Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients

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Short running head title: Cytokine mRNA in induced sputum in asthma

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Abbreviations:
ICS: inhaled corticosteroids
eNO: exhaled nitric oxide
FEV1: forced expiratory volume in 1 second
IFN- : interferon
IL: interleukin
mAb: monoclonal antibody
mRNA: messenger RNA
PC20: histamine dose resulting in a 20% decrease in FEV1
Th: T helper
SUMMARY

Background: Asthma is a chronic inflammatory disorder of the airways driven by T cell activation. Th2 cells and their cytokines are thought to play a role in the pathophysiology of allergic as well as non-allergic asthma.

Methods: We obtained airway cells by sputum induction in healthy (n=15) and asthmatic (n=39) individuals and studied airway T cell cytokine profiles (Interleukin (IL)-4, IL-5, IL-13, IL-10 and IFN-γ) at the mRNA level by real-time RT-PCR.

Results: Asthma patients had increased expression of IL-5 (p=0.001) and IL-13 (p=0.03) mRNA in sputum compared to the non asthmatic controls. IL-4 mRNA and IFN-γ mRNA were detectable in sputum of respectively 44% and 21% of patients but never in controls. Sputum IL-10 mRNA levels did not significantly differ between patients and controls. Sputum mRNA expression levels of IL-4, IL-5 and IL-13 significantly correlated with eosinophil percentages, and were higher in allergic asthmatics than in non-allergics (p=0.03, p=0.02 and p=0.0002 respectively), while they did not differ between mild and moderate-to-severe asthmatics. In contrast, the IFN-γ mRNA expression was higher in non-allergic than in allergic patients (p=0.04) and higher in the moderate-to-severe asthma patient group than in mild asthmatics (p<0.01). The sputum IL-5 mRNA levels (but not the other cytokine mRNA levels) also correlated with exhaled nitric oxide (eNO) and with bronchial hyper-reactivity expressed as the histamine dose resulting in a 20% decrease in FEV1.

Conclusion: Real-time RT-PCR analysis of mRNA in induced sputum confirms a predominance of Th2 cytokines in both allergic and non-allergic asthma. IL-5 reflects eosinophil infiltration as well as eNO levels and hyper-reactivity. The Th1 cytokine, IFN-γ, rather points to asthma severity. The technique is a promising tool in further studies on asthma severity and disease activity.
INTRODUCTION
Asthma is histologically characterised by inflammatory cell infiltration of the bronchial mucosa, by epithelial cell desquamation, goblet cell hyperplasia and thickening of the submucosa.[1] Local overproduction of T helper (Th) 2 cytokines (IL-4, IL-5, IL-9 and IL-13) by Th2 cells plays an important role in its pathophysiology.[1][2] Although initial studies showed that IFN-γ, a Th1 cytokine, was able to prevent airway inflammation [2], recent studies point to IFN-γ as a cytokine that causes severe airway inflammation.[3] Eosinophils recruited to the airways also produce cytokines, as well as several chemokines by which they maintain or even increase the airway hyper-reactivity.[1][2] Furthermore, mast cells and basophils contribute to the pathogenesis of airway inflammation by the release of cytokines and chemokines, whereas the epithelial cell damage can induce cytokine production by epithelial cells, which in turn elicits the recruitment of inflammatory cells.[1][2]
Analysis of induced sputum is a non-invasive technique to obtain viable cells from the lower airways to evaluate airway inflammation [4] and is useful for diagnosis and monitoring of asthma.[5] Sputum can be used to measure cytokines at the protein and at the mRNA level.[6][7] With this technique, IL-5 mRNA was detected by a semi-quantitative RT-PCR technique in more of the asthmatic subjects than in atopic non-asthmatic subjects or healthy controls.[7] The number of IL-4 and IL-5 mRNA expressing CD3⁺ cells detected by in situ hybridization on cytopsins of induced sputum was higher in asthmatic subjects than in healthy controls.[8] More recently quantitative RT-PCR techniques have been developed to measure cytokine production in cell cultures and/or tissues.[9]
We therefore combined the technique of sputum induction and this quantitative real-time RT-PCR to study differences in the expression of an extensive panel of T cell cytokines in the airways between healthy controls and asthmatic subjects. We further tried to define differences of cytokine mRNA expression in the airways of allergic and non-allergic asthmatics, and of mild and moderate-to-severe asthmatics, and to correlate mRNA expression with other parameters regularly used to study inflammatory activity (exhaled nitric oxide (eNO) and the histamine concentration that provoked a 20% decrease in FEV1 (PC20)).
MATERIAL AND METHODS

