) 398-1225

E-mail: golevae@njhealth.org

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Abstract

Background: Variability in response to inhaled corticosteroids may result in less than optimal asthma control and development of biomarkers assessing therapeutic efficacy of corticosteroids is important.

Objective: To examine whether *in vitro* peripheral blood mononuclear cell (PBMC) responses to corticosteroids relate to the clinical inhaled corticosteroid response.

Methods: PBMC were collected from 125 children with asthma (6-17 years) at enrollment (Visit 0, V0) and after one year of bimonthly guidelines-based management visits (Visit 6, V6). Difficult- versus easy-to-control asthma were defined as requiring daily therapy with ≥ 500 μg of fluticasone propionate (FLU) with/without a long-acting beta-agonist versus requiring ≤ 100 μg of FLU in at least 4 visits. mRNA levels of glucocorticoid receptor alpha (GRalpha), corticosteroid transactivation (FK binding protein 5 (FKBP5)) and transrepression markers (IL-8, TNFalpha) were measured by RT-PCR in freshly isolated cells and in response to 10^{-8} M FLU.

Results: Compared to easy-to-control, PBMC of difficult-to-control asthma had significantly lower GRalpha at V0 ($p=0.05$). A 30% increase in IL-8 suppression by FLU ($p=0.04$) and a trend for increased TNFalpha suppression by FLU between V0 and V6 ($p=0.07$) were observed in easy-to-control asthma. In contrast, no changes between V0 and V6 in the IL-8 and TNFalpha suppression by FLU were observed in difficult-to-control asthma. Corticosteroid-mediated transactivation (FKBP5 induction by FLU) increased in the PBMC of difficult- and easy-to-control asthma between V0 and V6 ($p=0.05$ and $p=0.03$ respectively).

Conclusions: PBMC of children with difficult-to-control asthma treated with guidelines-

63 based therapy and requiring high dose inhaled corticosteroids had reduced *in vitro*
64 responsiveness to corticosteroids.

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Key messages: This is the first study that has evaluated *in vitro* PBMC responsiveness to corticosteroids in children with asthma over the course of one year of guidelines-based therapy. In the children with difficult-to-control asthma, we observed reduced responsiveness to corticosteroids manifested by reduced GR α expression and a defect in GR-mediated inhibition of proinflammatory cytokines.

Capsule summary: PBMC based assays can be used to monitor *in vivo* responsiveness to inhaled corticosteroids. Children with difficult-to-control asthma have reduced levels of glucocorticoid receptor and less efficient *in vitro* inhibition of proinflammatory cytokines by corticosteroids despite optimized therapy.

Key words: asthma, steroid resistance, corticosteroids, glucocorticoid receptor.

Abbreviations: APIC - Asthma Phenotypes in the Inner City, GR α – glucocorticoid receptor α , FLU – fluticasone propionate, FKBP5 – FK binding protein 5, ICS – inhaled corticosteroids, IL-8 – interleukin 8, LABA – long-acting beta agonist, PBMC – peripheral blood mononuclear cells, TNF α – tumor necrosis factor α , V0 – Visit 0, V6 – Visit 6.

Introduction

Asthma is a heterogeneous disease, and for some patients, particularly those with severe disease, existing therapies have limited efficacy. Inhaled corticosteroids (ICS) are recommended as the main anti-inflammatory and controller medication for asthma management, with an escalation in dose, in combination with long-acting beta agonists (LABA) or leukotriene antagonists, as asthma becomes more severe¹. For most patients with mild to moderate asthma, disease control can be achieved with this approach. For patients with more severe disease, who continue to experience asthma symptoms, exacerbations and poor lung function while receiving ICS-containing controller therapies, there are numerous potentially contributing factors such as allergy, tobacco smoke exposure, co-morbidities, controller adherence^{2, 3}, and immunologic insensitivity to corticosteroids⁴.

No peripheral blood biomarkers exist that are capable of predicting clinical response to ICS in asthmatics. In addition, it is not known whether poor *ex vivo* corticosteroid responsiveness is associated with difficult-to-control asthma in children. The objective of this study was to validate a panel of *in vitro* assays based on a group of corticosteroid regulated gene targets in peripheral blood mononuclear cells (PBMC) recently developed by our research group⁵. Data generated from these assays were analyzed for associations with clinical inhaled corticosteroid responsiveness among a subgroup of participants in the NIAID-funded Inner City Asthma Consortium's Asthma Phenotypes in the Inner City (APIC) study^{2, 6, 7} that were evaluated at the National Jewish Health and University of Texas Southwestern Medical Center sites.

Methods

Study participants. 125 children with asthma (6-17 years) were recruited for the NIAID-funded, Inner City Asthma Consortium's APIC study at National Jewish Health (Denver, CO) and University of Texas Southwestern Medical Center (Dallas, TX) sites. Participants underwent a baseline assessment and received guidelines-based management for one year with visits every two months⁶. Inclusion criteria for this study included physician diagnosis of asthma as well as having had 2 or more episodes of short-acting beta-agonist administration within the past 12 months, exclusive of use associated with exercise-induced symptoms. All children had spirometry assessed at screening, but the results had no bearing on inclusion into the study. This definition of asthma allowed enrollment of children with a wide spectrum of asthma severity. At screening, study clinicians determined the appropriate asthma control regimen that each participant would receive during a 4 week run-in period using the participants medical history, physical exam results, symptoms, number of systemic steroid bursts, percent predicted FEV₁ and level of therapy at the time of enrollment into the study. A predefined, computerized NAEPP Expert Panel Report-3 based treatment algorithm for asthma was used by the study clinicians to adjust the participant's controller regimen at each visit based on asthma symptoms, spirometry results and recent exacerbations¹.

Difficult-to-control asthma was defined as requiring daily therapy with 500 µg of fluticasone propionate (FLU) or greater with or without LABA on at least 4 visits. Easy-to-control asthma was defined as requiring daily therapy with 100 µg of FLU or less assigned on at least 4 visits⁶. Children who did not fit in either of these two categories were assigned to a category termed indeterminate asthma⁶. Assessments involving

demographics, family history, allergy history, asthma history, environmental exposures, body mass index (BMI), stress, allergy sensitization, allergic inflammation, pulmonary physiology, asthma severity and rhinitis severity were also captured at baseline with some outcomes assessed longitudinally over the 12-month observational and management period. Blood samples were collected at study enrollment (Visit 0, V0) and at 12 months (Visit 6, V6). The study was approved by the Institutional Review Boards at National Jewish Health and University of Texas Southwestern Medical Center.

Specimen collection and culture. PBMC were isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation⁵. Cells were lysed immediately in RLT RNA lysis buffer (Qiagen, Valencia, CA) for the analysis of gene expression in freshly isolated cells or cultured in medium (RPMI containing 10% AB+ serum, 50µg/ml streptomycin, and 50 units/ml penicillin) with 10^{-8} M FLU or medium without FLU for 3 hours. The cells were then harvested and stored in the RNA lysis buffer at -80°C.

