Distinct epithelial gene expression phenotypes in childhood respiratory allergy

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Abstract

Background. Epithelial cell contribution to the natural history of childhood allergic respiratory disease remains poorly apprehended. Our aims were to identify epithelial pathways dysregulated in different phenotypes of respiratory allergy.

Methods. We established gene expression signature of nasal brushings from children with dust-mite-allergic rhinitis, associated or not with controlled or uncontrolled asthma. Supervised learning and unsupervised clustering were used to predict the different subgroups of patients, and define altered signalling pathways. These profiles were compared to those of primary cultures of human nasal epithelial cells, stimulated either by IL-4, IL-13, interferon-α, -β or -γ, or during in-vitro differentiation.

Results. A supervised method discriminated children with allergic rhinitis from healthy controls (prediction accuracy: 91%), based on 61 transcripts, including 21 Th2-responding genes. This method was then applied to predict children with controlled or uncontrolled asthma (prediction accuracy: 75%), based on 41 transcripts: 9 of them, down-regulated in uncontrolled asthma, are directly linked to interferon. This group also included GSDML, which is genetically associated with asthma.

Conclusions. Our data reveals a Th2-driven epithelial phenotype common to all dust-mite-allergic children. It highlights the influence of epithelialexpressed molecules on the control of asthma, in association with atopy and impaired viral response.

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Introduction

Asthma is a heterogeneous and complex disease that frequently co-occurs with allergic rhinitis. Classical pathogenic models characterize asthma and allergic rhinitis as inflammatory diseases associated with an increased Th2 response. Despite chronic inflammatory processes, several additional defects intrinsic to the airway epithelium may contribute to the pathogenesis. Airway remodeling observed in asthma can indeed appear several years before any clear clinical diagnosis, and even affect preschool asthmatic children (1). Asthma patients appear more susceptible to infections by respiratory viruses (2). Epithelial cells derived from asthma patients exhibit a reduced capacity to secrete fibronec tin, a well-known contributor of repair in damaged airway epithelium, together with defective anti-oxidant pathways and tight junctions (3-5). Some studies have attempted to associate specific gene expression profiles with asthma or allergic rhinitis, mostly in adults (3, 6-10). Information has also been gathered by genome wide association studies. Single nucleotide polymorphisms (SNPs) in the GSDML locus (near ORMDL3), a protein detected in epithelial tissues, are strongly associated with childhood-onset disease (11). Based on these findings, our rationale was that defects in the airway epithelium could correspond to primary defects, or early secondary events, which could favor a chronic inflammation, and lead to asthma and/or allergic rhinitis. We thus determined the transcriptional signatures of nasal brushings collected from paediatric patients with different clinical phenotypes of dust-mite respiratory allergy. Data analysis revealed altered behaviors of several signaling pathways that were further analyzed in an in-vitro model of differentiated human nasal epithelial cells (HNECs).
Materials and Methods

Subjects and samples

Patients were aged 6 to 17 years old (n=45). Thirty-three were being followed-up for a dust-mite-allergic rhinitis. Fourteen were also asthmatic, with either controlled asthma (group "a", n=7) or uncontrolled asthma (group "A", n=7). Nineteen had rhinitis without asthma (group "R", n=19). The control group (group "C") comprised 12 healthy children. Clinical diagnoses and asthma control staging corresponded to ARIA and pediatric NAEPP/EPR3 criteria (see supplementary file 1 for details). The project received the approval of the Nice University Hospital Ethics Committee and all of the volunteers’ parents gave written, informed consent.

Nasal respiratory epithelium sampling was performed by brushing the inferior turbinates.

Viral respiratory tract infection detection by Real-time quantitative PCR (qPCR) was performed according to the RealAccurate™ Respiratory Kit manufacturer’s instructions (PathoFinder, 6229 EV Maastricht, Netherlands). Seven children (1 asthmatic, 5 rhinitics without asthma, 1 control) positive for respiratory viruses were not included in further analyses. The characteristics of the patients and samples are summarized in Tables 1 and E1.

