CHAPTER 3

SPUTUM RNA SIGNATURE IN ALLERGIC ASTHMATICS

SUBMITTED FOR PUBLICATION

Rob GJA Zuiker [1*], Catherine Tribouley [2,3*], 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] , Jacobus Burggraaf [1], and Marcella K Ruddy [2,6].

**both authors contributed equally*

1. Centre for Human Drug Research, Leiden,
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 University Medical Center Groningen, Dept. of Clinical Pharmacy & Pharmacology and Dept. of General Practice and QPS, Groningen
5. Present affiliation: Janssen Biologics B.V., Leiden,
6. Present affiliations: EMD Serono, Rockland, Massachusetts, USA

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 MCG 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 FCERIA, 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 asthmatics. 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 [2;4-7].

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 beta2-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 2, 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 [1;18-20].

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 in identical metered dose inhalers (MDIs) and inhaled per single puff through an Aerochamber (Volumatic, GlaxoSmithKline, Zeist, The Netherlands).

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 that had caused a fall in FEV1 of at least 15% from baseline during the run-in period. Following diluent, incremental doubling concentrations (7.81 to 2,000 BU/mL) of HDM extract (Dermatophagoides pteronyssinus; SQ 503, ALK-BPT, ALK-Abelló, Almere, The Netherlands) in phosphate-buffered saline (PBS) were aerosolized by a calibrated jet-nebulizer (DeVilbiss 646, output 0.13 mL/MIN, Somerset, Pennsylvania, USA) and inhaled at approximately 12 MIN intervals, until the EAR was reached (defined as a decrease in FEV1 of > 15% from post-diluent baseline within 1 h post-allergen). Airway response to inhaled allergen was measured by FEV1 in duplicate on a calibrated spirometer (Vmax Spectra; Sensor Medics, Bilthoven, The Netherlands) according to standard procedures [21], at 10, 20, 30, 45, 60, 90 and 120 minutes and then hourly until 7 h after the last allergen inhalation. The highest, technically valid measurement was expressed as percentage decrease from post-diluent baseline FEV1 and included into the analysis.

SPUTUM INDUCTION, PROCESSING AND ANALYSIS ❦ Sputum induction was performed as previously described [20;22] using a DeVilbiss Ultraneb 2000 ultrasonic nebulizer (Tefa Portanje, Woerden, The Netherlands) connected to a 100-CM long plastic tube, with an internal diameter of approximately 22 MM, connected to a two-way valve (No.2700; Hans-Rudolf, Kansas City, MO, USA) with a mouthpiece. Hypertonic saline (NaCl 4.5%) was nebulised and inhaled through the mouth, with the nose clipped, during three periods of 5 minutes. At approximately 7 minutes following each induction, spirometry was performed as a safety measure.

The cell pellet was processed as a full sample according to guidelines [20;23]. The processing took place within 2 h of collection. A DTT 0.1% solution (Dithiothreitol, Calbiochem, La Jolla, CA, USA) was mixed with a protease inhibitor pill (Complete Protease Inhibitor Cocktail tablets, Roche Applied Science #11 697 498 001; 1 pill per 50 mL of solution). The volume of the entire sputum sample was determined and an equal amount of 0.1% DTT/protease inhibitor solution was added. Subsequently, the sample was mixed with a pipette and placed in a warm shaking bath for 15 minutes at 37 °C. The homogenized mixture was centrifuged at 390G (1500 RPM) during 10 minutes. The supernatant was removed.

To determine cell viability and the total cell count, the cell pellet was re suspended in 2 mL PBS and filtered; 50 µL of the suspension was mixed with 50 µL of Trypan Blue. Total cell counts were determined in a counting chamber (Bürker; Omnilab 402521) using a cell counter (Omnilab 7005333). Cytospin slides (50 µL/cytospin; Shandon Cytospin 4, Thermon Electron Corporation, Runcorn, UK) were prepared by diluting the cell suspension with PBS in order to obtain approximately 0.5x 106 cells per mL, and subsequently centrifuged for 3 minutes at 254 G. Differential cell counts of eosinophils, neutrophils, lymphocytes, macrophages, epithelial and squamous cells were performed on May-Grünwald-Giemsa-stained cytospins by a certified cytopathologist. In each sputum sample, at least 500 nucleated cells, excluding squamous cells, were counted twice and the average percentage of each cell type was determined and expressed as percentage of nonsquamous cells. If > 80% of the cell count consisted of squamous cells, the quality of the sputum sample was judged unsatisfactory and was excluded from analysis.