Real-time PCR. Total RNA was extracted using RNeasy Mini Kit (Qiagen), reverse-transcribed into cDNA, and analyzed by real-time PCR using the dual-labeled fluorogenic probe method (ABI Prism 7300 sequence detector by Applied Biosystems). The mRNA expressions of glucocorticoid receptor alpha (GRalpha), Vitamin D 24-Hydroxylase (cytochrome P450 Family 24 Subfamily A Member 1 (cyp24a)), FK506 binding protein 5 (FKBP5) (levels in freshly isolated cells and fold induction in response to 10^{-8} M FLU), interleukin 8 (IL-8) (levels in freshly isolated cells and fold suppression in response to 10^{-8} M FLU), tumor necrosis factor alpha (TNFalpha) (levels in freshly isolated cells and fold suppression in response to 10^{-8} M FLU) were analyzed. Standard curves for all targets were generated using the fluorescent data from two-fold serial

dilutions of 500 ng total RNA of the target sample. GRalpha standard curve was generated from ten-fold serial dilutions of the GRalpha plasmid⁸.

Statistical analysis. Within each group of study participants (difficult-to-control, easy-to-control and indeterminate), a one-sample Wilcoxon test was used to compare fold change between V0 and V6 in gene transcripts expression in PBMC. Gene transcripts expression in PBMC at V0 or fold change in gene transcripts expression in PBMC between V0 and V6 were compared between groups of interest using linear regression models that adjusted for age, race, sex, total serum IgE based on blood drawn at V0 (log-transformed), number of positive aeroallergen skin tests based on 12 common indoor and outdoor allergens, serum Vitamin D at V0 (log-transformed), cyp24A expression level at V0 (log-transformed), BMI percentile at Screening (1 month before V0) or V6 (as applicable), urine cotinine as measured by NicAlertTM test strips (Nymox Pharmaceutical Corp.) at Screening or V6 (as applicable), and anxiety level as measured by Beck Anxiety Scale at V0 or V6 (as applicable). NicAlert[®] was used in this study for the semi-quantitative determination of cotinine in urine to determine participant exposure to tobacco products such as cigarettes, pipes, or chewing tobacco within the past 48 hours. V0 PBMC expressions of the relevant biomarkers were also included as covariates in regression models of fold change between V0 and V6. Groups that were compared included easy-to-control versus difficult-to-control, easy-to-control versus indeterminate, difficult-to-control versus indeterminate, easy-to-control versus the combined difficult-to-control and indeterminate groups, and difficult-to-control versus the combined easy-to-control and indeterminate groups. Figures only report statistically significant group comparisons at a 0.05 significant level. No adjustment to the

178 significance level was made for multiple comparisons between groups.

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Results

Study participants

Out of the 125 children in the two APIC sites that participated in this study, 95 paired PBMC samples [easy-to-control (n=31), indeterminate (n=19), difficult-to-control (n=45)] from V0 and V6 were available for the analysis of the *in vitro* responsiveness of PBMC samples to FLU. Of the 30 participants without paired PBMC samples, blood samples were not collected at either V0 or V6 for 13 participants and data from 17 participants was excluded due to the low yield of PBMC or RNA at either V0 or V6. Clinical characteristics of the 95 participants included in the analysis are summarized in **Table 1** and **Table E3**. No statistically significant differences in clinical characteristics between the 95 participants included in the analysis and the 30 participants excluded from the analysis were observed (data not shown). The majority of participants were male Hispanics and non-Hispanic blacks. Fifty-five percent of the participants with easy-to-control asthma did not require any controller medication at V6, while only 23% of participants in this group did not require controller medication at V0. The majority of participants with difficult-to-control asthma required high dose ICS or ICS/LABA at V0 and V6. At V0 and V6, the majority of participants in the easy-to-control group had asthma control level 1, while only approximately half of those in the difficult-to-control group were at that control level.

Children with difficult-to-control asthma have significantly lower GRalpha at V0 compared to children with easy-to-control asthma

Analysis of GRalpha expression by freshly isolated PBMC revealed that difficult-

to-control had significantly lower expression of GRalpha mRNA at V0 than easy-to-control asthma ($p=0.05$) (**Fig. 1**). In addition, GRalpha expression at V0 in the combined difficult-to-control and indeterminate asthma groups was significantly lower than in easy-to-control ($p=0.037$). GRalpha mRNA expression at V0 was not associated with treatment step level at V0 ($p=0.72$; **Fig. E1**). Within difficult-to-control asthma, no difference was observed in GRalpha mRNA expression at V0 between participants with asthma control level 1 and 2 versus 3 and 4 ($p=0.37$; data not shown).

GRalpha mRNA expression decreased between V0 and V6 in easy-to-control participants (geometric mean of fold change= 0.76 ± 1.59 ; $p=0.004$) but remained the same over time in difficult-to-control and indeterminate groups ($p=0.052$ and $p=0.49$ respectively). The fold change in GRalpha mRNA expression between V0 and V6 was not associated with treatment step level at V6 ($p=0.92$; **Fig. E2**).

Improvement of corticosteroid-mediated transrepression in PBMC of children with easy-to-control asthma and lack thereof in difficult-to-control asthma at V6

Assessment of the GRalpha transrepression function in easy-to-control participants' PBMC revealed a 30% increase between V0 and V6 in FLU suppression of IL-8 ($p=0.04$; **Fig. 2**) and a trend for increased FLU suppression of TNFalpha between V0 and V6 ($p=0.07$, **Fig. 3**). In contrast, no changes between V0 and V6 in the IL-8 and TNFalpha suppression by FLU were observed in participants with difficult-to-control asthma ($p=0.27$, **Fig. 2**; $p=0.69$, **Fig. 3**). Compared to easy-to-control asthma, the fold change between V0 and V6 in IL-8 and TNFalpha fold suppression by FLU was significantly lower in difficult-to-control asthma ($p=0.023$, **Fig. 2**; $p=0.001$, **Fig. 3**). Within

difficult-to-control asthma, no difference was observed in the fold change between V0 and V6 in IL-8 and TNFalpha suppression by FLU in participants with asthma control levels 1 and 2 vs. 3 and 4 ($p=0.52$ and $p=0.65$ respectively; data not shown).

Selective changes in TNFalpha expression by PBMC in study participants at V6

TNFalpha expression by PBMC significantly decreased between V0 and V6 in both easy- and difficult-to-control groups ($p<0.001$; **Fig. 4**) but this decrease was not observed in the indeterminate group ($p=0.11$; **Fig. 4**). Compared to both easy- and difficult-to-control groups, the indeterminate group had a significantly higher fold change between V0 and V6 in TNFalpha expression ($p=0.035$ and 0.008 respectively; **Fig. 4**).

