The handle http://hdl.handle.net/1887/38706 holds various files of this Leiden University dissertation

**Author:** Zuiker, Rob  
**Title:** Development and use of biomarkers in clinical development of new therapies for chronic airway disease  
**Issue Date:** 2016-04-06
CHAPTER 3

SPUTUM RNA SIGNATURE IN ALLERGIC ASTHMATICS

Rob GJA Zuiker [1], Catherine Teknowsley [2,3], Zuzana Diamant [1,4], Didrik Boer [2,3], Adam P Cohan [1], K. Van Dieck [1,2], J. De Laeye [1,3], Viviana M Blasi [2], Wil Mehrotra [2], Jacqueline Burggraf [1,2], and Marcella K Ruddy [1,6].

*Both authors contributed equally.

3. Present affiliation: Novartis, New York, USA.
4. Dept. of Respiratory Medicine and Allergology, Lund, Sweden, and University Medical Center Groningen, Dept. of Clinical Pharmacy & Pharmacology and Dept. of General Practice and QPS, Groningen.
ABSTRACT

RATIONALE Inhaled allergen challenge is a validated exacerbation model of allergic asthma offering useful pharmacodynamic assessment of pharmacotherapeutic effects in a limited number of subjects.

OBJECTIVES To evaluate whether an RNA signature can be identified from induced sputum following an inhaled allergen challenge, whether a RNA signature could be modulated by limited doses of inhaled fluticasone, and whether these gene expression profiles would correlate with the clinical endpoints measured in this study.

METHODS Thirteen non-smoking, allergic subjects with mild to moderate asthma participated in a randomized, placebo-controlled, 2-period cross-over study following a single-blind placebo run-in period. Each period consisted of 3 consecutive days, separated by a wash-out period of at least three weeks. Subjects randomly received inhaled fluticasone ((FP) MDI; 500 μG BID x 5 doses in total) or placebo pre- until 24 H post-allergen. On day 2, house dust mite (HDM) extract was inhaled and airway response was measured by FEV1 at predefined time points until 7 H post-allergen. Sputum was induced by NaCl 4.5%, processed and analysed at 24 H pre- and 7 & 24 H post-allergen. RNA was isolated from eligible sputum cell pellets (<80% squamous of 500 cells), amplified according to Nugen technology and profiled on Affymetrix arrays. Gene expression changes from baseline and fluticasone treatment effect were evaluated using a mixed effects ANCOVA model at 7 and at 24 hours post allergen challenge.

RESULTS Inhaled allergen induced statistically significant gene expression changes in sputum, which were effectively blunted by fluticasone (adjusted p-value <0.025). 47 RNA signatures were selected from these responses for correlation analyses and further validation. This included TH2 cytokines, chemokines, high affinity IgE receptor FcεRI, histamine receptor HRH4, and enzymes and receptors in the arachidonic pathway. Individual messengers from the 47 RNA signature correlated significantly with lung function and sputum eosinophil counts.

CONCLUSION Our RNA extraction and profiling protocols allowed reproducible assessments of inflammatory signatures in sputum including quantification of drug effects on this response in allergic asthma. This approach offers novel possibilities for development of PD biomarkers in asthma.

INTRODUCTION

Inhaled allergen challenge can be applied to study the pathophysiology and the immune-biology to allergic stimuli within the airways. Allergen challenge is highly reproducible and serves as an integral disease model enabling the investigation of several features of asthma [1]. In drug development, allergen challenge is an established tool predicting clinical efficacy of novel anti-allergic and anti-asthma treatments [2]. Hypertonic saline-induced sputum [3] has been shown to yield reproducible increases in inflammatory cells and biomarkers following allergen-induced late asthmatic response (LAR) [4] with subsequent response to novel and existing anti-inflammatory therapies [24-7].

Microarray technology allows to profile gene expression of the entire genome and has been widely applied in several asthma studies [8,9]. A large majority of these gene profiling studies involved tissue obtained from asthmatics like airway epithelium [10,11], bronchial biopsies [12] or nasal mucosal cells [13]. Although gene expression has also been studied in fluids from asthmatics like blood [14], broncho-alveolar lavage [15], and induced sputum [16], little is published on extensive gene expression profiling on induced sputum cells following allergen challenge.