The remaining suspension was centrifuged a second time. The resulting cell pellet was resuspended in 1 ml of TRIzol® (Invitrogen, Cat. # 15596-018, Life Technologies, Carlsbad, California, USA). RNA was amplified using WT-Ovation® amplification technology (NuGEN, San Carlos, California, USA). The amplified material was labeled and hybridized using a standard Affymetrix protocol. Gene expression studies were performed using the Rosetta/Merck Human RSTA Custom Affymetrix 2.0 microarray (Affymetrix, Santa Clara, California, USA) containing 51,562 probe sets

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 Benjamini-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 311 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 TH2 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 Chemoattractant Protein (MCP)-4 [25], CCL17/ Thymus and Activation Regulated Chemokine (TARC) [26] and CCL26/ eotoxin-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 HRH4 which is specific for eosinophils and basophils [29]; FCERIA, 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 C_4 to D_4) [30]; ALOX15 (15-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. ILIRLI 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 NRG1, CCR2, CD1C, MAP2K6, IL26 were negatively correlated with FEV1 measurements at 7 hours. In the fluticasone treatment effect, probe sets for, NRG1, RUNX3, FLT3, negatively correlated to the FEV1 measurements at 7 hours and 24 hours. NRG1 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 LAR 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 globlet 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 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

	FDR < 0.025	
	# of probe sets	in common
Allergen Challenge Effect (ACE) at Hour 7	4,175	714
Fluticasone Treatment Effect (FTE) at Hour 7	1,001	
Allergen Challenge Effect (ACE) at Hour 24	1,143	311
Fluticasone Treatment Effect (FTE) at Hour 24	1,018	

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 LAR, FEV1 measure ii: % change in time weighed average of FEV1 during lar, fevi measure iii: % change in FEV1 at hour 24. Significant probe sets (p-values < 0.1 and correlation coefficient > 0.73) are displayed.

Effect of interest	Probe set	Gene symbol	Clinical endpoint	Correlation coefficient (90% confidence interval)	P-value
mRNA at hour 7 and clinical endpoint at hour 7					
Allergen challenge effect	100150696_TGI_at	NRG1	FEV1 Measure II[2]	-0.90 (-0.98, -0.62)	0.002
	100300593_TGI_at	NRG1	FEV1 Measure II[2]	-0.83 (-0.96, -0.44)	0.01
	100124067_TGI_at	CCR2	FEV1 Measure I[1]	-0.79 (-0.95, -0.32)	0.02
	100312593_TGI_at	NRG1	FEV1 Measure I[1]	-0.77 (-0.94, -0.28)	0.025
	100161022_TGI_at	CDIC	FEV1 Measure II[2]	-0.77 (-0.94, -0.27)	0.027
	100303601_TGI_at	CCR2	FEV1 Measure I[1]	-0.76 (-0.94, -0.26)	0.027
	100145467_TGI_at	CDIC	FEV1 Measure II[2]	-0.76 (-0.94, -0.26)	0.027
	100161022_TGI_at	CDIC	FEV1 Measure I[1]	-0.76 (-0.94, -0.25)	0.03
	100147484_TGI_at	CCR2	FEV1 Measure II[2]	-0.75 (-0.94, -0.24)	0.031
	100300556_TGI_at	MAP2K6	FEV1 Measure II[2]	-0.75 (-0.94, -0.24)	0.03
	100311406_TGI_at	CDIC	FEV1 Measure II[2]	-0.74 (-0.93, -0.21)	0.036
	100159528_TGI_at	IL26	FEV1 Measure II[2]	-0.74 (-0.93, -0.21)	0.036
	100303601_TGI_at	CCR2	FEV1 Measure II[2]	-0.74 (-0.93, -0.20)	0.038
Fluticasone treatment effect	100135727_TGI_at	CDIA	FEV1 Measure II[2]	0.87 (0.46, 0.97)	0.012
	100127751_TGI_at	NRG1	FEV1 Measure I[1]	-0.84 (-0.97, -0.39)	0.017
	100302360_TGI_at	NRG1	FEV1 Measure I[1]	-0.84 (-0.97, -0.37)	0.019
	100155853_TGI_at	RUNX3	FEV1 Measure I[1]	-0.77 (-0.95, -0.19)	0.044
	100302360_TGI_at	NRG1	FEV1 Measure II[2]	-0.76 (-0.95, -0.16)	0.049
	100309572_TGI_at	FLT3	FEV1 Measure I[1]	-0.75 (-0.95, -0.16)	0.051
	100133255_TGI_at	MMP1	FEV1 Measure III[3]	-0.74 (-0.91, -0.34)	0.01
mRNA at hour 7 and clinical endpoint at hour 24					
Allergen challenge effect	100148726_TGI_at	CCL17	FEV1 Measure III[3]	-0.80 (-0.95, -0.36)	0.016
	100133255_TGI_at	MMP1	FEV1 Measure III[3]	-0.74 (-0.93, -0.22)	0.035
Fluticasone treatment effect	100145401_TGI_at	MAOA	FEV1 Measure III[3]	0.80 (0.27, 0.96)	0.031
	100301747_TGI_at	ADAM19	FEV1 Measure III[3]	0.80 (0.26, 0.96)	0.031
	100127751_TGI_at	NRG1	FEV1 Measure III[3]	-0.75 (-0.95, -0.14)	0.054
	100155853_TGI_at	RUNX3	FEV1 Measure III[3]	-0.74 (-0.94, -0.12)	0.058
	100309572_TGI_at	FLT3	FEV1 Measure III[3]	-0.74 (-0.94, -0.12)	0.058
mRNA at hour 24 and clinical endpoint at hour 24					
Allergen challenge effect	100157709_TGI_at	ALOX15	FEV1 Measure III[3]	0.77 (0.28, 0.94)	0.025
	100149346_TGI_at	SOCS2	FEV1 Measure III[3]	-0.75 (-0.94, -0.24)	0.031