Subjects
This study was performed between September 2002 and August 2004 (with the exclusion of April, May and June because of the tree-pollen and grass-pollen season). Patients attended the outpatient clinic of either the Pneumology or the Allergy Departments of our University Hospital. Asthma diagnosis was based on prior or current proof of reversibility of FEV1 ≥12 after inhalation of Salbuterol. Healthy volunteers were recruited amongst the University students and co-workers. Thirty nine asthmatic subjects (16 women, 23 men), not taking systemic steroids and 15 healthy controls (8 women, 7 men) between 18 and 65 years were recruited; no significant differences in age distribution between patients and controls were found (p=0.2 for females and p=0.07 for males). All healthy controls were non-smokers, whereas 32 of the 39 asthmatics were non-smokers, two of the asthmatics were occasional smokers (less than three cigarettes a day), one smoked regularly (20 or more cigarettes a day) and no data on smoking habits were available for four asthmatics. Five of the 15 healthy controls had a clinical history suggestive for allergic rhinoconjunctivitis in the grass pollen season and had specific IgE antibodies for grass pollen but not to any of the other allergens tested (house-dust-mite, cat, dog, tree and weed pollen, and molds). Two others had a history of oral symptoms upon contact with a food allergen and specific IgE to hazelnut (n=1) and tomato (n=1) but not to the other allergens tested.

Disease activity was evaluated using asthma symptom scores (ASS)\[10\] and validated Dutch translated Juniper scores.\[11\] In subgroups of patients bronchial hyper-responsiveness was quantified as the histamine concentration that provoked a 20% decrease in FEV1 (PC20) (n=25) and exhaled nitric oxide (NO) was measured on-line based on the old ERS guidelines with Ecophysics CLD 700 AL MED (Düren, Switzerland) (n=16).\[10\] Healthy controls had normal spirometry, no present clinical symptoms of upper or lower airway disease, ASS of zero and did not use or used in the past 5 years any anti-asthma medication.

The study was approved by the local ethical committee of the Faculty of Medicine, Leuven. Informed consent was obtained from all study subjects.

Sputum induction
Sputum was induced with an aerosol of inhaled hypertonic saline in concentrations of 3%, 4% and 5% for 7 minutes, (unless a fall in FEV1 of greater than 10% occurred, in which case the procedure was stopped) generated by a De Vilbiss Nebulizer (Ultra-NebTm 2000 model 200HI) after pre-treatment with 400 µg of inhaled salbutamol.

Sputum examination
Sputum was processed by a modification of the technique described by Pizzichini et al.\[4\] All portions that appeared free of salivary contamination were selected, in order to minimise the possibility that cells were obtained from different airway compartments and to limit squamous cell contamination. A volume of Hanks’ Balanced Salt Solution containing 0.1% dithiothreitol (Sigma, St. Louis, MO, USA) and 3% Bovine Serum Albumin (Sigma) of four times the weight was added. Portions were agitated with a vortex, placed on a bench rocker for 5 min, filtered through a 70 µm Falcon cell strainer and centrifuged at 1500 rpm for 10 minutes. The cell pellet was resuspended in 1000 µl RPMI 1640 (Bio Whittaker Europe, Cambrex) containing 2mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) (Bio Whittaker Europe) and 10% bovine calf serum (BCS) (Hyclone, Logan, UT, USA). Trypan blue was used to evaluate the percentage of dead cells (Bio Whittaker Europe) in a Bürker chamber.

Differential cell count
Cytospins were prepared in 32 out of 39 patient samples and 11 control samples. The cell suspension was adjusted to $1.0 \times 10^6$ cells/ml and 50,000 cells were put in a Shandon 3 Cytocentrifuge (Techgen, Zellik, Belgium). Cytospins were air-dried and stained using May Grünwald Giemsa.