In this study Affymetrix 2.0 microarray technology was used to measure the gene expression levels of > 50.000 transcripts in induced sputum obtained from 13 allergic asthmatics before and after allergen challenge. In a refined set of 47 genes signatures we aimed to study: 1) the feasibility and reproducibility of quantification of gene expression in induced sputum at 7 and 24 hour post-challenge 2) their reversibility after a short course of inhaled fluticasone (FP) treatment, and 3) the correlation with lung function and eosinophil measurements.

METHODS

STUDY POPULATION AND DESIGN 🌟 Thirteen non-smoking subjects with clinically stable, mild to moderate allergic asthma [17] using prn short-acting beta-agonists only and with dual airway responses to inhaled house dust mite (HDM), documented during the single-blind placebo run-in screening period, participated in a double-blind, 2-way cross-over study. Each period consisted of 3 consecutive days, with ≥ 3 weeks washout between periods, Figure 1. The screening was identical to the subsequent treatment periods during which subjects randomly received inhaled FP (MDI; 500 μG BID, total of 5 doses) or matching placebo. On day 1, baseline measurements including, spirometry and subsequent sputum induction (3 x 5 MIN
NaCl 4.5%) were performed prior to study medication. On day 1, 1 h post-study medication, subjects underwent a titrated allergen challenge [1]. The subsequent airway response was repeatedly measured by FEV1 until 7 h post-allergen. At 24 h post-allergen (day 3), test procedures were repeated as on day 1. All test procedures were conducted according to standardized, validated methods and at the same time of the day (within 2 hours) during the different treatment periods [12-20].

A dual airway response to inhaled HDM extract consisted of an early (EAR) and a late asthmatic response (LAR) defined as a fall in FEV1 > 15% from baseline occurring between 0-3 h and 3-7 h post-allergen, respectively.

This study was part of an allergen study measuring allergen induced TH2-profile in sputum [7].

The study was approved by the Ethics Committee of Leiden University Medical Center, Leiden, The Netherlands, and all participants gave a signed informed consent (Eudract number 2007-003671-40).

**STUDY MEDICATION** Fluticasone 250 μg/puff (Allen & Hanburys, Glaxo Wellcome Ltd, Middlesex, UK) and matching placebo (Armstrong Pharmaceuticals Inc., Canton, MA, USA, packaged at Merck Frosst, Kirkland, Canada) were supplied by Wellcome Ltd, Middlesex, UK) and matching placebo (Armstrong Pharmaceuticals Inc., Canton, MA, USA, packaged at Merck Frosst, Kirkland, Canada) were supplied (Eudract number 2007-003671-40).

**ALLERGEN CHALLENGE** The allergen challenge was performed using the 2 minutes tidal breathing method that has been previously validated [1]. The run-in period served as a dose (range) finding procedure, while during study periods 1 & 2 each subject inhaled the same 2 or 3 cumulative doses of the allergen extract in period served as a dose (range) finding procedure, while during study periods 1 & 2 each subject inhaled the same 2 or 3 cumulative doses of the allergen extract

...
interrogating 50,159 human transcripts predominantly from refseq, GenBank, dbest and ensembl databases as described on the Gene Expression Omnibus website (https://http://www.ncbi.nlm.nih.gov/geo/). The accuracy of sample processing was monitored through quality metrics assessing rna yield, rna quality: 18S/28S ribosomal rna ratio, rna Integrity Number (rin) score, and hybridization parameters: 3’/5’ ratios for GAPDH mrna and scale factor. In addition, the amount of bacterial rna contamination was evaluated by calculating the area under the curve for the 16S and 23S (bacterial) versus the 18S and 28S (eukaryotic) ribosomal rna peaks using a bio analyzer electropherograms (Agilent, Santa Clara, California, USA). Specimens with more than 80% bacterial contamination were removed from the analysis. Data were normalized using the Robust Multichip Average (rma) algorithm prior to statistical analysis.

STATISTICAL MODEL FOR DATA ANALYSIS A mixed effect ancova model was selected including terms for baseline gene expression, treatment, sequence and period as fixed effects and subject nested in sequence as a random effect. Gene expression change from the appropriate baseline was used as the dependent variable. The baselines for each of the periods were used as covariates.