[1]% change in maximal drop of FEV1 during LAR; [2]% change in time weighed average of FEV1 during LAR; [3]% change in FEV1 at hour 24

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

Effect of interest	Probe set	Gene symbol	Clinical endpoint	Correlation coefficient (90% confidence interval)	P-value
MRNA at hour 7 and clinical endpoint at hour 7					
Allergen challenge effect	100302783_TGI_at	ILIRL1	Eosinophil counts	0.92 (0.70, 0.98)	0.001
	100161010_TGI_at	HRH4	Eosinophil counts	0.91 (0.67, 0.98)	0.002
	100149346_TGI_at	SOCS2	Eosinophil counts	0.91 (0.66, 0.98)	0.002
	100312840_TGI_at	ILIRL1	Eosinophil counts	0.88 (0.56, 0.97)	0.004
	100301491_TGI_at	GATA2	Eosinophil counts	0.87 (0.53, 0.97)	0.005
Fluticasone treatment effect	100148162_TGI_at	ILIRL1	Eosinophil counts	0.98 (0.92, 1.00)	< 0.001
	100312840_TGI_at	ILIRL1	Eosinophil counts	0.98 (0.88, 1.00)	< 0.001
	100302783_TGI_at	ILIRL1	Eosinophil counts	0.97 (0.85, 0.99)	< 0.001
	100142511_TGI_at	CCL26	Eosinophil percentage	0.97 (0.84, 0.99)	< 0.001
	100148726_TGI_at	CCL17	Eosinophil percentage	0.96 (0.80, 0.99)	< 0.001
	100161010_TGI_at	HRH4	Eosinophil counts	0.96 (0.79, 0.99)	< 0.001
	100134267_TGI_at	CRLF2	Eosinophil percentage	0.93 (0.69, 0.99)	0.002
	100135727_TGI_at	CD1A	Eosinophil percentage	0.92 (0.64, 0.98)	0.004
	100148210_TGI_at	ILIRL1	Eosinophil counts	0.90 (0.59, 0.98)	0.005
	100157709_TGI_at	ALOX15	Eosinophil counts	0.89 (0.52, 0.98)	0.008
	100302151_TGI_at	ILIRL1	Eosinophil counts	0.88 (0.52, 0.98)	0.008
	100162200_TGI_at	GATA2	Eosinophil percentage	0.88 (0.52, 0.98)	0.008
	100125222_TGI_at	IL13	Eosinophil percentage	0.88 (0.51, 0.98)	0.008
	100163032_TGI_at	VDR	Eosinophil counts	0.88 (0.51, 0.98)	0.008
	100302151_TGI_at	ILIRL1	Eosinophil percentage	0.88 (0.50, 0.98)	0.009
	100142202_TGI_at	CD1B	Eosinophil percentage	0.87 (0.48, 0.97)	0.01
	100124660_TGI_at	GGT5	Eosinophil counts	0.87 (0.46, 0.97)	0.011
	100143473_TGI_at	HDC	Eosinophil counts	0.87 (0.46, 0.97)	0.012
MRNA at hour 7 and clinical endpoint at hour 24					
Allergen challenge effect	100135727_TGI_at	CD1A	Eosinophil percentage	0.91 (0.65, 0.98)	0.002
	100132327_TGI_at	ADAM19	Eosinophil percentage	0.87 (0.55, 0.97)	0.004
	100136515_TGI_at	CD209	Eosinophil percentage	0.87 (0.53, 0.97)	0.005
Fluticasone treatment effect	100148210_TGI_at	ILIRL1	Eosinophil counts	0.93 (0.67, 0.99)	0.003
	100309438_TGI_at	MAOA	Eosinophil counts	-0.92 (-0.98, -0.64)	0.003
	100309708_TGI_at	PTGS1	Eosinophil counts	0.92 (0.65, 0.98)	0.003
	100124660_TGI_at	GGT5	Eosinophil counts	0.92 (0.63, 0.98)	0.004
	100156386_TGI_at	PTGS1	Eosinophil counts	0.89 (0.53, 0.98)	0.008
	100302151_TGI_at	ILIRL1	Eosinophil counts	0.88 (0.51, 0.98)	0.009
	100136515_TGI_at	CD209	Eosinophil percentage	0.88 (0.49, 0.97)	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. IS: induced sputum.