Improvement in corticosteroid-mediated transactivation in PBMC of children with easy- and difficult-to-control asthma at V6

No difference in FKBP5 mRNA expression was found in PBMC of easy- and difficult-to-control asthma patients at V0 (ng FKBP5/ng 18s RNA geometric mean $\pm$ SD, 1123.01 ± 1.77 vs. 973.22 ± 2.32 for easy- and difficult-to control asthma). A significant decrease between V0 and V6 in FKBP5 expression by freshly isolated PBMC was observed in both the easy- and difficult-to-control asthma (geometric mean of fold change= 0.57 ± 2.37 , $p=0.002$ and 0.53 ± 2.53 , $p<0.001$, respectively; **Fig. E3**). At the same time, both groups had significant increases between V0 and V6 in FKBP5 expression in response to FLU ($p=0.03$ and $p=0.05$, respectively; **Fig. 5**). No change between V0 and V6 in FKBP5 induction by FLU was observed in indeterminate asthma

($p=0.57$; **Fig. 5**). Compared to the combined easy to-control and indeterminate asthma groups, the fold change between V0 and V6 in FKBP fold induction by FLU was significantly higher in difficult-to-control asthma ($p=0.04$; **Fig. 5**). Within difficult-to-control asthma, the fold change between V0 and V6 in FKBP5 induction by FLU was not significantly different in those with asthma control levels 1 and 2 versus 3 and 4 ($p=0.18$; data not shown).

Discussion

Clinical trials have highlighted the potential for blood-based biomarkers to guide treatment for patients with asthma⁹⁻¹¹. Such an approach could allow a transition of treatment guidelines from recommendations based on asthma severity to biomarker-based recommendations that increase treatment precision. Identification of asthma endotypes with a distinct functional or pathobiological mechanism would provide a rational basis for new and personalized treatments¹²⁻¹⁵.

Pediatric severe asthma is considered to be a spectrum, which includes patients with refractory, exacerbation prone and therapy resistant asthma^{16, 17}. To date there are no standardized approaches on how to define severe therapy resistant pediatric asthma^{18, 19}. Clinical steroid responsiveness can be measured in many ways, but currently, there is no accepted definition for pediatric asthma. It is estimated that up to 5% of pediatric asthma patients have severe steroid resistant asthma^{18, 19}. Studies emphasize the utility of multidomain assessment of severe pediatric asthma patients for further phenotyping and molecular profiling of their disease²⁰.

Corticosteroids are used as first line anti-inflammatory medications in asthma and other inflammatory diseases. Chronic use of high-dose corticosteroids is commonly associated with adverse side effects²¹. Patients treated with corticosteroids may develop reduced corticosteroid sensitivity and even resistance²¹. At the cellular level, corticosteroids mediate their function through intracellular glucocorticoid receptor alpha (GRalpha), which belongs to a large family of transcriptional factors called nuclear hormone receptors⁴. In the absence of corticosteroids, GRalpha is maintained in the cytoplasm as a heterocomplex with molecular chaperons. Corticosteroids diffuse across

the plasma membrane and bind GR α , which induces rearrangement of the GR α heterocomplex, GR α homodimerization and nuclear translocation²².

Anti-inflammatory actions of corticosteroid medications are GR α -mediated, which suppresses inflammation through inhibitory mechanisms known as transactivation or transrepression²². In the presence of corticosteroids, GR α translocates into the nucleus and binds to DNA at glucocorticoid responsive elements to regulate the expression of various anti-inflammatory genes (transactivation) such as glucocorticoid-induced leucine zipper (GILZ), mitogen-activated kinase phosphatase-1 (MKP-1), the β 2-adrenoceptor, or inhibitor of NF- κ B (I κ B α), FK binding protein (FKBP5) among others²³. Transrepression results from a direct interaction and tethering of activated GR α with transcription factors such as NF- κ B, AP-1, interferon regulatory factor-1 in the nucleus, thus repressing the transcription of pro-inflammatory genes²². Activated GR α also regulates gene repression by competing with co-activators and/or recruiting the transcriptional co-repressor, HDAC2, which favors condensation of the chromatin structure through the deacetylation of histones^{24, 25}.

Despite the significant progress made in the field, the exact molecular mechanisms mediating corticosteroid insensitivity in patients with severe asthma are not known. Potential mechanisms have been described mostly in immune cells including alveolar macrophages or circulating PBMC derived from patients with corticosteroid insensitivity. These studies demonstrated that immune cells from patients with corticosteroid resistant or severe asthma exhibit an *ex vivo* diminished sensitivity to corticosteroid treatment^{5, 24, 26-29}. GR α phosphorylation, which occurs on three major serine residues (ser203, ser211, and ser226) located on its N-terminus, regulates key

GR α functions, including receptor turnover, subcellular trafficking, and activity in a cell- and gene-specific manner³⁰. Corticosteroid-induced GR α phosphorylation on ser211 appears to be essential for driving its transcriptional activity, while phosphorylation at ser203 or ser226 represses GR α function³¹. This insensitivity was evidenced by corticosteroid failure to stimulate GR α nuclear translocation²⁶ and histone acetylation²⁴, a reduction in GR α binding activity, possibly due to GR α phosphorylation via the p38 MAPK pathway or a decreased level of GR α . Inhibition of GR α activity by the dominant negative isoform GR β ²⁶, by cytokine-activated transcription factors STAT5 and AP-1³² or decreased HDAC2 recruitment to GR α has been documented²⁵. However, the exact mechanisms involved in corticosteroid insensitivity in patients with severe asthma are still undefined.

In our study, we followed the expression of corticosteroid-regulated targets and *in vitro* response to FLU treatment in PBMC of children with asthma at baseline and after a year of guidelines-based therapy. We have selected gene targets that are known to be regulated by corticosteroids through transactivation and transrepression mechanisms to estimate whether there was selective changes in the GR α -mediated transactivation and transrepression in easy- vs. difficult-to-control asthma patients. To our knowledge, this is the first study that has performed such an analysis. We found that the expression of corticosteroid-regulated targets in PBMC significantly differs between easy- and difficult-to-control asthma and may be reflective of corticosteroid insensitivity in the children with difficult-to-control asthma (**Table 2**).

Several significant observations were made. Compared to easy-to-control asthma, we observed reduced GR α expression in PBMC at V0 of children with

difficult-to-control asthma. This reduced GR α mRNA expression was not related to the inhaled corticosteroid medication burden at V0, as no difference in GR α expression was observed across different step levels of treatment. Reduced GR α levels may influence efficacy for corticosteroid-mediated transcription in difficult-to-control asthma. GR α levels are a major factor in determining sensitivity to corticosteroids and a critical factor regulating corticosteroid mediated transcriptional programs. Studies have demonstrated that cells containing higher GR α levels are primed, through DNA-loading of preformed GR α dimers, to respond to corticosteroids at much lower concentrations. This response is due to the increased affinity and cooperative corticosteroid-binding brought by corticosteroid-independent dimerization of GR α ³³. It has also been reported that the rate of GR α nuclear import is significantly slower at low GR α concentrations in the presence of ligand, while at the same time, there is an increased rate of GR α export out of nuclei at low GR α concentrations³⁴. This implies that tissues with high concentrations of GR α respond more rapidly to corticosteroids due to their increased rate of nuclear import and maintain nuclear localization for longer following corticosteroid withdrawal because of their slower rate of nuclear export. Compared to tissues with low GR α concentrations, these features contribute to enhanced sensitivity to corticosteroids³⁵. GR α expression decreased between V0 and V6 in the easy-to-treat asthma group but there was no observed difference in GR α expression between V0 and V6 in the indeterminate- and difficult-to-control groups, despite significantly higher inhaled corticosteroid medication burden at V6. Whether or not this is a result of long-term maintenance high-dose ICS administration, type of steroid used or other stressors is

unclear. GR α promoter regulation, GR α protein expression and its phosphorylation study in easy-and difficult-to-control asthma patients require further investigation to understand this further.