ANALYSIS OF TREATMENT EFFECTS For each time point, 7 hours and 24 hours, the allergen challenge effect and the fp treatment effect were calculated. The allergen challenge effect (ace) was calculated as the change from baseline when the subject received placebo treatment. The fp treatment effect (fte) was calculated as the difference in change from baseline between the fp treatment group and the placebo group. P-values for each gene in each treatment effect were adjusted using the Benjamin–Hochberg’s procedure with a false discovery rate (fdr) level pre-specified at 0.025 to select significant genes.

CORRELATION ANALYSES Pearson correlation coefficient and the associated p-value were computed for correlation between the estimated individual subject-level effect, separately for allergen challenge effect and fp treatment effect, for a given clinical endpoint and gene of interest. Assuming no period or sequence effect, subject-level allergen challenge effect was calculated as the log-transformed change from baseline, for a clinical endpoint or gene of interest, when the subject received placebo treatment. Similarly, subject-level fp treatment effect was calculated as the difference in change from baseline for a clinical endpoint or gene of interest when the subject received fluticasone vs placebo. Type I error of 10% (two-sided) was used to select significant results, and no multiplicity adjustment was applied for declaring statistical significance.

RESULTS
Sputum specimens were collected from asthmatic subjects who provided a baseline specimen in period 1 and period 2 and which passed the quality control. The reproducibility of the sputum induction and collection procedures for rna profiling after hybridization on microarrays, were evaluated by comparing individual gene expression intensities in combination with hierarchical clustering using Pearson correlation coefficients [24], Figure 2. The results of this cluster analysis revealed that 14 out of 18 sputum specimens clustered appropriately in subject specific pairs, validating our sputum collection and isolation protocol.

The whole microarray contained 51,562 probe sets. At 7 hours post allergen challenge, and applying a false discovery rate of < 0.025, a total of 4,175 and 1,001 statistical significant probe sets were identified for the allergen effect (ace) and the fp treatment effect (fte), respectively. Likewise, 1,143 and 1,018 statistical probe sets were identified at 24 hours post allergen for the allergen effect and the fp treatment effect, respectively. 714 probes sets were regulated by both the allergen challenge effect and fp treatment at 7 hours and 31 probe set at 24 hours post challenge, Table 1. All the genes regulated by both the allergen challenge and fp at each time point were reversed from their allergen induced levels in presence of fluticasone, Figure 3. In other words, fluticasone effectively blunted the response to the allergen challenge at the gene expression level.

Quantification of the individual genes that contribute to the key cytokines of the Th1, Th2 and Th17 pathways was performed by displaying the change from baseline in gene expression at 7 hours and 24 hours following allergen challenge in presence or absence of fp treatment (Figure 4). This analysis revealed the up-regulation by the allergen challenge and the down-regulation by fp treatment of the gene expression for several key Th1 cytokines (Interleukin (il)-4, il-5 and il-13) and an absence of an effect on key Th1 cytokines (Interferon (inf) -γ and Tumor Necrosis Factor (tnf)). Chemokine ligand 13 (ccl13)/Monocyte Chemotactrant Protein (mcp)-4 [25], ccl17/Thymus and Activation Regulated Chemokine (tarc) [26] and ccl26/eotaxin-3 [27] are Inflammatory chemokines mediating Th2 cell recruitment and known to be induced by il-4. Their gene expressions were up-regulated by the allergen challenge and down-regulated by fp treatment following a similar pattern as the Th2 cytokines (Figure 5). Likewise, the same pattern was observed for genes belonging to pathways controlling the release of inflammatory parameters like: Hdc (histidine decarboxylase) known to catalyze the production of histamine [28]; histamine receptor 4 h1r4 which is specific for eosinophils and basophils [29]; fcer1A, the alpha subunit of the high affinity IgE receptor which directly binds IgE and through crosslinking induces the release of preformed histamine and proteases as...
well as the generation of leukotrienes and prostaglandins; the messengers for the enzyme GGT5 (gamma glutamyl transferase 5, which converts leukotrienes C4 to D4) [32]; ALOX5 (5-lipoxygenase) and the receptor PTGER3 (prostaglandin receptor 3) were also up regulated by the allergen challenge and down regulated by FP treatment. In most of the cases, the fold change from baseline was higher at 7 hours versus 24 hours and the p-values smaller. This suggests that the 7 hours’ time point provides the most useful readouts of the strict inflammatory response following an allergen challenge.