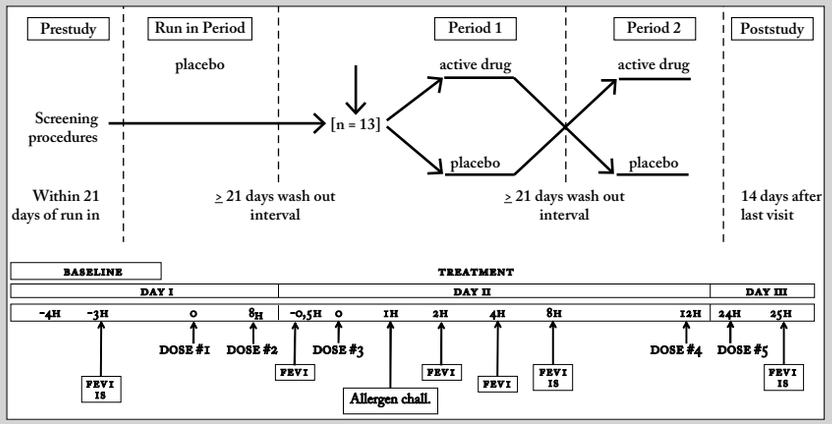


Figure 2 Hierarchal cluster assessment of sputum microarray data. Numbers refer to subject allocation numbers. Log 10 ratios of intensity estimates versus the average of all intensities are displayed. Dark color refers to probe sets that are up-regulated in reference to the pool of all specimens analyzed 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

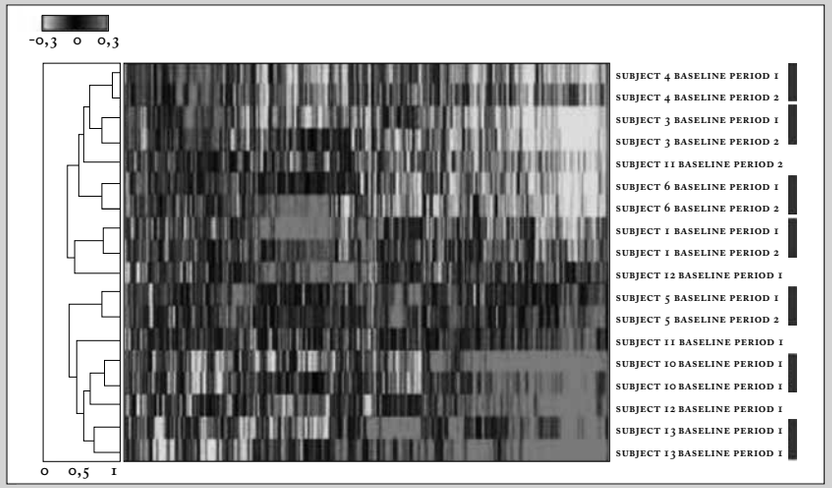


Figure 3 Log 10 Estimates of gene expression changes for the significant genes identified from contrast analysis at 7 hours and 24 hours with an FDR < 0.025. ACE; allergen challenge effect, estimates of changes from baseline in the placebo group. FTE: fluticasone effect, estimates of differences in change from baseline between the placebo and the fluticasone groups

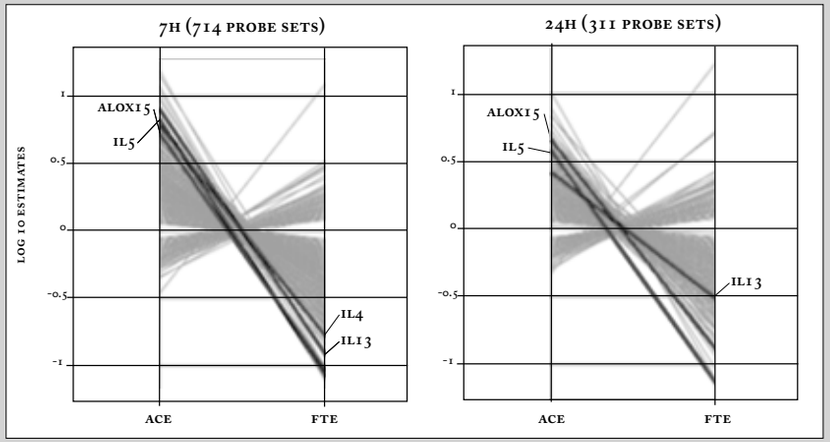


Figure 4 Fold change from baseline in gene expression. Th2 cytokines (IL4, IL5, IL13), Th1 cytokines (IFNG and TNF), Th17 cytokines (IL22, IL26). Fold change from baseline for the placebo group is represented on the left in each bar. Fold change from baseline for the fluticasone group is represented on the right in each bar. P-values are adjusted p-values, error bars represent 90% confidence