Isolation and culture of primary HNECs

Primary cultures of HNECs derived from the nasal mucosa of inferior turbinates were performed according to (12). Each experiment was performed using three different donors. Before treatment with cytokines, HNECs were cultured 4 weeks in an air-liquid interface to obtain a well-differentiated epithelium (12). Tested agents: IL-4 (BD Pharmingen, San Diego, CA 92121, United States), IL-13 (PeproTech, Rocky Hill, NJ 08553, United States): 10 ng/mL for 24 h; IFN-α/IFN-β1a (Sigma-Aldrich): 1000 UI/mL for 6 h and IFN-γ (BD-Pharmigen): 100 ng/mL for 6 h were added on the basolateral side.

Gene expression analyses
Microarray analyses were performed on the GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA 95051, USA), according to the manufacturer's instructions. Microarray data are archived in GEO under reference GSE19190 and GSE22147, and on the MEDIANTE database developed by the laboratory (13, 14). The qPCR procedure is detailed in supplementary file 1, with the primers and probes listed in Table E2.

**Biostatistics**

Most of statistical calculations and prediction analyses were performed using limma and randomForest, two packages of the R statistical environment (15). We used the Functional Annotation Clustering report available from DAVID (16). Hierarchical clusterings were done with the MultiExperiment Viewer (MeV) program version 4.3, using a Manhattan distance metric and average linkage (17). Biological networks were built using Ingenuity Pathway Analysis software™ (IPA™). Gene Set Enrichment Analysis (GSEA) was used to determine whether an *a-priori* defined set of genes can characterize differences between two biological states (18, 19). ENCODE functional genomics data were used to determine potential binding sites for interferon regulatory factors.

**Immunohistochemistry**

Immunohistochemistry methods are described in supplementary file 1.

**ELISA assay**

The level of Muc5AC released by HAECs in response to IL-13 stimulation was measured using ELISA assay following manufacturer’s instructions (EIAab & USCNLIFE, Wuhan EIAab Science Co., Ltd).
Results

Genes differentially expressed in the nasal epithelial cells of children with dust mite allergy

The transcriptional signature of dust-mite-allergic rhinitis children (n=27) was compared with the one of healthy children (n=11). We identified 169 differentially expressed transcripts (adjusted p-value<0.05) (data on request). Among them, 24 transcripts were modulated with an absolute value of log₂(ratio) greater than 1 (Table 2). Functional annotation by DAVID is provided on Table E3. Variation of some genes (CST1, POSTN, NTS, CD44, GSN, ALOX15) was confirmed by qPCR (Figure E1).

Supervised clustering was used to predict a status to each of the 38 patients (allergic or healthy). Thirty-five of the 38 patients were correctly predicted (prediction accuracy of 91%) (Fig 1A). Specificity (Sp) and Sensitivity (Se) are summarized in a ROC curve (Figure E2A), showing an area under ROC equal to 0.88. The Random Forest approach that was used allowed the identification of the most influential genes for the prediction, leading to a set of 61 transcripts that was common to 20 independent cross-validations. Most genes were up-regulated in the allergic conditions (Figure 1B). Sixteen of them had been linked previously to asthma or allergic rhinitis by gene expression profiling (Table E4) (3, 6-10). The signature was linked to the allergic status, but independent of the asthma status. Indeed, supervised clustering was unable to discriminate the 13 asthmatic patients from the 14 allergic rhinitis patients without asthma. No differential expression was detected between these two groups of patients (not shown).

Epithelial Th2 signature discriminates allergic children from healthy controls

We observed that several transcripts dysregulated in patients allergic to dust mites were also linked by the literature to IL-4 or IL-13 (20). We mimicked in vitro this situation by a
stimulation of primary HNECs with IL-4 or IL-13. We confirmed with this model that IL-13 stimulation increased the expression (Fig E3A) and the secretion of Muc5AC, a goblet cell marker (Fig E3B). We also observed in parallel the decreased expression of FoxJ1, a ciliated cell marker (Fig E3A). Forty-six airway epithelial transcripts were selected, based on their high response to Th2 cytokines and their detection in highly differentiated airway epithelial cells (Table E5). Twenty-one transcripts of this “Th2 signature” were among the 61 transcripts predicting allergic or healthy children (Figure 1). The importance of the Th2 regulation was assessed by a normalized enrichment score above 1 (1.61, with nominal p-value and FDR q-value being <0.001), as determined by GSEA (18, 19). Interestingly, we noticed that the transcripts associated with the Th2 signature displayed a larger log2(ratio) in uncontrolled (A1:A6) than in controlled (a1:a7) asthma (Figure 1). This led us to investigate the existence of a specific expression profile associated with uncontrolled asthma.