**Quantitative cytokine mRNA**

RNA could be isolated from 34 out of 39 patient sputum samples and 11 control samples with the Qiagen Mini Rneasy kit (Maryland, USA). The condition of differential cell count and cytokine mRNA could be measured in 29 of the patients. RNA was transcribed to cDNA with the Ready-to-go T-primed First Strand Kit (Amersham Pharmacia biotech, Uppsala, Sweden). Real-time quantitative RT-PCR was performed for interleukin (IL)-4, IL-5, IL-13, IL-10, IFN-γ and β-actin in the ABI prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA) as described.[9] The primer and probe sequences for IL-4, IL-10 and IFN-γ were previously published.[9] The primer and probe sequences for IL-5 and IL-13 were designed with the computer program Primer Express (Applied Biosystems) and are shown in table 1.

**Table 1: Primer and probe sequences of IL-5 and IL-13**

<table>
<thead>
<tr>
<th>IL-5 forward primer (FW)</th>
<th>5’CCCACAAGTGCATTGGTGAA3’</th>
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<tbody>
<tr>
<td>IL-5 reverse primer (RV)</td>
<td>5’CCTCAGAGTCTCATGCTATCAG3’</td>
</tr>
<tr>
<td>IL-5 Taqman probe (TP)</td>
<td>5’FAM-AGACCTTGGCACTGTTTCTACTCATCGAA-TAMRA3’</td>
</tr>
<tr>
<td>IL-13 FW</td>
<td>5’GAAGGCTCCCGCTCTGCAAT3’</td>
</tr>
<tr>
<td>IL-13 RV</td>
<td>5’ACACGTTGATCAGGGATTCGA3’</td>
</tr>
<tr>
<td>IL-13 TP</td>
<td>5’FAM-CCTGACAGCTGGCATTACTGTGCAGC-TAMRA3’</td>
</tr>
</tbody>
</table>

PCR amplifications were performed in a total volume of $25 \mu l$ containing $5 \mu l$ cDNA, 12.5 $\mu l$ Universal PCR Master Mix, no AmpErase® UNG (Applied Biosystems), 100-300 nM concentrations of each primer and 200 nM concentrations of the corresponding detection probe (Applied Biosystems or Eurogentec, Belgium). Each PCR amplification was performed in duplicate wells using the following conditions: 94°C for 10 min, followed by 40 cycles at 94°C for 15 s and 60°C for 1 min. cDNA plasmid standards, consisting of purified plasmid DNA specific for each individual target, were used to quantify the target gene in the unknown samples, as described.[9] All results were normalised to β-actin to compensate for differences in the amount of cDNA.

**Statistics**

Statistical analyses were performed with the help of GraphPad Prism (GraphPad Software Inc., San Diego, USA) by using the two-tailed Mann-Whitney U test. One-tailed MWU was only used to test differences amongst allergic and non-allergic subjects. Kruskall-Wallis test was used to study differences between three or more groups and Dunn’s Multiple Comparison Test was used as post-test. Normality was analysed with the Kolmogorov Smirnov test. Correlations were studied by Spearman non-parametric test or Pearson test where appropriate. Contingency tables were analysed by Fishers’ exact test. A difference was considered to be significant when $p \leq 0.05$. 
RESULTS
Patients' characteristics
Table 2 shows patient characteristics and medication use. Asthma severity was scored on the basis of the Global Initiative for Asthma (GINA) criteria.[12] Mild intermittent (n=11) and mild persistent (n=12) asthma patients were grouped as mild asthmatics and moderate (n=8) and severe (n=8) asthmatics were grouped as moderate-to-severe asthmatics. Patients were allowed to continue their usual treatment. Thirteen patients regularly used inhaled corticosteroids (ICS): <500 µg/day Beclomethason Dipropionate (BDP) or equivalent (n=1), 500-1000 µg/day BDP or equivalent (n=5) and >1000 µg/day BDP or equivalent (n=7); “non-users” either had never used ICS or did not use them since at least 3 months. A significantly larger proportion of patients with moderate-to-severe asthma used ICS in comparison to patients with mild asthma (Fishers’ exact test: p<0.0001).

Among asthmatic subjects, 21 were classified as allergic according to the presence of serum specific IgE antibodies for house-dust-mite (n=19), pets (n=12), molds (n=2) and/or for pollen (grass or tree) (n=17) and a clinical history suggestive of allergic responses to those allergens (analysis was not performed in one patient; none of the patients had specific IgE to weed pollen). No differences in FEV1% (Fishers’ exact test: p=0.48), asthma severity (GINA classification) (Fishers’ exact test: p=0.49) and ICS use (Fishers’ exact test: p=0.28) between the allergic and the non-allergic asthmatics were found.