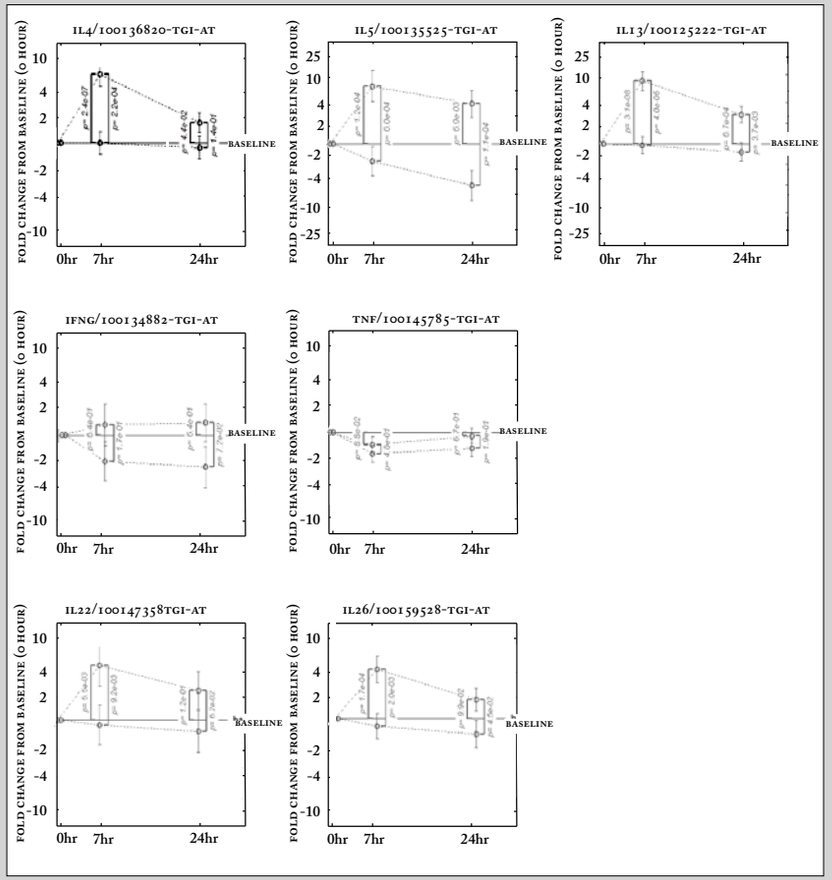


Figure 5 Fold change from baseline in gene expression. Inflammatory chemokines (CCL13, CCL17, CCL26), molecules controlling the release of histamine (HDC, HRH4, FCER1A) prostaglandins and leukotrienes (PTGER3, ALOX15, GGT5). Fold change from baseline for the placebo group is represented on the left in 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.