Control of asthma affects gene expression signature of nasal epithelial cells

The next analysis compared the two groups of subjects with uncontrolled or controlled asthma. Table 1 describes the characteristics of these patients. The expression of 160 genes differed significantly between the two groups (Table E6). Functional annotation is reported in Table E7. The Random Forest approach correctly predicted the class for 12 of 14 samples, with a 75.4% prediction accuracy (Figure 2A). Specificity (Sp) and Sensitivity (Se) are summarized in a ROC curve (Figure E2B), showing an area under ROC equal to 0.833. It also identified a minimal set of the 41 most influential genes for the prediction (Fig 2B). Up-regulation of POSTN, FETUB, or DPP4 (also belonging to “Th2 signature”, Table E5) in the uncontrolled group is coherent with the larger log2(ratio) observed for Th2 responding transcripts in Figures 1 and 2. We also noticed a down-regulation of several interferon stimulated genes (ISGs), such as CXCL10, MX2, IFIT2, DUOX2, GBP2, and GBP5 among a
larger cluster of down-regulated genes (Figure 2). This was illustrated by Ingenuity Pathways Analyses (Figure E4). Our cluster of down-regulated genes also included GSDML, a gene located in a region of chromosome 17q21, recently linked to juvenile asthma in several genome-wide association studies (11). The differential expression of CXCL10, MX2, IFIT2, DUOX2 and GSDML was confirmed by qPCR (Figure E1). GSDML and DUOX2 proteins were immunodetected by histochemistry in nasal and bronchial airway epithelia (Figure 3).

**IFN-response analysis in patients and in cultured HNECs**

The interferon regulation was explored in vitro by stimulating primary HNECs with IFN-α, IFN-β or IFN-γ. We selected IFN-responsive transcripts (Table E8). GSEA approach revealed significant normalized enrichment scores for all subtypes of interferons in the uncontrolled/controlled asthma comparison (1.7 for IFN-α, 1.64 for IFN-β and 1.56 for IFN-γ). The capacity of interferons to induce the expression of MX1, ST8SIA4 and GSDML was confirmed by qPCR on primary cultures of HNECs. A peak of GSDML induction was observed after 12 hours of IFN-α stimulation, while the peak of induction for MX1 and ST8SIA4 was reached at 6h (Figure 4A). A survey of the genomic context for MX2, IFIT2, CXCL10, GSDML, ST8SIA4 and DUOX2 revealed the presence of several consensus binding sequences for IFN regulatory factors (full data on request) (Figure E5). Altogether, these observations suggest a direct regulation of these different genes by IFN. Importantly, the transcript levels of IFN-β, IFN-λ1, IFN-λ2/3, IFN-α1 and IFN-α2 were decreased in uncontrolled asthmatics compared to either controlled asthmatics or healthy subjects (Figure 4B). Endogenous production of epithelial interferons, rather than defective IFN signaling pathways, is therefore likely involved in these processes.

**Airway epithelial regeneration and severity of allergic diseases**
Impaired capacity of repair of the airway epithelium is a hallmark of allergic airway diseases (5). We have recently established gene expression signatures during in-vitro HNECs regeneration and differentiation (12). The “terminal differentiation” signature was used to enrich the annotation derived from the expression profiles obtained with Th2 cytokines and interferons experiments. As expected, the classification of the patients was principally linked to the effects of Th2 cytokines and IFNs. However, several transcripts associated to the disease were also linked to the “terminal differentiation” signature (Table E9). A first group of genes, including PLK4, CDC20B, GSDML and CYFIP2, was clearly induced during differentiation whilst being down-regulated in patients with uncontrolled asthma. This group puts together important markers of multiciliogenesis (PLK4, CDC20B) (12) and molecules associated to asthma in genome-wide analyses (GSDML, CYFIP2). A second group, which includes POSTN, SPINK5, CD44, TFF3, ITLN1, FOXA3 and SPDEF, was clearly associated with basal or mucous cells (21). Most of them were decreased during in vitro differentiation while all were up-regulated in patients with uncontrolled asthma.
Discussion