We also found that over the one-year guidelines-based therapy, glucocorticoid-mediated transactivation increased in the cells of children with difficult-to-control and easy-to-control asthma, as shown by increased FKBP5 induction by FLU between V0 and V6. FKBP5 is an immunophilin chaperon protein, a subunit of the multiprotein GR α complex that resides in the cytoplasm before hormone binding. It plays a role in the intracellular trafficking of GR α maintaining the complex in the cytoplasm and impairs GR α nuclear translocation³⁶. FKBP5 is induced potently by corticosteroids and may function as a negative-feedback loop to limit GR α signaling. In the abundance of FKBP5, GR α /chaperone/FKBP5 complex has reduced interaction with the cytoskeletal filaments that are required for GR α nuclear translocation in response to corticosteroids and therefore GR α complex is retained in the cytoplasm³⁶. Of interest, high baseline expression of FKBP5 in bronchial epithelial cells prior to treatment with corticosteroids was associated with reduced clinical responsiveness to corticosteroids *in vivo*³⁷. In our study, we did not observe a significant difference in FKBP5 expression between easy- and difficult-to-control asthma groups at V0. However, a significant reduction in FKBP5 expression between V0 and V6 was observed in both easy- and difficult-to-control groups. This provides supporting evidence for enhanced GR α nuclear translocation in response to agonists even in difficult-to-control asthma.

Despite increased GR α nuclear translocation, as evidenced by enhanced

GR α transactivation at V6 (compared to V0) in difficult-to-control asthma (FKBP5 induction by FLU), glucocorticoid-mediated transrepression did not change, as no changes in TNF α and IL-8 suppression by FLU between V0 and V6 were observed. The study by Ito et al.²⁵ demonstrated that cells with reduced histone deacetylase 2 expression, despite normal GR α nuclear translocation in response to steroid agonist and normal GR α -mediated transactivation, did not suppress NF- κ B, a transcriptional factor that regulates a production of many proinflammatory mediators, including IL-8 and TNF α . Histone deacetylase-2 was required to deacetylate GR α in order to enable NF- κ B suppression²⁵. The function of histone deacetylases in difficult-to-control asthma warrants further investigation.

At the same time, a significant increase between V0 and V6 in IL-8 suppression by FLU and a trend for increase in TNF α suppression by FLU was observed in easy-to-control asthma. Despite their small magnitude, these changes in IL-8 and TNF α suppression by FLU are probably of biologic relevance as they suggest that cells from children with easy-to-control asthma may better handle insults from allergens, viruses, etc. by allowing suppression of inducible cytokines by corticosteroids.

Elevated serum IL-8 was recently reported to be associated with difficult-to-control asthma in African American children study population examined by the Inner City Asthma Consortium¹⁵. Importantly, we found that during the guidelines based therapy, constitutive TNF α expression by PBMC decreased in easy-to-control and difficult-to-control asthma but did not change in participants with indeterminate asthma. To understand why TNF α levels decrease in difficult-to-control patients after guidelines-based therapy, a systematic assessment of cellular responses to various

agonists (e.g., toll like receptors or cytokine receptors) is required.

GRalpha expression level may be an important determinant of the glucocorticoid response in difficult-to-control asthma. Glucocorticoid levels are strongly elevated in response to a variety of physical and psychological stressors. A recent publication reported that there is greater methylation in the GR promoter in children that are exposed to stress³⁸, which may result in reduced GRalpha expression. The region that is methylated is a putative site for a nerve growth factor-inducible protein A, NGFI-A (gene name - early growth response 1, EGR1). Of interest, EGR1 expression was found to be significantly upregulated in bronchioalveolar lavage samples from adult steroid resistant asthma patients by our research group in the past²⁷. GRalpha promoter methylation, levels of growth factors, like NGF and EGR1, and stress and environmental exposures (like smoking) in children with difficult-to-control asthma require further investigation and may be linked to reduced GRalpha expression in this group.

Some may question the APIC controller step level algorithm used (Table E1); in particular, Step Level 4: fluticasone 250 mcg twice daily (i.e., NAEPP-defined high-dose ICS) prior to adding LABA^{1, 6}. At the time of APIC study development, approval and the start of study enrollment in August 2011, there was greater concern about LABA safety in children (e.g., FDA black box warning) than there is today. There was also evidence in a NHLBI-sponsored "PACT" study that, different than studies in adults, higher-strength ICS (fluticasone 100 mcg BID) was more effective than combination ICS-LABA (fluticasone 100 mcg QD + salmeterol BID) in children with mild-moderate persistent asthma³⁹. This suggested at the time that there may be more to gain by increasing ICS than adding LABA.

Subsequently, the NHLBI-sponsored “BADGER” study demonstrated that, in children with uncontrolled asthma while receiving fluticasone 100 mcg BID, adding LABA was generally more effective than stepping up ICS dose to fluticasone 250 mcg BID⁴⁰. In 2016, the VESTRI study demonstrated LABA safety when combined with ICS⁴¹. In practice today, there is good reason to consider ICS-LABA combination therapy ahead of high-dose ICS. There is also good reason, in children who are not well controlled and continue to have severe exacerbations despite ICS-LABA combination therapies (i.e., difficult-to-control asthma), to consider in-depth evaluations and biologic therapeutic options. For example, other Inner City Asthma Consortium studies have contributed to the understanding of the efficacy, safety and exacerbation-protective mechanisms of action of omalizumab in 6 – 20 year old children⁴²⁻⁴⁵.

It is notable in this APIC study that children with difficult-to-control asthma [i.e., required high-dose ICS (fluticasone 500 mcg/day or higher) through the course of the study] had significantly more day and night symptoms and exacerbations, and persistent low lung function, appearing clinically insensitive to ICS benefits when compared with their easy-to-control counterparts⁶. Importantly, these children also had molecular evidence of corticosteroid insensitivity as demonstrated in this investigation. With recent and ongoing developments and clinical availability of more controller therapeutic options for children with difficult-to-control asthma, the APIC study provides additional insights to phenotype- and endotype-directed care^{7, 15}.

In summary, this is the first study to evaluate corticosteroid responsiveness in PBMC of children with asthma in relation to how much inhaled corticosteroid was needed to achieve acceptable asthma control over the course of one year of guidelines-

based therapy. The study demonstrated reduced responsiveness to FLU in PBMC of children with difficult-to-control asthma *in vitro* even after one year of therapy and identified novel potential biomarkers, including GR α and corticosteroid-mediated transrepression, for monitoring corticosteroid responsiveness in patients with difficult-to-control asthma.

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Table 1. Characteristics of the study participants over 12 months of treatment in APIC study.