Table 2: Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/ Female</td>
<td>23/16</td>
</tr>
<tr>
<td>Allergic/ Non-allergic</td>
<td>21/17</td>
</tr>
<tr>
<td>%FEV1&lt;70% of expected value</td>
<td>3 (1 on CS)</td>
</tr>
<tr>
<td>%FEV1 70-90% of expected value</td>
<td>9 (3 on CS)</td>
</tr>
<tr>
<td>Mild intermittent asthma</td>
<td>11 (0 on CS)</td>
</tr>
<tr>
<td>Mild persistent asthma</td>
<td>12 (1 on CS)</td>
</tr>
<tr>
<td>Moderate persistent asthma</td>
<td>8 (5 on CS)</td>
</tr>
<tr>
<td>Severe persistent asthma</td>
<td>8 (7 on CS)</td>
</tr>
</tbody>
</table>

a FEV1 expressed as % of the expected value for age, length and weight, b Disease severity based on revised GINA criteria[12]

Comparison of Th1/Th2 cytokine mRNA levels in induced sputum from asthmatic patients and healthy controls
mRNA was extracted from the samples of induced sputum, transcribed to cDNA, and real-time RT-PCR was performed. All values were normalised to β-actin mRNA in order to compensate for variations in cell numbers and RNA quantity between the samples. IL-4 and IFN-γ were selected as the typical representatives of Th2 and Th1 cytokines respectively. None of the healthy volunteers had values of IL-4 mRNA above 1 or IFN-γ mRNA above 10, while 44% and 21% of the patients had values above this cut-off respectively (figure 1A-B). This resulted in a significant difference for IL-4 by Fishers’ exact test between controls and asthmatics (cut-off copies IL-4/β-actin mRNA ≥1: p=0.02; cut-off copies IFN-γ/β-actin ≥10: p=0.07). A significant correlation between the sputum IL-4 and IFN-γ mRNA levels in the patients was found (p=0.03, Spearman r=0.387) (data not shown). All patients (except one) with increased sputum IFN-γ mRNA levels also had increased sputum IL-4 mRNA levels, demonstrating that both Th2 and Th1 cytokines might simultaneously be elevated in the airways of asthmatic patients. With regard to the other Th2 cytokines, there was a
significantly higher expression of IL-5 and IL-13 mRNA in sputum of patients compared to healthy controls (figure 1C-D). IL-5 and IL-13 mRNA levels correlated significantly with each other (p=0.0001, r=0.6162); whereas no correlation was found between IL-4 and either IL-5 or IL-13 mRNA (p=0.18, r=0.234 and p=0.74, r=0.06 respectively). IL-10 mRNA levels in sputum were comparable between healthy controls and asthmatics (figure 1E).

IL-4, IFN-γ, IL-5, IL-13 and IL-10 sputum mRNA levels did not significantly differ in the group of steroid naïve patients versus the group of steroid-treated patients (compare open and closed symbols in the patients group in figure 1).

**Correlation of the eosinophil count with cytokine mRNA levels in asthmatic patients**

Inflammatory cell subtypes were counted (in %) on cytospins of 11 healthy controls and 32 asthmatics. A significant increase in the percentage of sputum eosinophils of asthmatics compared to healthy controls was found (figure 1F). In approximately half of the patients, no or few eosinophils were found; most of them were treated with inhaled corticosteroids. No differences were found in the percentages of lymphocytes and macrophages between patients and controls (data not shown). Cytokine mRNA levels in induced sputum were compared with the percentage of sputum eosinophils. As indicated in figure 2, the levels of IL-5, as well as IL-4 and IL-13 mRNA correlated significantly with the percentage of airway eosinophils. IL-5 and IL-13, but not IL-4, mRNA levels also correlated with airway eosinophils in the subgroup of steroid naïve patients (figure 2A-C) but in the subgroup of steroid users these correlations were not found (open symbols).