The airway epithelium is a complex physical barrier, playing an intrinsic role in innate and adaptive immunity, through the production of numerous cytokines and chemokines. Our work highlights the importance of IFN- and Th2-driven epithelial responses in pathogenesis of allergic respiratory diseases. Our findings were based on biostatistical approaches, such as Random Forest, Ingenuity and GSEA, which take into account not only the number of patients, but also the size of gene clusters that can be regulated by these cytokines. These approaches increase in an important manner the statistical significance of our observations.

The choice to perform our investigations on upper, rather than on lower, airway epithelium was motivated by its non-invasive character, easily applicable to young children. It allows the recruitment of true controls exempt of pulmonary disease - a situation which is clearly not possible in studies using bronchoscopic sampling (1). It also allows investigating patients with severe or uncontrolled asthma phenotypes without stopping their inhaled corticosteroids long term-treatment for asthma, as nasal deposition can be considered as negligible with the devices used in this study (22). Interestingly, we observed a good overlap between our “nasal epithelial” gene lists and several gene expression profiling performed on bronchial epithelium, notwithstanding differences existing between nasal and bronchial epithelial cells (23, 24), such as the quasi absence of remodeling in nasal epithelium (25). In this context, the use of alternative techniques to harvest lower airway cells during general anesthesia could be worth investigating (26). Our inability to identify ad hoc biomarkers for discriminating asthmatic and rhinitis patients can be due to the choice of sampling material. If true, a comprehensive comparison of bronchial and nasal epithelial cells from the same patients would help exploring this issue. This was unfortunately not planned in the present study. The relatively small size of the study (45 samples) could also be limiting. A third explanation could be that the major differences discriminating asthma from allergic rhinitis
would, rather, depend on other cell types (dendritic or mesenchymal cells…). From that perspective, progression from allergic rhinitis to asthma could not be detected in the epithelium *per se*.

Our study establishes epithelial activation by Th2 cytokines as a central trait of the airway allergic respiratory disease. This is in line with the observation that IL-13 can recapitulate most of the characteristics of asthma in several experimental models of the disease (27). Our clinical and *in-vitro* results clearly demonstrate the major impact of Th2 cytokines on the allergic subjects, reflecting the strength of the atopic status. Several “Th2-induced” genes such as POSTN, ITLN1, ALOX15, CD44 and SERPINB4, have previously been linked to asthma. Others could represent additional relevant biomarkers, due to their large and consistent variations in the allergic group. A first example is CST1, a cysteine protease inhibitor, already associated with pulmonary fibrosis in systemic sclerosis patients (28). A second example is NTS which has been implicated in wound healing in chronic colitis (29).

An enhanced up-regulation of Th2-responsive genes (characterized by larger log2ratios) was observed in uncontrolled asthma. In fact, asthma severity in children has already been associated with atopy in epidemiological studies (30). This association seems specific for childhood asthma, as an inverse relationship has been reported in adults (31). Interestingly, IL-13 has been shown to induce a profibrotic bronchial phenotype *via* a continuous secretion of TGF-β2 (32). Strikingly, the first Gene Ontology theme in the comparison between uncontrolled and controlled asthma corresponds to the term “structural constituents of extracellular matrix”, with enhanced expression of POSTN, TNC, FBN2, FBLN2, FMOD and COL4A6. It is tempting to speculate that these dysregulations could be directly related to the structural modifications and remodeling of the epithelium preferentially observed in severe asthma (33). At the same time, POSTN has been reported to induce reentry
of differentiated mammalian cardiomyocytes into the cell cycle, in link with tissue repair (34). Mucus hypersecretion and goblet cell metaplasia are important traits of asthma, also linked with IL-13 dysregulation. We also noticed that TFF3, expressed in mucous cells, and which can promote the differentiation of ciliated human airway epithelial cells (21), was upregulated in allergic patients. This illustrates the complexity of the mechanisms leading to abnormal repair and remodeling.