In order to facilitate the correlation analyses, the union of the genes affected by the allergen challenge and fluticasone 7 hours or 24 hours post-challenge was reduced to a set of 47 rna signatures based on statistical significance, intensity of the change from baseline, biological relevance and classified based on druggable structural and functional categories (Figure 6). All the genes represented in the 47 rna signatures harbor robust expression changes, and the large majority of them is up-regulated after 7 hours with the exception of FLT3 and CRLF2, which are regulated only after 24 hours.

The 47 rna signature set was then used to identify genes correlating with lung function measurements (Table 2) and eosinophil cell counts and percentages (Table 3). Allergen challenge and FP treatment-mediated correlations were independently assessed for each probe set in the signature by estimating correlations at the subject level at 7 and 24 hours post allergen challenge. Correlation plots for the most significant probe sets from each correlation analysis type are represented in Figure 7. High correlation for some of the probe sets, e.g. ILIR1 and HRH4 and the eosinophil counts from the allergen challenge and the fluticasone treatment effect were observed, with correlation coefficients greater than 0.9 and p-value between < 0.001-0.002. In the allergen challenge effect analysis, probe sets for NRGI, CCR2, CD1C, MAPK6, IL26 were negatively correlated with FEV1 measurements at 7 hours. In the fluticasone treatment effect, probe sets for NRGI, RUNX3, FLT3, negatively correlated to the FEV1 measurements at 7 hours and 24 hours. NRGI was the most significant gene consistently negatively correlated to lung function measurements at 7 hours in both the allergen effect and the fluticasone effect analysis with p-values of and coefficients of correlations in the range of -0.75 (p-value 0.054) to -0.90 (p-value 0.002).

**DISCUSSION**

In this study a rna signature in sputum induced by the allergen challenge and reversed with fluticasone was identified. A subset of these genes, known to regulate the key inflammatory responses associated with allergic asthma, correlated with clinical endpoints and may constitute potential PD biomarkers of response to fluticasone.

TH2 responses have been traditionally described as playing a central role in the pathophysiology of asthma, although not all patients share a TH2 inflammatory pattern [31]. It is striking that in our study the shift toward the TH2 differentiation pathway is a major element of the transcriptional response to the HDM challenge in sputum and is down regulated following response to fluticasone treatment in the mild asthmatic atopic subjects enrolled in this study. The implications of these results are several-fold.

First, the screening of subjects for dual EAR and LAB responses and the strong homogeneity of our results are consistent with the concept of clustering of clinical asthma phenotypes in which presence of eosinophilic infiltration was identified as one of the key variables [32]. Furthermore, clinical phenotypes of asthma have been linked to molecular signatures and pathways in a study where TH2 “high” and “low” phenotypes, characterized by differences in airway responsiveness, eosinophilia and airway remodeling, could be differentiated at the molecular level [33]. The observed low variability and high effect size obtained for the gene expression measurements in this study is likely due to the careful selection of a homogeneous allergic, corticosteroid responsive subject population characterized by eosinophilic inflammation in response to an allergen challenge.

Second, our results also suggest that gene expression measurements collected in such an allergen challenge platform could guide the development of novel quantitative assays. For instance, one direct application of this technology could be the quantification of the rnas that correlate the best with eosinophil numbers as a surrogate to the standard sputum eosinophil cell count assays. Another application of our technology would be the selection of PD biomarkers of response to anti-inflammatory treatment in asthma identified from a set of markers that correlate with clinical endpoints.