**Differences in the cytokine and chemokine pattern between allergic and non-allergic asthmatics**

We further compared the sputum mRNA pattern in allergic and non-allergic asthma. Two asthmatic individuals, allergic to grass pollen only, have been excluded for the comparison between allergic and non-allergic asthma, as asthma symptoms, outside the pollen season, in those patients were probably not related to their allergy. We found a significantly higher percentage of eosinophils in the sputum from allergic asthmatics when compared to non-allergic asthmatics (table 3). Allergic asthmatics had significantly higher sputum IL-4, IL-5 and IL-13 (table 3) mRNA levels compared to the non-allergic asthmatics. IFN-γ levels, on the other hand, were significantly higher in the non-allergic group (table 3). When comparing with control subjects, sputum IL-4 and IL-13 mRNA levels were significantly elevated in allergic individuals only (p=0.03 and p=0.0001 respectively) and not in non-allergic individuals (p=0.4 and p=0.3 respectively) (data not shown), while IFN-γ levels were significantly increased in non-allergic asthmatics only and not in allergic asthmatics (p=0.05 and p=0.6 respectively). Sputum IL-5 mRNA levels differed significantly from control levels in both the allergic and the non-allergic group of patients (p=0.0001 and p=0.03 respectively). Sputum IL-10 mRNA levels did not statistically differ between both groups (table 3) and levels were comparable to those in controls for both groups.

**Table 3: Comparison of the airway eosinophil count and cytokine pattern between allergic and non-allergic asthmatics**

<table>
<thead>
<tr>
<th></th>
<th>Allergic P25%-median-P75%</th>
<th>Non-Allergic P25%-median-P75%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>%eosinophils/leukocytes</td>
<td>1.4 - 4.4 - 10.3</td>
<td>0 - 0.4 - 2.1</td>
<td>0.02: *</td>
</tr>
<tr>
<td>Copies IL-4/β-actin.10⁴</td>
<td>0.0 - 0.7 - 4.4</td>
<td>0 - 0 - 0.2</td>
<td>0.03: *</td>
</tr>
</tbody>
</table>
Copies IFN-\(\gamma\)/\(\beta\)-actin.\(10^4\) | 0 – 1.1 – 16.2 | 1.9 – 2.4 – 9.9 | 0.04: * 
Copies IL-5/\(\beta\)-actin.\(10^4\) | 29.8 – 80.4 – 260.7 | 2.8 – 26.7 – 86.5 | 0.02: * 
Copies IL-13/\(\beta\)-actin.\(10^4\) | 4.4 – 7.1 – 17.3 | 0.1 – 0.5 – 3.1 | 0.0002: *** 
Copies IL-10/\(\beta\)-actin.\(10^4\) | 15.8 – 91.3 – 244.0 | 20.5 – 86.7 – 225.1 | 0.89: ns 

\(^c\) Cells were isolated from induced sputum of allergic asthmatic (n=17) and non-allergic asthmatic patients (n=14). Eosinophil count and cytokine mRNA levels were determined as explained in figure 1. Comparisons were performed using the one-tailed Mann-Whitney U test. P=percentile and ns= not significant.

**Correlation of the cytokine mRNA levels in induced sputum with exhaled NO, airway hyper-responsiveness, FEV1, and asthma severity**

We furthermore studied whether cytokine mRNA levels could reflect airway inflammation. To that aim, exhaled NO levels were compared to sputum cytokine mRNA expression. As shown in figure 3A, there was a significant correlation between eNO levels and sputum IL-5 mRNA levels. No correlation between IFN-\(\gamma\), IL-13, IL-4 or IL-10 mRNA with the eNO levels could be found. We also found that eosinophil levels correlated significantly with eNO levels (in a subgroup of 16 patients, data not shown).

To study whether sputum cytokine mRNA levels reflect airway hyper-responsiveness, the log (PC20) values of histamine provocation tests were correlated with sputum cytokine mRNA levels in a subgroup of 25 patients. As indicated in figure 3B, there was a significant inverse correlation of the IL-5 mRNA levels and the log (PC20). The correlation was even stronger in steroid naïve patients (figure 3B). Thus, high sputum IL-5 mRNA levels correlate with increased airway hyper-responsiveness. In steroid naïve patients, we furthermore confirmed a significant correlation between airway hyper-responsiveness (PC20) and eNO levels (r=-0.82; p=0.007) (data not shown) [10] and found a significant correlation between PC20 and eosinophil counts (r=-0.68, p=0.004) (data not shown). No correlation between sputum IFN-\(\gamma\), IL-4, IL-13 or IL-10 mRNA and the airway hyper-responsiveness (PC20) could be found (in the total patient group nor in the different subgroups related to their treatment) (data not shown).