The major impact of Th2 cytokines probably masks weaker, intrinsic epithelial features. We believe that this represents a confounding factor when analyzing epithelial susceptibility with the microarray technique. In order to dampen the impact of the Th2 response, we have compared subgroups of patients with uncontrolled or controlled asthma.

Another information derived from our work is the existence of an altered IFN response in nasal epithelium of uncontrolled asthmatic patients: this trait may be more-directly related to an intrinsic epithelial susceptibility. The IFN-response was reduced in the uncontrolled asthma group relative to the controlled asthma and “healthy” groups. We excluded the possibility that these effects were caused by viral infections: (i) a viral PCR assay, able to detect 90% of respiratory tract viral infections, eliminated cases of viral infection; (ii) a careful evaluation of CXCL10 transcript levels in “non-infected” patients identified additional infected samples.

ISGs that are decreased in patients with uncontrolled asthma indeed represent a quarter of the genes identified by our second supervised analysis. Unexpectedly, we identified GSDML in that cluster. Our study reports several additional observations suggesting a direct association of this gene with the abnormalities affecting the paediatric asthmatic epithelium: first, the existence of several response elements for IFN regulatory factors in its promoter region; second, its transcriptional induction by epithelial IFN in HNEC; third, its protein
expression in airway epithelial cells. This is in line with the fact that one of its SNPs (rs7216389) has been genetically associated with early-onset of asthma, acute severe exacerbation (80% of which are virus-induced in children) and asthma severity (11, 35, 36). Moffatt et al. (37) initially noted an association of childhood asthma risk with modified expression of ORDML3, located in the immediate vicinity of GSDML. The identification of a variant in a splice site for GSDML in strong linkage disequilibrium with ORMDL3 has recently suggested that GSDML would be the causative gene associated with asthma (38).

Decreased levels of IFN-α, IFN-β, IFN-λ1 and IFN-λ2/3 paralleled the down-regulation of IFN-stimulated genes observed in patients with uncontrolled asthma. This confirms and extends findings by de Blic et al (39), showing a correlation between asthma symptoms and the IFN-response in a cohort of 28 children with difficult-to-treat asthma. In their study, lower levels of IFN-γ were associated with persistent symptoms (39). Deficient epithelial cell IFN-β and IFN-λ production during rhinovirus-induced asthma exacerbations was associated with increased viral load, defective apoptosis and cell death bias (2). Epidemiological studies have associated the onset of asthma with reduced IFN-γ production by peripheral blood mononuclear cells at nine months of age, whilst a normalization of IFN-γ production is observed in children showing a resolution of asthma symptoms (40, 41).

Regardless of the exact molecular mechanisms leading to impaired IFN production, one could anticipate from our results some benefits of IFN treatments in patients with uncontrolled asthma, as suggested in recent in vitro experiments with IFN-β (42). As the association between defective epithelial cell IFN pathways and the lack of asthma control in patients could be specific to pediatric asthma, careful evaluation of therapeutic options based on the development of innate immunity would require the organization of clinical trials addressing this specific issue in a pediatric population. Finally, our work well illustrates some specificities of severe paediatric asthma, which not only results from the development of T-
cell-mediated biased adaptive responses, but also from an inefficient epithelial innate immunity.
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Legends to Tables and Supplementary Tables

Table 1. Subject characteristics.
Statistical comparisons were performed in Allergic(A+a+R) versus Healthy control group (C) and in uncontrolled (A) versus controlled (a) asthmatic group using t-test and khi2. Only significant results are shown (*).

*p-value<0.005, uncontrolled (A) versus controlled (a) asthmatic group (t-test).
Abbreviations: PMN, polymorphonuclear cells; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; FEF 25-75%, forced mid-expiratory flow rate 25-75%. Statistical comparisons were performed using t-test: *, p-value<0.005.

Table 2. Genes differentially expressed in nasal epithelial brushings from dust-mite-allergic rhinitis patients versus healthy subjects (microarray analysis).
Genes were ranked according to decreasing log2(Allergic/Healthy). Only genes with a log2(ratio) greater than 1 and a significant p-value (p-value<0.05) after a Benjamini-Hochberg correction for multiple tests were selected. Gene names are shown in red where they were induced in vitro with a value of log2(ratio) greater than 1 by both IL-4 and IL-13 in HNECs. A more detailed table is provided as Supplementary Table E3.