Characteristic	Easy-to-control (n=31)		Indeterminate (n=19)		Difficult-to-control (n=45)		p-value ¹	
Age (years) Mean $\pm$ SD	11.1 $\pm$ 3.2		9.7 $\pm$ 3.1		10.5 $\pm$ 3.0		0.27	
Race and ethnicity Hispanic Black, non-Hispanic White, non-Hispanic Other/Mixed	20 (64.5%) 8 (25.8%) 2 (6.5%) 1 (3.2%)		11 (57.9%) 7 (36.8%) 0 (0.0%) 1 (5.3%)		21 (46.7%) 22 (48.9%) 2 (4.4%) 0 (0.0%)		0.24	
Sex Female Male	14 (45.2%) 17 (54.8%)		5 (26.3%) 14 (73.7%)		15 (33.3%) 30 (66.7%)		0.39	
Treatment²	V0	V6	V0	V6	V0	V6	V0	V6
Step 0. No controller meds; albuterol	7 (22.6%)	17 (54.8%)	1 (5.3%)	0 (0.0%)	1 (2.2%)	0 (0.0%)	<0.001	<0.001
Step 1. Montelukast	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (10.5%)	0 (0.0%)	0 (0.0%)		
Step 2. Flovent Diskus 50 mcg bid	10 (32.3%)	9 (29.0%)	5 (26.3%)	5 (26.3%)	2 (4.4%)	0 (0.0%)		
Step 3. Flovent Diskus 100 mcg bid	9 (29.0%)	2 (6.5%)	3 (15.8%)	5 (26.3%)	3 (6.7%)	2 (4.4%)		
Step 4. Flovent Diskus 250 mcg bid	3 (9.7%)	3 (9.7%)	5 (26.3%)	3 (15.8%)	10 (22.2%)	10 (22.2%)		
Step 5. Advair Diskus 250 mcg/50 mcg bid	2 (6.5%)	0 (0.0%)	5 (26.3%)	3 (15.8%)	14 (31.1%)	14 (31.1%)		
Step 6. Advair Diskus 500 mcg/50 mcg bid	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (5.3%)	15 (33.3%)	19 (42.2%)		
Asthma control level³	V0	V6	V0	V6	V0	V6	V0	V6
1	28 (90.3%)	23 (74.2%)	17 (89.5%)	14 (73.7%)	23 (51.1%)	22 (48.9%)	0.005	0.049
2	2 (6.5%)	5 (16.1%)	1 (5.3%)	1 (5.2%)	10 (22.2%)	8 (17.8%)		
3	1 (3.2%)	3 (9.7%)	1 (5.3%)	2 (10.5%)	6 (13.3%)	5 (11.1%)		
4	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (10.5%)	6 (13.3%)	10 (22.2%)		

1. Continuous variables are compared using a Kruskal-Wallis test while categorical variables are compared using a Pearson χ^2 test or Fisher's exact test, as appropriate.

2. See Table E1 for a detailed description of APIC treatment steps.

3. See Table E1 for a detailed description of asthma control levels.

Table 2. Summary of the expression levels of steroid response biomarkers in PBMC of study participants over 12 months of guideline based treatment.

Biomarker	Visit	Easy-to-control	Indeterminate	Difficult-to-control
GRalpha	V0		Lower than easy-to-control	Lower than easy-to-control
TNFalpha suppression by Fluticasone	V0 to V6	Marginally increased	No change*	No change*
IL-8 suppression by Fluticasone	V0 to V6	Increased	No change*	No change*
FKBP5 induction by Fluticasone	V0 to V6	Increased	No change	Increased ⁺
TNFalpha	V0 to V6	Reduced	No change**	Reduced

* Change between V0 and V6 is lower than easy-to-control

⁺ Change between V0 and V6 is higher than indeterminate

** Change between V0 and V6 is higher than easy- and difficult-to-control

Figure Legends

Figure 1. Expression level of GRalpha at V0 in easy-to-control (green), indeterminate (orange) and difficult-to-control (blue) asthmatic children. Geometric means and 95% confidence intervals are denoted by horizontal lines. Adjusted p-values obtained from pairwise comparisons between groups are provided at the top of the figure.

Figure 2. Fold change between V0 and V6 in fold suppression of IL-8 at 3 hours post-Fluticasone in easy-to-control (green), indeterminate (orange) and difficult-to-control (blue) asthmatic children. Geometric means and 95% confidence intervals are denoted by horizontal lines. Adjusted p-values obtained from pairwise comparisons between groups are provided at the top of the figure. P-values comparing expression levels between V0 and V6 within each group are provided below the corresponding geometric mean.

Figure 3. Fold change between V0 and V6 in fold suppression of TNFalpha at 3 hours post-Fluticasone in easy-to-control (green), indeterminate (orange) and difficult-to-control (blue) asthmatic children. Geometric means and 95% confidence intervals are denoted by horizontal lines. Adjusted p-values obtained from pairwise comparisons between groups are provided at the top of the figure. P-values comparing expression levels between V0 and V6 within each group are provided below the corresponding geometric mean.

Figure 4. Fold change between V0 and V6 in TNFalpha levels in easy-to-control (green), indeterminate (orange) and difficult-to-control (blue) asthmatic children. Geometric means and 95% confidence intervals are denoted by horizontal lines. Adjusted p-values obtained from pairwise comparisons between groups are provided at the top of the figure. P-values comparing expression levels between V0 and V6 within each group are provided below the corresponding geometric mean. Only p-values less than or equal to 0.05 are provided.

Figure 5. Fold change between V0 and V6 in fold induction of FKBP5 at 3 hours post-Fluticasone in easy-to-control (green), indeterminate (orange) and difficult-to-control (blue) asthmatic children. Geometric means and 95% confidence intervals are denoted by horizontal lines. Adjusted p-values obtained from pairwise comparisons between groups are provided at the top of the figure. P-values comparing expression levels between V0 and V6 within each group are provided below the corresponding geometric mean.