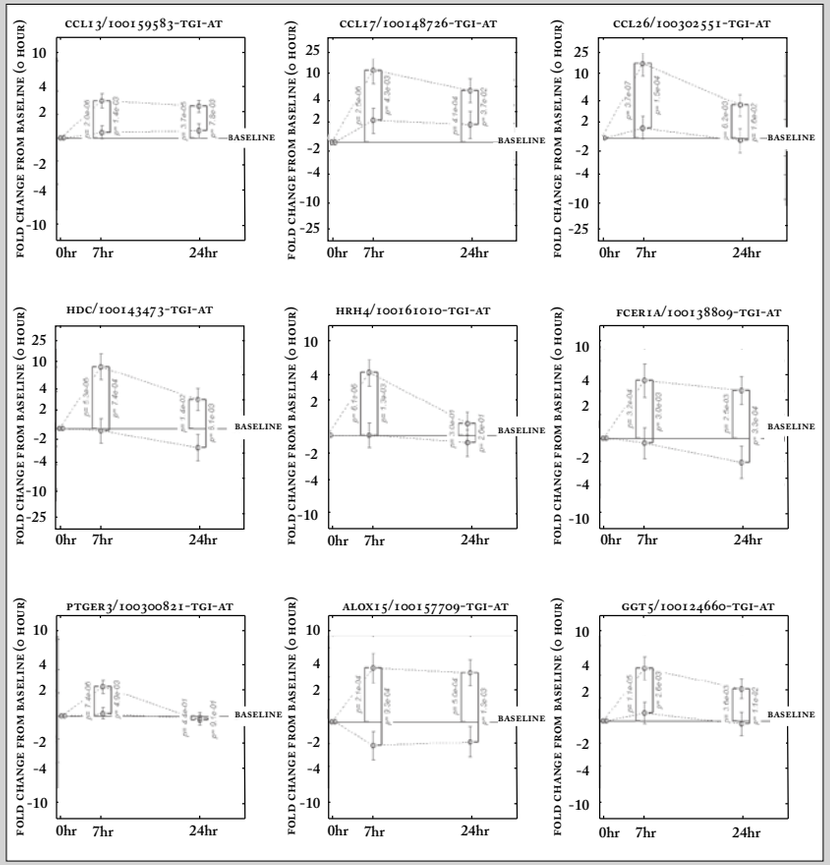


Figure 6 Fold changes over baseline (point estimate and 90% confidence intervals) for the 47 RNA signatures. Light bars represent the change from baseline in the placebo group and dark bars in the fluticasone group. P-values for the allergen challenge effect (ACE) and the fluticasone treatment effect (FTE) are represented. P1: Th2 cytokines, P2: chemokines and chemokine receptors, P3: FCER1 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"

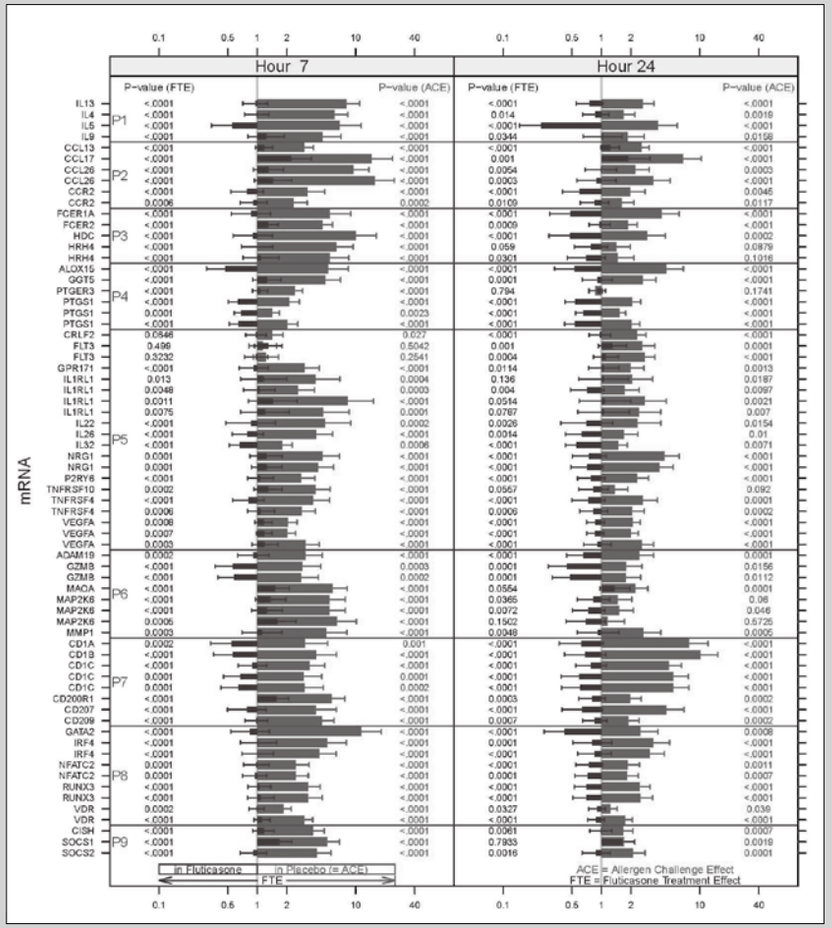
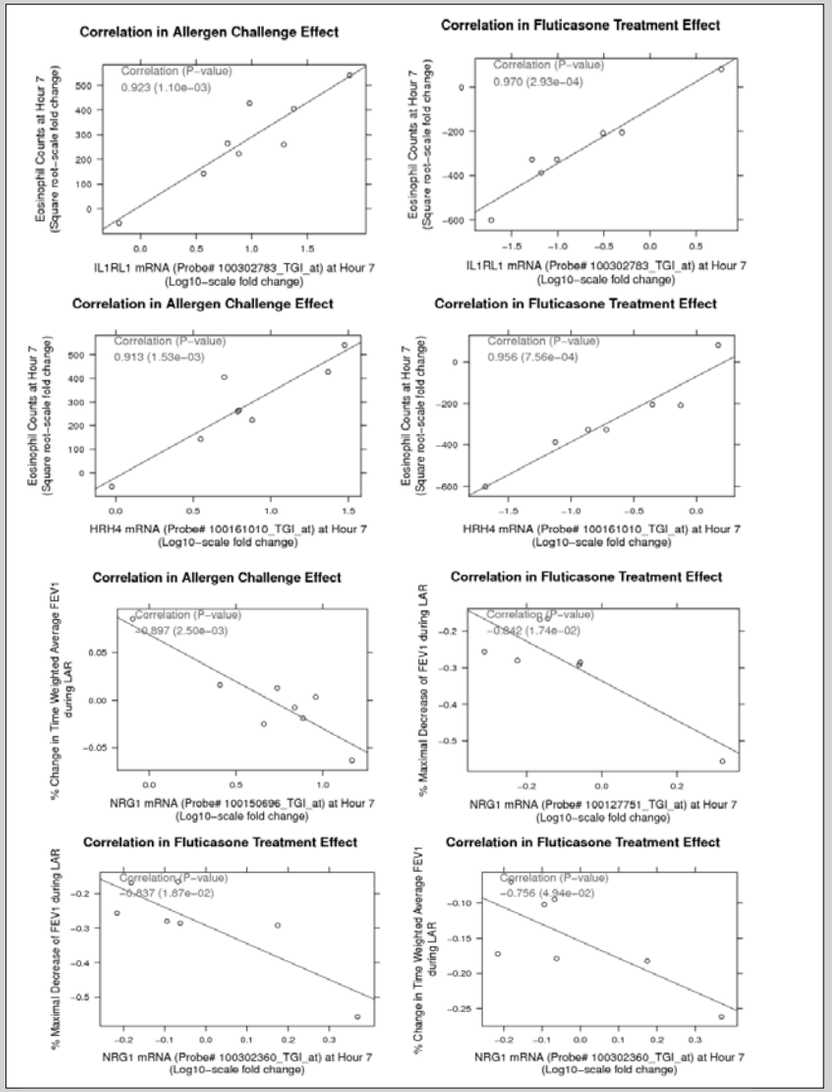