Supplementary Table E1. Subject characteristics.
Abbreviations: a, dust-mite-allergic rhinitis with controlled asthma; A, dust-mite-allergic rhinitis with uncontrolled asthma; R, dust-mite-allergic rhinitis without asthma; C, healthy subject; WC, well controlled; NWC, not well controlled; VPC, very poorly controlled; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity

Supplementary Table E2. Sequences of primers and probes designed with Primer Express®.

Supplementary Table E3. Functional annotation clustering of the 169 genes regulated in allergic patients compared with healthy subjects.
Annotation was performed according to DAVID Bioinformatics functional annotation clustering. The most significant theme for each functional annotation group with group enrichment scores greater than 1 is listed. For each theme, the number of differentially-expressed genes and the number of genes on the microarray are represented. The probability of obtaining the number of genes in a certain theme in the list of our differentially-expressed genes was compared with the representation of the same theme among all genes on the array and was calculated as a p-value. “Count” defines the number of transcripts in a list of interest that belong to a given pathway. “Pop hits” defines the number of transcripts in the whole transcriptome belonging to the same pathway. Genes up-regulated are shown in red and genes down-regulated in green. Abbreviations: CC: cellular component, BP: biological process, MF: molecular function.

Supplementary Table E4. Literature analysis of former microarray studies.

The Random Forest approach allows the identification of a set of 61 transcripts in the comparison allergic rhinitis versus healthy controls. They were, then, analyzed in available, previously-published microarray analysis data from studies of bronchial or nasal epithelia with asthma or allergic rhinitis (3, 6-10)

Supplementary Table E5. 46 genes differentially induced in vitro by IL-4 and IL-13 in primary HNECs.

Genes were ranked according to decreasing log2(IL-4/control). Only genes with an absolute value of log2(ratio) greater than 1 in both IL-4 (10 ng/mL for 24 h) and IL-13 (10 ng/mL for 24 h) stimulation conditions are listed.

Supplementary Table E6. 160 genes differentially expressed in nasal epithelial brushings (patients with uncontrolled asthma versus patients with controlled asthma).
Genes were ranked according to decreasing log₂(uncontrolled asthma/controlled asthma).

Only genes with a significant p-value (p-value<0.05) using the Benjamini-Hochberg correction are listed.

**Supplementary Table E7. Functional annotation of the 160 genes regulated in patients with uncontrolled, compared with controlled, asthma.**

Annotation was performed according to DAVID Bioinformatics functional annotation clustering. Most significant theme for each functional annotation group with a group enrichment score greater than 1 is listed. For each theme, the number of differentially-expressed genes and the number of genes on the microarray are represented. The probability of obtaining the number of genes in a certain theme in the list of our differentially-expressed genes was compared with the representation of the same theme among all genes on the array and was calculated as p-value. “Count” defines the number of transcripts in a list of interest that belong to a given pathway. “Pop hits” defines the number of transcripts in the whole transcriptome belonging to the same pathway. Genes up-regulated are shown in red and genes down-regulated in green. Abbreviations: CC: cellular component, BP: biological process, MF: molecular function.

**Supplementary Table E8. 316 genes differentially expressed in primary HNECs treated with IFN-α, IFN-β or IFN-γ.**

Genes were ranked according to decreasing log₂(IFN-α/control). Only genes with absolute values of log₂(ratio) greater than 1 in one of the conditions of stimulation: IFN-α, IFN-β (1000 UI/mL) or IFN-γ (100 ng/mL) for 6 h, are listed.