The validated Dutch translated Juniper scores [11] measuring asthma symptoms and the ASS commonly used in our hospital [10] significantly correlated with each other in 21 patients interviewed for both (p=0.0005: ***, r=0.694) (data not shown). ASS were obtained at the time of sputum induction in nearly all patients. No correlation was found between the ASS and sputum IL-4, IL-5, IL-10, IL-13 or IFN-\(\gamma\) mRNA levels in the total patient group nor in the steroid naïve group (data not shown).

**Correlation of the cytokine mRNA levels in induced sputum with asthma severity based on GINA criteria**

In the last set of analysis, we compared differences between asthmatics classified according to the revised GINA criteria of severity.[12] The sputum cytokine mRNA patterns were compared amongst these patient groups. Kruskall-Wallis test revealed no significant differences in IL-4, IL-10 or IL-13 mRNA between the groups (data not shown). IL-5 mRNA levels are similarly increased in mild asthmatics as in moderate-to-severe asthmatics (figure 4A). Sputum IFN-\(\gamma\) mRNA levels, on the other hand, were significantly increased in moderate-to-severe asthmatics above levels in healthy controls or mild asthmatics (figure 4B).
DISCUSSION
In this study, we have used induced sputum to analyse the cells and cytokines in the airways of asthmatic patients and healthy controls. Induced sputum has the advantage, in comparison with bronchoscopy performed for obtaining biopsies or with broncho-alveolar lavage fluid, of being a non-invasive, easy and well-tolerated technique. We here combined the technique of sputum induction with real-time RT-PCR, a technique that has recently been developed to quantify cytokine mRNA levels in research settings. We were able to demonstrate that mRNA levels for several cytokines differ between allergic and non-allergic asthma patients and that some correlate with disease activity and severity.

First, sputum IL-4 mRNA was only detectable in asthmatics (detected in 44% of the patients) and never in healthy controls. But also, increased IFN-γ mRNA levels (present in 21% of the patients only) were again found in asthmatics only, which is consistent with recent findings by other authors.[13] If patients were classified according to the revised GINA criteria [12], we found that the increase in sputum IFN-γ levels occurs predominantly in the asthmatics with moderate-to-severe asthma. IFN-γ is a classical Th1 cytokine. Initially, impaired Th1 function was proposed as the underlying factor in atopy and/or asthma. Most individuals with increased IFN-γ mRNA levels however also showed increased IL-4 mRNA levels and a significant correlation between IL-4 and IFN-γ levels was found. Thus both Th1 and Th2 cytokines can be elevated in parallel in the airways and both might contribute to disease development. We furthermore found a significantly higher sputum expression of the mRNA for both IL-5 and IL-13 in the asthmatics, and IL-5 and IL-13 mRNA levels correlated significantly with each other. Increased IL-5 and IL-13 mRNA levels have also been found by others on bronchial biopsies [14][15] and on cells obtained by induced sputum from asthmatic patients.[8] Increased IL-5 and IL-13 protein levels have been found in induced sputum from asthmatics.[16][17] Komai-Koma et al reported that the IL-13 production was downregulated in corticoid-treated patients.[17]

Importantly, we demonstrate in this manuscript that allergic and non-allergic asthmatics appear to have slight differences in airway inflammation. It is debated whether or not allergic and non-allergic asthma are distinct inflammatory diseases.[18] Some authors state that even non-allergic asthma might be caused by inhalation of a known or unknown allergen or, as the opposite view, that the allergic status is associated with but not required for asthma development.[18][19][20] Others have stressed the differences between allergic and non-allergic asthma.[21] We first show a higher percentage of eosinophils in allergic asthmatics compared to non-allergics. Amin et al, similar to us, found higher numbers of eosinophils in allergic patients compared to non-allergic patients.[22] Our results are also in accordance with results from an epidemiologic study in which atopic subjects with bronchial hyper-reactivity were found to have higher levels of serum ECP and a higher number of blood eosinophils than nonatopic subjects with bronchial hyper-reactivity.[23] However, not all studies are concordant on this, and other authors reported a similar increase in the number of eosinophils in allergic and non-allergic asthmatics.[18] [24] At the level of sputum mRNA expression, our results demonstrate a significantly higher IL-4, IL-5 and IL-13 mRNA expression in induced sputum from allergic asthmatics compared to non-allergic asthmatics, whereas non-allergics have higher IFN-γ mRNA levels. In comparison to control subjects, IL-4 and IL-13 mRNA levels were increased in the allergic patient group only, whereas IFN-γ levels were increased in the non-allergic group. A dysregulation of IL-4 production [25] or a change in IL-4 responsiveness [26] have been suggested to be basic underlying abnormalities in atopy. In allergic asthma, the number of IL-4 mRNA copies detected in bronchial biopsies correlated with the serum IgE levels.[27] This indirectly suggested that allergies should indeed have higher IL-4 mRNA levels in the airways. However, the same authors did not observe differences in IL-4 mRNA levels amongst allergic and non-allergic asthmatics.[19] Our study
is the first to show such a difference, and confirms that there are, besides the many similarities, also quantitative differences in the cellular and cytokine profiles between allergic and non-allergic asthmatics.