The results presented here also raised important questions. We identified from our data set two cytokines, IL-22 and IL-26, induced by the allergen challenge and reverted to baseline by fluticasone, which have been associated with the TH17 pathway. IL-22 is preferentially produced by TH17 cells in psoriatic skin and mediates the epithelium hyperplasia induced by IL-23 [34]. IL-26 is often co-expressed together with IL-17 and IL-22 by activation of TH17 cells, however, its function remains to be further investigated [35]. Despite the significance of IL-22 and IL-26, we were however unable to detect any up or down-regulation of the cytokines IL-17A and IL-17 F, as well as other genes associated with the TH17 pathway [36], therefore providing more support to the concept of a dominant TH2 response in this study.
Another question is whether the observed signature in sputum is due to i) changes in cell counts, in particular eosinophil cell counts since this cell type is predominantly increased in sputum following a segmental allergen challenge, ii) up or down-regulation of messengers within a given cell type or, iii) a combination of the above. The only way to address this question is to profile individual cell types isolated from sputum, however, the results from our analysis indicated some changes in gene expression that were correlated with cell type specific eosinophil cell counts and some that are not, therefore supporting option iii). On the one hand, we have identified two genes IL1RL1 and HRH4 that correlate extremely precisely with eosinophil cell counts (correlation coefficients > 0.9, p-values < 0.002) and are known to be expressed predominantly in eosinophils, basophils and mast cells. RNAs for both genes therefore appear to be excellent surrogates of eosinophil measurements in sputum. Interestingly, polymorphisms in the HRH4 gene were found to be associated with atopic dermatitis [37], while variants of the IL1RL1 gene have been associated with atopic dermatitis and atopic asthma [38]. Given the important role that IL1RL1 has in eosinophil function as a receptor for IL-33, this gene might therefore also represent a promising drug target in inflammatory diseases characterized by a strong eosinophilic component correlating with disease symptoms. Then again, we have identified from this study multiple examples of genes that display similar expression pattern upon allergen challenge and fluticasone treatment and which are known to have very different cell type specificity. In particular chemokines CCL13 and CCL17 have a dendritic specific expression while CCL26 is epithelial specific; similarly CD1A and CD1B are T-cell specific markers. However, as the expression of those genes is up-regulated by the allergen challenge and down-regulated by fluticasone, this suggests that the identified signature cannot be explained uniquely by variations in eosinophil cell counts or percentages and also reflects major transcriptional changes in a large variety of cell types. An analysis of the transcriptional signatures of isolated sputum cell types in combination with the identification of transcriptional modules of genes co-expressed in asthma as previously described in blood [39] could map the relative contribution of each gene and cell type to the inflammatory response.

Finally, we also identified from our analysis a set of RNAs that uniquely correlates with classical lung function measurements. At 7 hours, chemokines or chemokine receptors (CCL13, CCL17, CCL26, and CCR2) and membrane bound glycoproteins such as CD1B, CD1C and CD209 correlate to lung function measurements. NRG1, the gene that most significantly correlated to FEV1 measurements at 7 hours, is a member of the neuregulin family, which signals through tyrosine kinases of the ErbB3 family. NRG1 induces the expression of the goblet cell mucin proteins MUC5AC and MUC5B in human airway epithelium [40]. Its inhibition may therefore represent a novel therapeutic approach for decreasing mucus hypersecretion in respiratory diseases. In conclusion, our RNA extraction and profiling protocols allowed sensitive assessments of allergen-induced inflammatory signatures in sputum and precise quantification of drug effects on this response in allergic asthmatics. This approach offers novel possibilities for development of pharmacodynamic biomarkers in asthma.
Table 2 Correlations between gene expression measurements from the 47 RNA signatures and various FEV1 measurements. FEV1 measure I: % change in maximal drop of FEV1 during LAB, FEV1 measure II: % change in time weighed average of FEV1 during LAB, FEV1 measure III: % change in FEV1 at hour 24. Significant probe sets (p-values < 0.1 and correlation coefficient > 0.73) are displayed.