**Supplementary Table E9. Information about a selection of 215 genes showing an absolute average log₂(fold change) above 0.5 relative to control.** Genes were grouped according to their response to Th2 cytokines, interferons, or throughout the time course of in vitro differentiation. The different columns indicate the reference to Affymetrix Probe set ID,
the Gene Symbol, the existence of a link between the gene and asthma in PubMed, the chromosomal localization, the mRNA accession number, and the Gene Ontology annotations. Average log2(ratio) relative to control is indicated for TH2 cytokines, IFN, differentiation, and then for controlled asthma (a), uncontrolled asthma (A), isolated allergic rhinitis (R).
Figure legends

**Fig.1** Discrimination by Random Forest of dust-mite-allergic rhinitis children from healthy children and heatmap representation of the 61 most discriminant genes. A. Histogram showing, for each patient, the percentage of classification as a dust-mite-allergic rhinitis (red) or healthy (blue) child. B. Non-supervised, hierarchical clustering of the same patients using the set of 61 genes common to all Random Forest classifiers (see details in materials and methods). Each square represents the expression level of a given gene in a given sample relative to the average expression level in controls. A red to green color scale indicates gene expression levels above (red) or below (green) the average level of expression in healthy subjects for the same transcript. Clustering was performed using an average linkage method, using a Manhattan distance. The red color on gene names (right column) indicates genes induced *in vitro* by IL-4 and IL-13.
FIGURE 1
Fig. 2 Discrimination by Random Forest of children with uncontrolled, versus controlled, asthma and heatmap representation of the 41 most discriminant genes. A. Histogram showing, for each patient, the percentage of classification as uncontrolled asthma (red) and controlled asthma (orange). B. Non-supervised, hierarchical clustering of the same patients using the 41 probes common to all Random Forest classifiers (see materials and methods for details). Each square represents the expression level of a given gene in a given sample. A red to green color scale indicates gene expression levels above (red) or below (green) the average level of the healthy controls for the same transcript. Clustering was performed using an average linkage method, using a Manhattan distance. Red-colored gene names indicate transcripts induced by IL-4 and IL-13 (left column) or by interferons (right column) (corresponding to a log2(ratio) greater than 1 in HNECs). Gene names colored in green indicate transcripts down-regulated by IL-4 and IL-13 (left column) (log2(ratio) less than -1 in HNECs).
FIGURE 2
Fig.3. Immunohistochemical staining of sinus and bronchial biopsies. Representative immunostainings of GSDML and DUOX2 in surface epithelium and sub-mucosal glands for healthy adults are shown. Bars indicate 100 µm. Positive immunostainings were observed for the two proteins in sinus and bronchial sections. Strong signals were also detected in submucosal glands for the different markers.
FIGURE 3
**Fig. 4 Characterization of the interferon response.** A. Measurement by qPCR of the induction of GSDML, ST8SIA4, and MX1 by IFNα in HNECs. Levels of GSDML, ST8SIA4, and MX1 were assessed in differentiated HNECs 3 h, 6 h, 12 h, and 24 h after stimulation by IFNα. Measurements were performed using Sybrgreen. Results are expressed as fold change relative to the non-stimulated HNECs ± SEM. Statistical comparisons were performed using Student’s t-test (*, p<0.05; **, p<0.01;***, P<0.005) and SEM. B. Measurement of interferons by quantitative real-time PCR (qPCR) in patients. Levels of IFN-α1, IFN-α2, IFN-β, IFN-λ1 and IFN-λ2/3 transcripts in patients with uncontrolled asthma, controlled asthma, and healthy controls. Data are expressed relative to an average of 11 healthy controls. Measurements were performed using Taqman probes. Results are expressed as fold change relative to the healthy control group ± SEM. Statistical comparisons were performed using Student’s t-test (*, p<0.05; **, p<0.01;***, P<0.005) and SEM.
FIGURE 4
Fig. E1 qPCR validation for CST1, POSTN, NTS, CD44, GSN, ALOX15, MX2, GSDML, IFIT2, DUOX2 and CXCL10. qPCR validation of a selection of genes showing differential expression in nasal epithelial brushings between dust-mite-allergic rhinitis children and healthy children, or between uncontrolled and controlled asthma. The fold inductions by microarray and by qPCR were statistically significant for all genes (p-value<0.05, Benjamini-Hochberg corrected for microarray and p-value<0.05, Student’s t-test for qPCR).

Fig. E2. ROC (receiver operating characteristic) curves for the Random Forest supervised learning approach.

Graphical plot of the true positive rate (or sensitivity), versus. false positive rate (1−specificity ). A. Allergic versus Healthy: area under ROC was estimated to 0.88. B. Uncontrolled versus controlled asthma: area under ROC was estimated to 0.833.