Figure 1

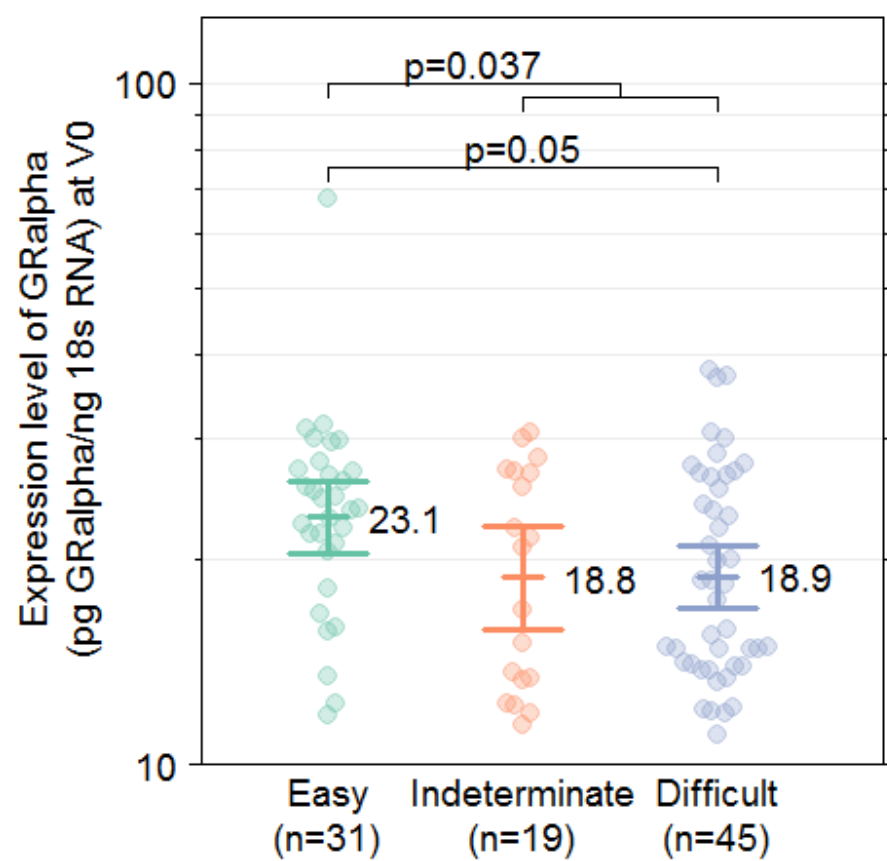


Figure 2

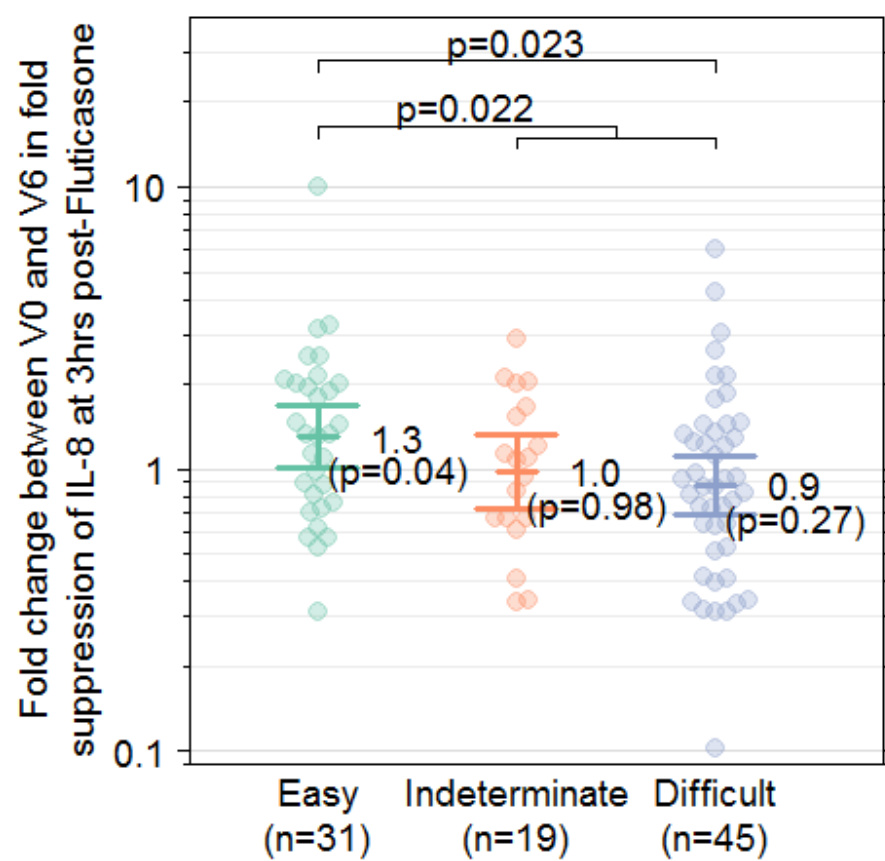


Figure 3

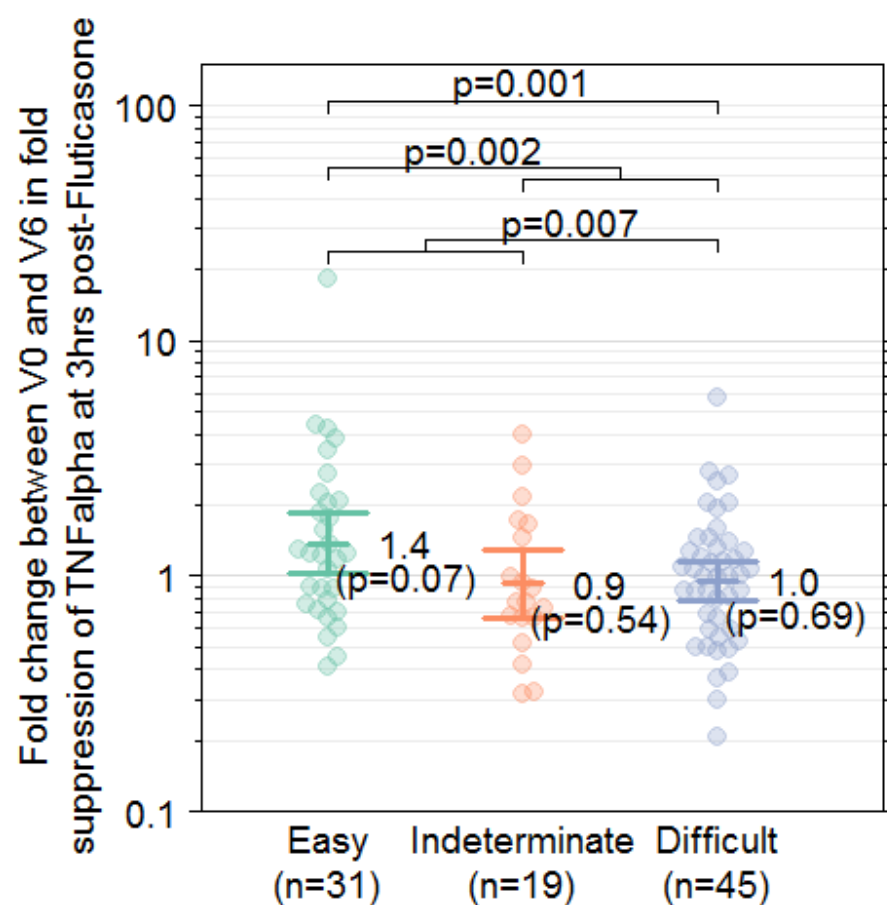


Figure 4

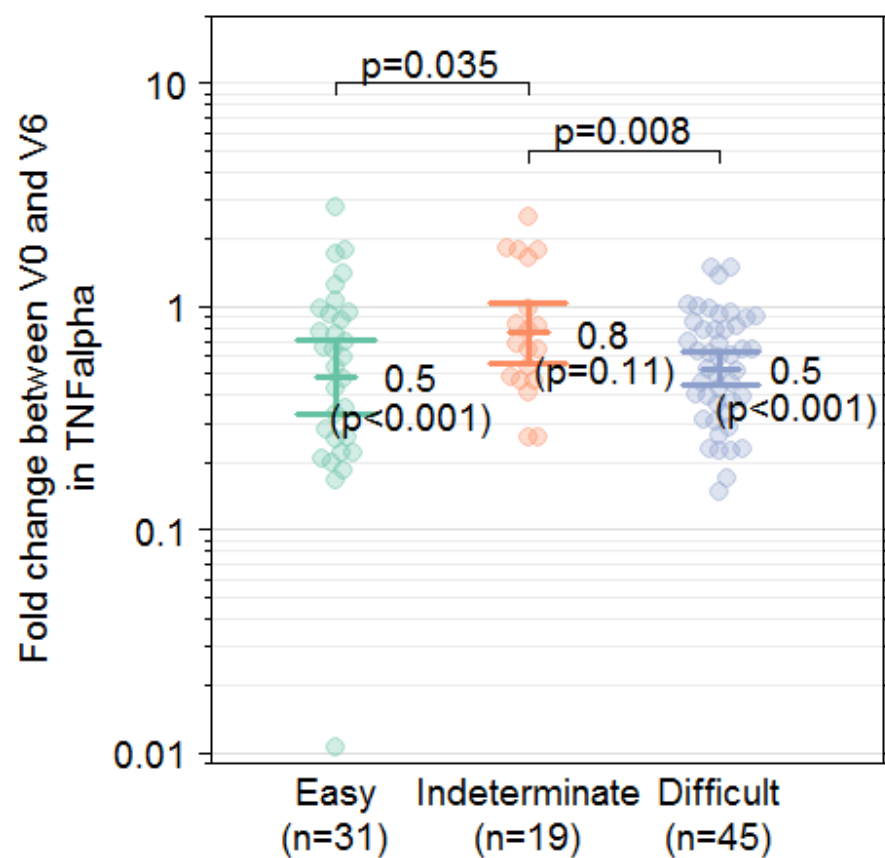
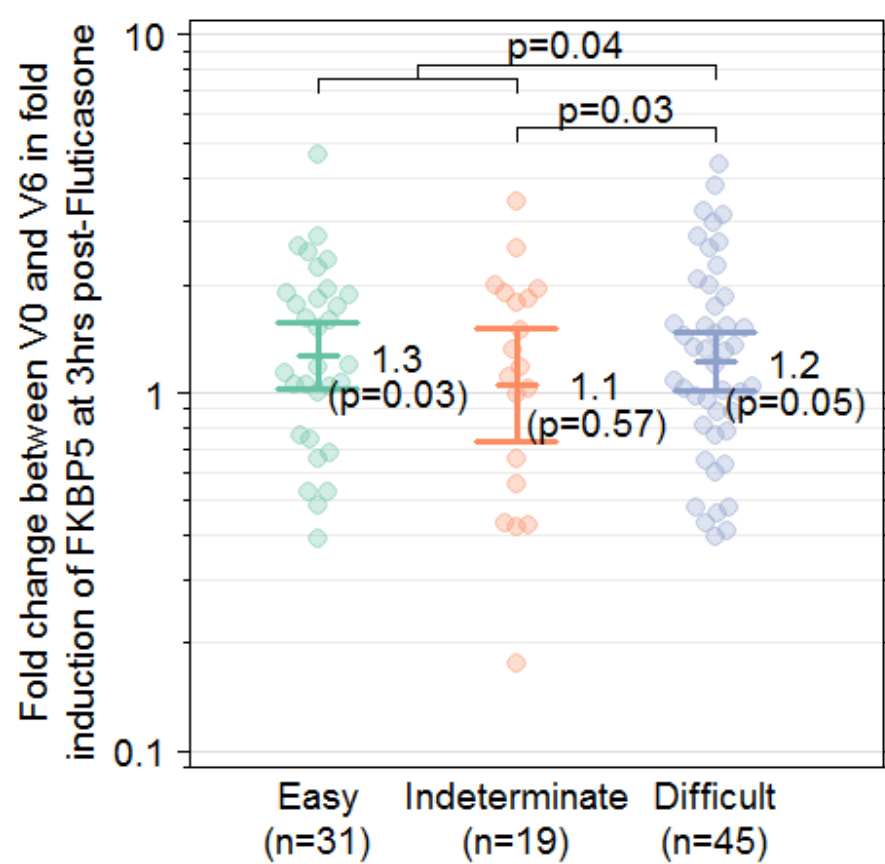


Figure 5



E Figure Legends

Figure E1. Expression level of baseline GRalpha at V0 by treatment step at Visit 0. GRalpha levels were compared in all study patients by distributing them in accordance to their treatment level. Geometric means and 95% confidence intervals are denoted by a solid line and given at the top of the figure. The adjusted p-value testing the association between treatment step level at Visit 0 and baseline GRalpha expression levels at Visit 0 is provided in the bottom right corner of the figure. Refer to Table E1 and Table E2 for definitions of asthma treatment step and asthma control levels, respectively.

Figure E2. Fold change between V0 and V6 in baseline GRalpha by treatment step at Visit 6 across all participants. Geometric means and 95% confidence intervals are denoted by a solid line and given at the top of the figure. The adjusted p-value testing the association between treatment step level at Visit 6 and fold change between V0 and V6 in baseline GRalpha is provided in the bottom right corner of the figure. Refer to Table E1 and Table E2 for definitions of asthma treatment step and asthma control levels, respectively.

Figure E3. Fold change between V0 and V6 in FKBP5 levels in easy-to-control (green), indeterminate (orange) and difficult-to-control (blue) asthmatic children. Geometric means and 95% confidence intervals are denoted by horizontal lines. P-values comparing expression levels between V0 and V6 within each group are provided below the corresponding geometric mean.