Figure 7 Correlation plots of the most significant probe sets to individual subject clinical measurements for the allergen challenge effect and the FP treatment effect. Correlation coefficients and corresponding p-values in parenthesis are listed in grey. HRH4 (histamine receptor 4); IL1RL1 (IL33 receptor); NRG1 (neuregulin 1)



REFERENCE LIST

1 Diamant Z, Gauvreau GM, Cockcroft DW et al. Inhaled allergen bronchoprovocation tests. J Allergy Clin Immunol. 2013; 132: 1045-1055

2 Boulet LP, Gauvreau G, Boulay ME, O'Byrne P, Cockcroft DW. The allergen bronchoprovocation model: an important tool for the investigation of new asthma anti-inflammatory therapies. Allergy 2007; 62: 1101-1110

3 Bakakos P, Schleich F, Alchanatis M, Louis R. Induced sputum in asthma: from bench to bedside. Curr Med Chem. 2011; 18: 1415-1422

4 Gauvreau GM, Watson RM, O'Byrne PM. Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation. Am J Respir Crit Care Med 1999; 160: 640-647

5 Diamant Z, Timmers MC, van d, V, Page CP, van der Meer FJ, Sterk PJ. Effect of inhaled heparin on allergen-induced early and late asthmatic responses in patients with atopic asthma. Am J Respir Crit Care Med 1996; 153: 1790-1795

6 Parameswaran K, Inman MD, Watson RM et al. Protective effects of fluticasone on allergen-induced airway responses and sputum inflammatory markers. Can.Respir J 2000; 7: 313-319

7 Zuiker RG, Ruddy MK, Morelli N et al. Kinetics of TH2 biomarkers in sputum of asthmatics following inhaled allergen. Eur Clin Respir J 2015; 2

8 Faiz A, Burgess JK. How can microarrays unlock asthma? J Allergy (Cairo.) 2012; 2012: 241314

9 Rolph MS, Sisavanh M, Liu SM, Mackay CR. Clues to asthma pathogenesis from microarray expression studies. Pharmacol Ther 2006; 109: 284-294

10 Lee JH, Kaminski N, Dolganov G et al. Interleukin-13 induces dramatically different transcriptional programs in three human airway cell types. Am J Respir Cell Mol Biol 2001; 25: 474-485

11 Lilly CM, Tateno H, Oguma T, Israel E, Sonna LA. Effects of allergen challenge on airway epithelial cell gene expression. Am J Respir Crit Care Med 2005; 171: 579-586

12 Laprise C, Sladek R, Ponton A, Bernier MC, Hudson TJ, Laviolette M. Functional classes of bronchial mucosa genes that are differentially expressed in asthma. BMC Genomics 2004; 5: 21

13 Guajardo JR, Schleifer KW, Daines MO et al. Altered gene expression profiles in nasal respiratory epithelium reflect stable versus acute childhood asthma. J Allergy Clin Immunol. 2005; 115: 243-251