Fig E3. Impact of IL-13 treatment on ciliated and goblet cell marker expression in HAECs. Differentiated HAECs were stimulated in presence of IL-13 (10 ng/ml) for 72 hours. A. Quantitative RT-PCR were carried out on extracted total RNA. The abundance of the target genes relative to that of control gene UBC was calculated as \( \Delta\Delta Ct = (\Delta Ct \text{ of target genes}) - (\Delta Ct \text{ of UBC}) \). IL-13 stimulation reduced the expression of the ciliated cell marker Foxj1 whereas it increased the expression of the goblet cell marker Muc5AC (a). B. Muc5AC protein level released in extracellular medium was measured by ELISA. IL-13 stimulation increased the Muc5AC level released by HAECs (b).

Fig. E4. Ingenuity Pathway Analysis identifies a network of ISGs genes modulated in the uncontrolled versus controlled asthma comparison. This network got the highest “network score” in the comparison. The network score was calculated with a right-tailed Fisher’s exact test. The network is displayed graphically as nodes (genes/gene products) and edges (the biological relationships between the nodes). Red and green nodes correspond to up- and down-regulated genes in the allergic versus healthy children comparison. As described in the
legend provided, nodes are displayed using various shapes that represent the functional class of the gene product. Edges are displayed with various labels that describe the nature of the relationship between the nodes. Grey nodes were identified by the pathway analysis as part of the network, but were not found to be differentially expressed in our comparison.

**Fig.E5. Genomic context of GSDML which belongs to the cluster of genes downregulated in uncontrolled asthma.** Binding sites for interferon regulatory factors are shown GSDML. Genomic regions and Encyclopedia of DNA Elements (ENCODE) functional genomics data were retrieved and displayed using the UCSC Genome Browser on Human March 2006 (NCBI36/hg18) Assembly (http://genome.ucsc.edu/). H3K4Me1 histone marks (monomethylated histone H3 at lysine 4) are associated with enhancer and promoter regions. ENCODE information about histone was derived from 8 cell lines. Histone H3K4Me3 (trimethylated histone H3 at lysine 4) binding is associated with promoter regions. ENCODE Histone Mark H3K4Me3 was derived from 9 cell lines. Hypersensitivity to DNAse I treatment is associated with accessible chromatin regions. The lane “DnaseI Clusters” represents DNAse I hypersensitivity clusters according to a greyscale, where the darker zones have the highest sensitivity to DNAseI. The lane “IRF4 ChIP-seq” reports the detection of IRF4 binding sites by chromatin immunoprecipitation followed by deep-sequencing in the GM12878 Cell Line. High affinity binding sites for IRF4 are indicated by the darkest greys. The lane “Mammal Conservation” indicates the conservation within 28 species according to a grayscale where darkest zones exhibit the highest conservation according to phastCons score (PHylogenetic Analysis with Space/Time Conservation).
Bibliography


Table 1. Subject characteristics.

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<th>Group &quot;a&quot; (n=7)</th>
<th>Group &quot;A&quot; (n=6)</th>
<th>Group &quot;R&quot; (n=14)</th>
<th>Group &quot;C&quot; (n=11)</th>
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<tr>
<td>Dust-mite-allergic rhinitis</td>
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<td>Yes</td>
<td>Yes</td>
<td>No</td>
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<td>Asthma</td>
<td>Controlled</td>
<td>Uncontrolled</td>
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<td>No</td>
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<td>Age</td>
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<td>9.1 ± 0.6</td>
<td>11.3 ± 2.8</td>
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<td>Epithelial cells %</td>
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<td>86.5 ± 6.5</td>
<td>86.9 ± 8.7</td>
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<td>PMN %</td>
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<td>5 ± 3.2</td>
<td>7.2 ± 7.8</td>
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<td>Lymphocytes %</td>
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Statistical comparisons were performed in Allergic(A+a+R) versus Healthy control group (C) and in uncontrolled (A) versus controlled (a) asthmatic group using t-test and khi2. Only significant results are shown (*).

*p-value<0.005, uncontrolled (A) versus controlled (a) asthmatic group (t-test).
Table 2. Genes differentially expressed in nasal epithelial brushings from dust-mite-allergic rhinitis patients versus healthy subjects (microarray analysis).

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