Table E1. Controller therapy steps in APIC study

Step	Medication Equivalent
0	No controller medication; Ventolin® HFA prn
1	Singulair® 5 mg PO once per day 6 to 14 years of age 10 mg PO once per day ≥ 15 years of age
2	Flovent® Diskus® 50 mcg bid
3	Flovent® Diskus® 100 mcg bid
4	Flovent® Diskus® 250 mcg bid
5	Advair™ Diskus® 250/50 mcg bid
6	Advair™ Diskus® 500/50 mcg bid

Table E2. Definitions of asthma control levels in APIC study participants.

Control Level*	Maximum of 1) # days with asthma symptoms/ two weeks and 2) # days with rescue albuterol use/ two weeks**	Maximum of 1) # nights of sleep disruption due to asthma/ two weeks and 2) # nights use of albuterol for awakening/ two weeks**	FEV ₁ (% predicted, personal best)	Courses of systemic steroids *** since last visit
1	0-3 days	0-1 night	≥ 85	No
2	4-9 days	2 nights	80 - < 85	Yes
3	10-13 days	3-4 nights	70 - < 80	-
4	14 days	5-14 nights	< 70	-

* Control level was defined using the highest control level across all four categories.

** Determined from participant recall, based on the 2-week interval directly preceding the study visit.

*** Defined as a prescription of a course of systemic steroids by a clinician or initiation of a course of systemic steroids by a participant to prevent a serious asthma outcome. If a participant initiates and completes a course of systemic steroids without clinician involvement, this course will be counted only if it meets the following minimum dosage: prednisone, prednisolone, or methylprednisolone at ≥ 20mg per day for 3 of any 5 consecutive days; or dexamethasone at ≥ 10mg per day for ≥ 1 day.