14 Singh A, Yamamoto M, Kam SH et al. Gene-metabolite expression in blood can discriminate allergen-induced isolated early from dual asthmatic responses. PLoS One 2013; 8: e67907

15 Goleva E, Hauk PJ, Hall CF et al. Corticosteroid-resistant asthma is associated with classical antimicrobial activation of airway macrophages. J Allergy Clin Immunol 2008; 122: 550-559

16 Peters MC, Mekonnen ZK, Yuan S, Bhakta NR, Woodruff PG, Fahy JV. Measures of gene expression in sputum cells can identify TH2-high and TH2-low subtypes of asthma. J Allergy Clin Immunol. 2014; 133: 388-394

17 From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA). Available from: http://www.ginasthma.org/ [cited 4 Jan 2016]. 2015.

18 ATS/ers recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide. Am J Respir.Crit.Care Med 2005; 171: 912-930

19 Crapo RO, Casaburi R, Coates AL et al. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. Am J Respir Crit Care Med 2000; 161: 309-329

20 Djukanovic R, Sterk PJ, Fahy JV, Hargreave FE. Standardised methodology of sputum induction and processing. Eur Respir J Suppl. 2002; 37: 1S-2S

21 Miller MR, Hankinson J, Brusasco V et al. Standardisation of spirometry. Eur Respir J 2005; 26: 319-338

22 Pizzichini E, Pizzichini MM, Leigh R, Djukanovic R, Sterk PJ. Safety of sputum induction. Eur Respir J Suppl. 2002; 37: 9S-18S

23 Efthimiadis A, Spanevello A, Hamid Q et al. Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation. Eur Respir J Suppl. 2002; 37: 19S-23S

24 Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 1998; 95: 14863-14868

25 Mendez-Enriquez E, Garcia-Zepeda EA. The multiple faces of CCL13 in immunity and inflammation. Inflammopharmacology. 2013; 21: 397-406

26 Sekiya T, Yamada H, Yamaguchi M et al. Increased levels of a TH2-type CC chemokine thymus and activation-regulated chemokine (TARC) in serum and induced sputum of asthmatics. Allergy 2002; 57: 173-177

27 Larose MC, Chakir J, Archambault AS et al. Correlation between CCL26 production by human bronchial epithelial cells and airway eosinophils: Involvement in patients with severe eosinophilic asthma. J Allergy Clin Immunol 2015; 136: 904-913

28 Ohtsu H. Pathophysiologic role of histamine: evidence clarified by histidine decarboxylase gene knockout mice. Int Arch Allergy Immunol 2012; 158 Suppl 1: 2-6

29 Hartwig C, Munder A, Glage S et al. The histamine H4-receptor (H4R) regulates eosinophilic inflammation in ovalbumin-induced experimental allergic asthma in mice. Eur J Immunol 2015; 45: 1129-1140

30 Han B, Luo G, Shi ZZ et al. Gamma-glutamyl leukotrienase, a novel endothelial membrane protein, is specifically responsible for leukotriene D(4) formation in vivo. Am J Pathol 2002; 161: 481-490

31 Gauthier M, Ray A, Wenzel SE. Evolving Concepts of Asthma. Am J Respir Crit Care Med 2015; 192: 660-668
32 Haldar P, Pavord ID, Shaw DE et al. Cluster analysis and clinical asthma phenotypes. Am J Respir Crit Care Med 2008; 178: 218-224
33 Woodruff PG, Modrek B, Choy DF et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. Am J Respir Crit Care Med 2009; 180: 388-395
34 Zheng Y, Danilenko DM, Valdez P et al. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature 2007; 445: 648-651
35 Donnelly RP, Sheikh F, Dickensheets H, Savan R, Young HA, Walter MR. Interleukin-26: an IL-10-related cytokine produced by TH17 cells. Cytokine Growth Factor Rev 2010; 21: 393-401
36 Newcomb DC, Peebles RS, Jr. TH17-mediated inflammation in asthma. Curr Opin Immunol 2013; 25: 755-760
37 Yu B, Shao Y, Zhang J et al. Polymorphisms in human histamine receptor H4 gene are associated with atopic dermatitis. Br J Dermatol. 2010; 162: 1038-1043
38 Reijmerink NE, Postma DS, Bruinenberg M et al. Association of IL1RL1, IL18R1, and IL18RAP gene cluster polymorphisms with asthma and atopy. J Allergy Clin Immunol 2008; 122: 651-654
39 Chaussabel D, Quinn C, Shen J et al. A modular analysis framework for blood genomics studies: application to systemic lupus erythematosus. Immunity 2008; 29: 150-164
40 Kettle R, Simmons J, Schindler F et al. Regulation of neuregulin rbeta1-induced MUC5AC and MUC5B expression in human airway epithelium. Am J Respir Cell Mol Biol 2010; 42: 472-481.