<table>
<thead>
<tr>
<th>Effect of interest</th>
<th>Probe set</th>
<th>Gene symbol</th>
<th>Clinical endpoint</th>
<th>Correlation coefficient (90% confidence interval)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluticasone treatment effect</td>
<td>100150696_tgi_at</td>
<td>nrg1</td>
<td>FEV1 Measure II</td>
<td>-0.90 (-0.98, -0.62)</td>
<td>0.002</td>
</tr>
<tr>
<td>Fluticasone treatment effect</td>
<td>100300593_tgi_at</td>
<td>nrg1</td>
<td>FEV1 Measure III</td>
<td>-0.83 (-0.96, -0.44)</td>
<td>0.01</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100124067_tgi_at</td>
<td>ccr2</td>
<td>FEV1 Measure I</td>
<td>-0.79 (-0.95, -0.32)</td>
<td>0.02</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100312593_tgi_at</td>
<td>nrg1</td>
<td>FEV1 Measure I</td>
<td>-0.77 (-0.94, -0.28)</td>
<td>0.025</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100161022_tgi_at</td>
<td>cd1c</td>
<td>FEV1 Measure II</td>
<td>-0.76 (-0.94, -0.26)</td>
<td>0.027</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100303601_tgi_at</td>
<td>ccr2</td>
<td>FEV1 Measure II</td>
<td>-0.76 (-0.94, -0.26)</td>
<td>0.027</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100145467_tgi_at</td>
<td>cd1c</td>
<td>FEV1 Measure II</td>
<td>-0.76 (-0.94, -0.25)</td>
<td>0.03</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100147484_tgi_at</td>
<td>ccr2</td>
<td>FEV1 Measure II</td>
<td>-0.75 (-0.94, -0.24)</td>
<td>0.031</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100300556_tgi_at</td>
<td>map2k6</td>
<td>FEV1 Measure II</td>
<td>-0.75 (-0.94, -0.24)</td>
<td>0.03</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100311406_tgi_at</td>
<td>cd1c</td>
<td>FEV1 Measure II</td>
<td>-0.74 (-0.93, -0.21)</td>
<td>0.036</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100159528_tgi_at</td>
<td>il26</td>
<td>FEV1 Measure II</td>
<td>-0.74 (-0.93, -0.20)</td>
<td>0.038</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100148726_tgi_at</td>
<td>ccl17</td>
<td>FEV1 Measure III</td>
<td>-0.80 (-0.96, -0.36)</td>
<td>0.012</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100133255_tgi_at</td>
<td>mmp1</td>
<td>FEV1 Measure III</td>
<td>-0.84 (-0.97, -0.39)</td>
<td>0.017</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100157709_tgi_at</td>
<td>alox15</td>
<td>FEV1 Measure III</td>
<td>-0.84 (-0.97, -0.37)</td>
<td>0.019</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100149346_tgi_at</td>
<td>socs2</td>
<td>FEV1 Measure III</td>
<td>-0.77 (-0.95, -0.19)</td>
<td>0.044</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100155853_tgi_at</td>
<td>runx3</td>
<td>FEV1 Measure III</td>
<td>-0.76 (-0.95, -0.16)</td>
<td>0.049</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100309572_tgi_at</td>
<td>flt3</td>
<td>FEV1 Measure III</td>
<td>-0.75 (-0.95, -0.16)</td>
<td>0.051</td>
</tr>
<tr>
<td>Allergen challenge effect</td>
<td>100132355_tgi_at</td>
<td>mmp1</td>
<td>FEV1 Measure III</td>
<td>-0.74 (-0.91, -0.34)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 1 Number of statistically significant probe sets identified from each contrast analysis, allergen challenge effect and fluticasone treatment effect, at 7 hours and 24 hours. The analysis was conducted on the whole microarray containing 50,159 human transcripts. The number of probe sets in common between the allergen challenge effect and the fluticasone effect analyses at a given time point are displayed on the right hand side. FDR: false discovery rate

<table>
<thead>
<tr>
<th>FDR &lt; 0.025</th>
<th># of probe sets in common</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allergen Challenge Effect (ACE) at Hour 7</td>
<td>4,175</td>
</tr>
<tr>
<td>Fluticasone Treatment Effect (FTE) at Hour 7</td>
<td>1,001</td>
</tr>
<tr>
<td>Allergen Challenge Effect (ACE) at Hour 24</td>
<td>1,244</td>
</tr>
<tr>
<td>Fluticasone Treatment Effect (FTE) at Hour 24</td>
<td>1,038</td>
</tr>
</tbody>
</table>
Table 3: Correlations between the gene expression measurements from the 47 RNA signatures and eosinophils (cell counts and percentages). Significant probe sets (p-values < 0.1 and correlation coefficients > 0.86) are displayed.