Another issue is whether sputum cytokines could be helpful in evaluating disease severity, symptom control and in monitoring therapy. We found IL-5, IL-4 and IL-13 mRNA levels to be significantly correlated with airway eosinophil percentages, and the latter is known to correlate with airway inflammation in steroid naïve patients.[5] We could demonstrate that bronchial hyper-responsiveness, measured by histamine provocation, and exhaled NO levels, which reflect airway inflammation [28] significantly correlated with the IL-5 mRNA level (but not with the other cytokines) in steroid naïve patients. This indicates that in the steroid naïve group, patients with increased airway hyper-reactivity and asthmatics with more active disease have higher IL-5 mRNA levels. Along the same line, Humbert et al demonstrated that the level of IL-5 mRNA expression in bronchial biopsies from patients with asthma inversely correlated with their FEV1 value, also leading to the conclusion that higher IL-5 values were found in more severe asthmatics.[27] Exhaled NO levels in steroid naïve asthma patients have been shown to correlate with their eosinophil count and airway hyper-responsiveness.[10] [29] It is therefore not surprising that we found also a correlation between IL-5 mRNA levels and exhaled NO in steroid naïve patients. In our study, sputum IL-5 mRNA levels were still increased in the subgroup of steroid-treated asthma patients, which makes the quantification of IL-5 mRNA levels an interesting tool to evaluate residual airway inflammation in steroid-treated patients, even if eosinophils are no longer present in the airways. This might in fact be very interesting to help to adapt the treatment protocol but requires further investigation. Recently a similar finding regarding serum IL-5 levels in patients treated with corticosteroids has been reported.[30] However, we should stress that the method of sputum induction fails in some patients and the quantification of cytokine mRNA from the samples is time and money consuming. It could therefore perhaps be reserved to difficult-to-treat asthma patients only. Also it is important to mention that cytokine mRNA levels correlated poorly with asthma symptoms as measured by ASS and Juniper score. An intriguingly high proportion of patients indeed had high ASS but only low cytokine mRNA levels. We can only speculate that symptoms reported by the patients might persist, even if the inflammation is less prominent, or that the inflammation in patients with high ASS and low cytokine mRNA expression has different characteristics. Another explanation could be that ASS correlate with airway hyper-reactivity, which could be dissociated from airway inflammation.[31]

Because we studied cytokine mRNA expression in induced sputum, we were not able to determine the cellular source of these cytokines. Cytokines are produced by several cell types, besides T cells. Cho et al have demonstrated by intracellular staining that at least sputum CD4⁺ and CD8⁺ cells producing IL-4 and IL-5 as well as CD4⁺ and CD8⁺ cells producing IFN-γ are increased in asthmatic airways.[13] Eosinophils were shown to be the main cell type expressing IL-4 and IL-5 mRNA in the nasal mucosa upon allergen challenge [32] and their role as Th2 cytokine producers in the lower airways is becoming more clear.[31] Moreover, airway basophils are shown to produce IL-4 upon allergen provocation.[33] There recently has been a lot of interest in IL-10 as a immunomodulatory cytokine in allergy.[34] It was suggested that IL-10 produced by regulatory T cells is essential for immune homeostasis and that this mechanism might be defective in asthmatics.[34] Decreased IL-10 expression both at the protein level and at the mRNA level has indeed been demonstrated on BAL fluids of asthmatics in comparison to healthy control individuals.[35] Opposite to these findings, we were not able to demonstrate decreased IL-10 mRNA expression in the airways of the asthmatic patients. In fact, a tendency towards increased IL-10 mRNA expression was found in steroid-treated patients, which indicates that the reduction of IL-10 mRNA expression in steroid-naïve patients is a consequence of the treatment and not a characteristic of the disease itself. This is also consistent with the observation that IL-10 production by alveolar macrophages is increased in steroid-treated patients compared to steroid-naïve patients. [31, 35] It is therefore important to consider that the quantification of cytokine mRNA expression in induced sputum might not reflect the activity of the disease itself, but rather the effect of the treatment.
10 expression in the airways of asthmatic patients was found. Robinson et al in accordance to our findings showed increased IL-10 mRNA levels in asthmatic airways.[36]