Table E3. Comparison of characteristics included in regression models among easy-to-control, indeterminate and difficult-to-control participants. Summary statistics include mean (standard deviation) for continuous variables and frequency (percentage) for categorical variables.

Characteristic	Easy-to-control (n=31)	Indeterminate (n=19)	Difficult-to-control (n=45)	p-value
BMI Percentile at V6 Mean $\pm$ SD	68.3 $\pm$ 31.2	72.6 $\pm$ 25.4	72.3 $\pm$ 29.5	0.80 ¹
NicAlert test result at V6 0-10 ng/ml 10-30 ng/ml 30+ ng/ml Missing	9 (29.0%) 18 (58.0%) 2 (6.5%) 2 (6.5%)	4 (21.1%) 10 (52.6%) 5 (26.3%) 0 (0.0%)	12 (26.7%) 23 (51.1%) 9 (20.0%) 1 (2.2%)	0.43 ²
Beck Anxiety Scale at V6 Mean (SD) Missing	43.9 $\pm$ 8.7 2 (6.5%)	44.9 $\pm$ 7.3 4 (21.1%)	47.6 $\pm$ 11.6 3 (6.7%)	0.40 ¹
Total serum IgE (kU/L) Mean $\pm$ SD	651.0 $\pm$ 1103.4	622.8 $\pm$ 943.8	612.2 $\pm$ 733.6	0.73 ¹
Number of positive skin tests Mean $\pm$ SD	4.7 $\pm$ 3.1	5.1 $\pm$ 3.4	4.6 $\pm$ 3.0	0.83 ¹
Serum Vitamin D (ng/ml) Mean $\pm$ SD	22.9 $\pm$ 7.5	24.2 $\pm$ 7.2	21.9 $\pm$ 6.2	0.51 ¹

1. P-value obtained from a Kruskal-Wallis test.

2. P-value obtained from a Fisher's exact test.

Figure E1

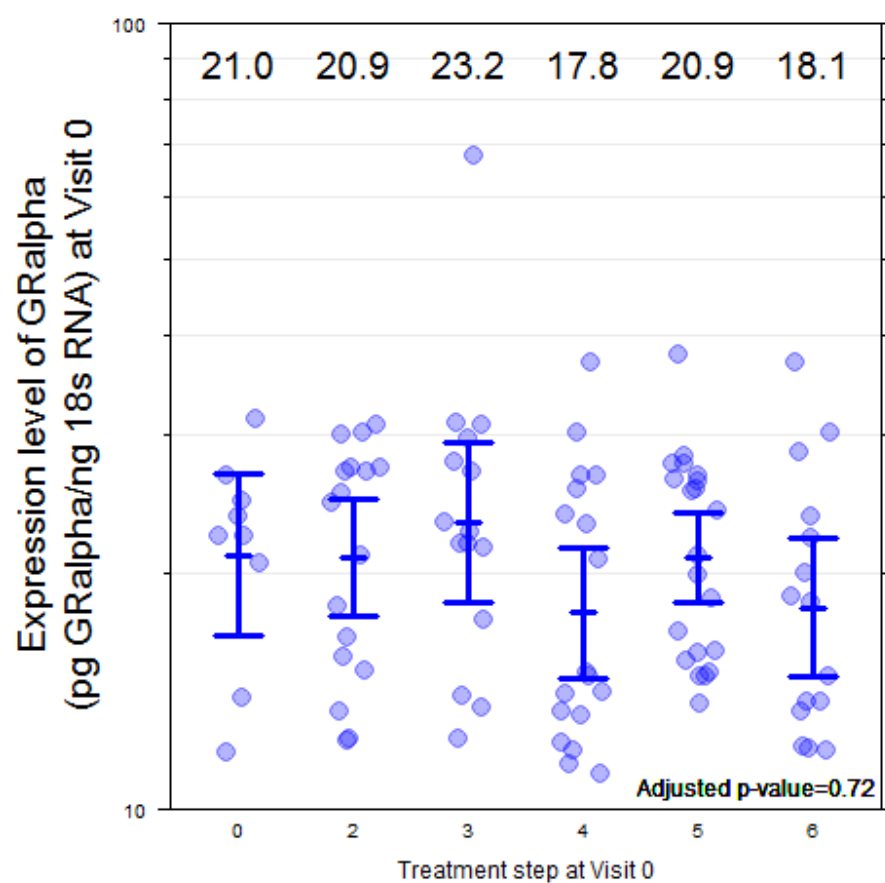


Figure E2

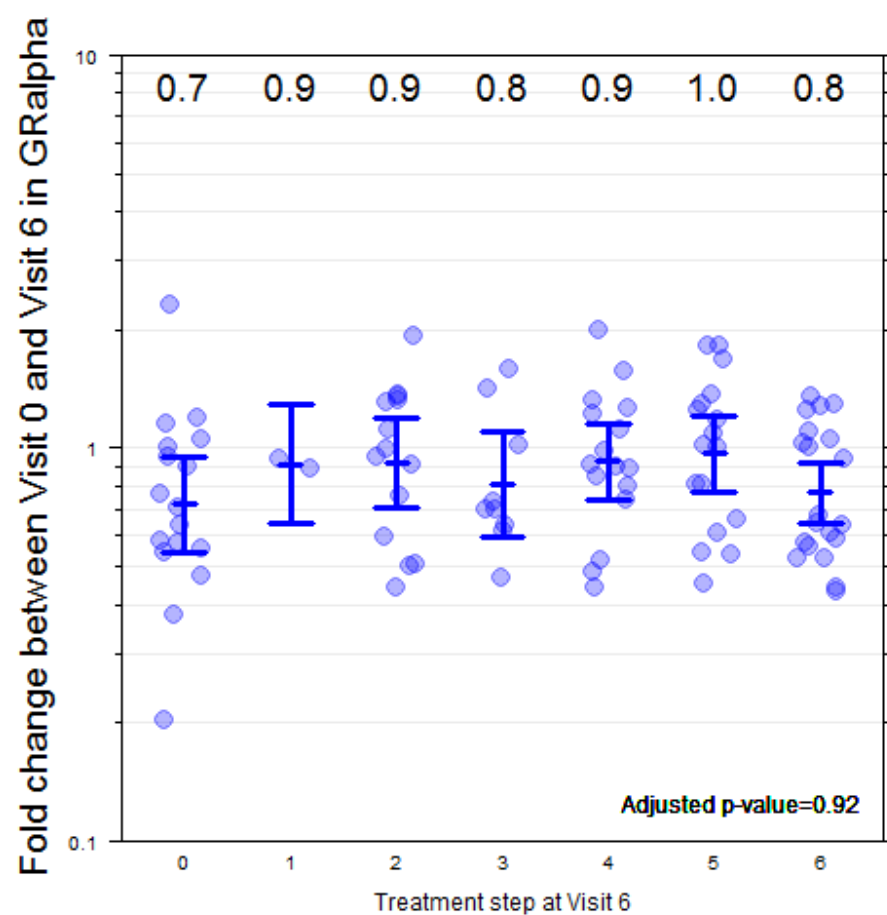


Figure E3