<table>
<thead>
<tr>
<th>Effect of interest</th>
<th>Probe set</th>
<th>Gene symbol</th>
<th>Clinical endpoint</th>
<th>Correlation coefficient (90% confidence interval)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allergen challenge effect</strong></td>
<td>100102783_TGI_at</td>
<td>IL1RL1</td>
<td>Eosinophil counts</td>
<td>0.92 (0.70, 0.98)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>100116100_TGI_at</td>
<td>HBB4</td>
<td>Eosinophil counts</td>
<td>0.91 (0.67, 0.98)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>100149146_TGI_at</td>
<td>SOCS2</td>
<td>Eosinophil counts</td>
<td>0.91 (0.66, 0.98)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>100121840_TGI_at</td>
<td>IL1RL1</td>
<td>Eosinophil counts</td>
<td>0.88 (0.56, 0.97)</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>100101949_TGI_at</td>
<td>GATA2</td>
<td>Eosinophil counts</td>
<td>0.87 (0.53, 0.97)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

| Fluticasone treatment effect | 100148162_TGI_at | IL1RL1 | Eosinophil counts | 0.98 (0.92, 1.00) | < 0.001 |
| | 100132840_TGI_at | IL1RL1 | Eosinophil counts | 0.98 (0.88, 1.00) | < 0.001 |
| | 100102783_TGI_at | IL1RL1 | Eosinophil counts | 0.97 (0.85, 0.99) | < 0.001 |
| | 1001241511_TGI_at | CCL26 | Eosinophil percentage | 0.97 (0.84, 0.99) | < 0.001 |
| | 100148726_TGI_at | CCL26 | Eosinophil percentage | 0.96 (0.80, 0.99) | < 0.001 |

**MENA at hour 7 and clinical endpoint at hour 2**

**Allergen challenge effect**

- 100102783_TGI_at (IL1RL1) Eosinophil counts 0.92 (0.70, 0.98) p-value 0.001
- 100116100_TGI_at (HBB4) Eosinophil counts 0.91 (0.67, 0.98) p-value 0.002
- 100149146_TGI_at (SOCS2) Eosinophil counts 0.91 (0.66, 0.98) p-value 0.002

**Fluticasone treatment effect**

- 100148162_TGI_at (IL1RL1) Eosinophil counts 0.98 (0.92, 1.00) p-value < 0.001
- 100132840_TGI_at (IL1RL1) Eosinophil counts 0.98 (0.88, 1.00) p-value < 0.001
- 100102783_TGI_at (IL1RL1) Eosinophil counts 0.97 (0.85, 0.99) p-value < 0.001
- 1001241511_TGI_at (CCL26) Eosinophil percentage 0.97 (0.84, 0.99) p-value < 0.001
- 100148726_TGI_at (CCL26) Eosinophil percentage 0.96 (0.80, 0.99) p-value < 0.001

**MENA at hour 7 and clinical endpoint at hour 24**

**Allergen challenge effect**

- 100135727_TGI_at (CD1A) Eosinophil percentage 0.91 (0.65, 0.98) p-value 0.002
- 1001132327_TGI_at (ADAM19) Eosinophil percentage 0.87 (0.55, 0.97) p-value 0.004
- 1001136515_TGI_at (CD40) Eosinophil percentage 0.87 (0.53, 0.97) p-value 0.005

**Fluticasone treatment effect**

- 100148210_TGI_at (IL1RL1) Eosinophil counts 0.93 (0.67, 0.99) p-value 0.003
- 100109438_TGI_at (MAD2A) Eosinophil counts 0.92 (0.98, 0.64) p-value 0.003
- 100124666_TGI_at (G6PD) Eosinophil counts 0.92 (0.63, 0.99) p-value 0.004
- 100135896_TGI_at (PTGS1) Eosinophil counts 0.89 (0.53, 0.98) p-value 0.008
- 100102151_TGI_at (IL1RL1) Eosinophil percentage 0.88 (0.51, 0.98) p-value 0.009
- 1001136515_TGI_at (CD40) Eosinophil percentage 0.88 (0.49, 0.97) p-value 0.01

---

Figure 1: Study design. Overview of the single-blind placebo run-in period and double-blind cross-over study periods 1 and 2 (upper section). Overview of study assessments (lower section). Time zero is time of first study medication dosing. The single-blind placebo run-in screening period and the subsequent study periods 1 & 2 were identical. 1x: induced sputum.

**Figure 2:** Hierarchal cluster assessment of sputum microarray data. Numbers refer to subject allocation numbers. Log 2 ratios of intensity estimates versus the average of all intensities are displayed. Dark color refers to probe sets that are up-regulated in reference to the pool of all specimens analyzed and light to the probe sets that are down-regulated. Left dark rectangles: link specimens from the same subject that co-cluster on the dendrogram.
Figure 3  Log 2 Estimates of gene expression changes for the significant genes identified from contrast analysis at 7 hours and 24 hours with an FDR < 0.025. ACE: allergen challenge effect, estimates of changes from baseline in the placebo group. FTE: fluticasone effect, estimates of differences in change from baseline between the placebo and the fluticasone groups.

Figure 4  Fold change from baseline in gene expression. Th2 cytokines (IL4, IL5, IL13). Th1 cytokines (IFNg and TNF). Fold change from baseline for the placebo group is represented on the left in each bar. Fold change from baseline for the fluticasone group is represented on the right in each bar. P-values are adjusted p-values, error bars represent 90% confidence.
Figure 5  Fold change from baseline in gene expression. Inflammatory chemokines (CCL23, CCL17, CCL26), molecules controlling the release of histamine (HDC, HRH4, FCER1), prostaglandins and leukotrienes (PTGIR3, ALOX15, GGT5). Fold change from baseline for the placebo group is represented on the left in each bar. Fold change from baseline for the fluticasone group is represented on the right in each bar. P-values are adjusted p-values, error bars represent 90% confidence intervals.

Figure 6  Fold changes over baseline (point estimate and 90% confidence intervals) for the 47 mRNA signatures. Light bars represent the change from baseline in the placebo group and dark bars in the fluticasone group. P-values for the allergen challenge effect (ACE) and the fluticasone treatment effect (VTR) are represented. P1: Th2 cytokines, P2: chemokines and chemokine receptors, P3: FCERT and histamine signaling, P4: Enzymes and signaling molecules in prostaglandin, leukotriene pathways, P5: Other cytokines, growth factors and their receptors, P6: Other enzymes, P7: Membrane bound glycoproteins, P8: Transcription factors, P9: Regulators of the inflammatory response.
Figure 7: Correlation plots of the most significant probe sets to individual subject clinical measurements for the allergen challenge effect and the fluticasone treatment effect. Correlation coefficients and corresponding p-values in parenthesis are listed in grey. hrh4 (histamine receptor 4); il1rl1 (il33 receptor); nrg1 (neuregulin 1)

Reference list

11. Lilly CM, Tateno H, Oguma T, Israel E, Sonna LA. Changes to asthmatic pathogenesis from microarray expression studies. Pharmacol Ther 2008; 120: 184-194
18. ATLS recommendations for standardized procedures for the offline and online measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide. Am J Respir Crit Care Med 2005; 171: 192-195

Chapter 4
Sputum Induction with Hypertonic Saline Reduces Fractional Exhaled Nitric Oxide in Chronic Smokers and Non-Smokers

Rob GJA Zuiker [1,*], Catherine Tribouley [2,*], Zuzana Diamant [1,4], J. Diderik Boot [1,5], Adam F Cohen [1], K. Van Dyck [2], I. De Lepeleire [2], Veronica M Rivas [2], Vlad Malkov [2], Jacob Burggraaf [5], and Marcella K Ruddy [2,6].

*Both authors contributed equally
1. Centre for Human Drug Research, Leiden, Netherlands
2. Merck Research Laboratories, New Jersey, USA and Brussels, Belgium
3. Present affiliation: Novartis, New York, USA
4. Dept. of Respiratory Medicine and Allergology, Lund, Sweden and Hanover Medical School
Groningen, Dept. of Clinical Pharmacy & Pharmacology and Dept. of General Practice and QPS, Groningen
5. Present affiliation: Janssen Biologics b.v., Leiden, Netherlands
6. Present affiliations: EMD Serono, Rockland, Massachusetts, USA