In conclusion, the most interesting of the analysed cytokines for further studies at the mRNA level are IL-4 as differences between allergic and non-allergic asthmatics exist; IFN-γ as a cytokine that is more prominent in more severe asthma; and IL-5 as a cytokine that correlates with eosinophilic inflammation, as well as with eNO and airway hyper-reactivity. These conclusions should now be further validated in larger patients groups and in prospective studies in order to evaluate effects of treatment.
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LEGENDS TO FIGURES

Figure 1: Eosinophils and cytokine mRNA levels in induced sputum: comparison between healthy controls and asthmatics.
Cells were isolated from induced sputum of healthy controls (n=11) and asthmatic patients (n=33). RNA was extracted and one µg RNA was transcribed to cDNA, which was used in duplicate to perform real time RT-PCR using AB-universal Mastermix, with either β-actin (A-E) or cytokine IL-4 (A), IFN-γ (B), IL-5 (C), IL-13 (D) or IL-10 (E) specific primers and VIC-(β-actin) or FAM-(cytokines) labelled specific probes. Results were quantified by the use of a cDNA plasmid-standard (A-E). Cytospins were prepared, stained with May Grünwald Giemsa and the percentage of eosinophils amongst the leukocytes was counted (F). Open symbols represent patients treated with inhaled corticosteroids (+ICS), closed symbols represent patients without inhaled corticosteroid-treatment (-ICS). Comparisons were performed using two-tailed Mann-Whitney U test, as parameters did not pass normality test. The median is indicated with a horizontal line. ns= not significant

Figure 2: Correlation of sputum cytokine mRNA levels with the sputum eosinophil count
Analysis of cells and of cytokine mRNA from induced sputum were performed in parallel in 27 asthmatic patients. Cytokine mRNA levels and eosinophil counts were determined as explained in figure 1. Open symbols represent patients treated with inhaled corticosteroids and closed symbols represent patients without inhaled corticosteroid-treatment. Spearman non-parametric correlation studies were performed as parameters did not pass normality test. ns= not significant

Figure 3: Correlation of cytokine mRNA levels with the exhaled NO levels and the airway hyper-reactivity to histamine expressed as log (PC20) in asthmatic patients
Cells were isolated from induced sputum of asthmatic patients. IL-5 mRNA levels were determined as explained in figure 1. Exhaled NO was measured (A) in 13 patients and PC20 was determined as the log of the histamine concentration (in mg/ml) resulting in a 20% decrease of FEV1 in 21 patients (B). Open symbols represent patients treated with inhaled corticosteroids and closed symbols represent patients without inhaled corticosteroid treatment. All parameters passed normality test and Pearson linear correlation studies were performed. ns= not significant

Figure 4: Cytokine mRNA levels amongst the different groups classified according to asthma severity
Cells were isolated from induced sputum of asthmatic patients (n=34) and patients were classified following the revised GINA criteria.[12] Mild intermittent and mild persistent asthmatics were grouped (mild) and moderate and severe persistent asthmatics were also grouped (moderate-to-severe asthmatics). Cytokine mRNA levels were determined as explained in figure 1. Open symbols represent patients treated with inhaled corticosteroids, closed symbols represent patients without inhaled corticosteroid treatment. Kruskall-Wallis non-parametric test with Dunn’s Multiple Comparison Test as post test was used as parameters did not pass normality test. The median is indicated with a horizontal line. ns= not significant.
REFERENCES
A. IL-5 vs eNO

![Graph showing the relationship between IL-5 and eNO](image)

$r = 0.62$

$p = 0.02: *$

**without ICS**

**with ICS**

B. IL-5 vs PC20

![Graph showing the relationship between IL-5 and PC20](image)

$r = -0.439$

$p = 0.046: *$

**all patients**

$r = -0.687$

$p = 0.007: **$

**steroid-naïve**
Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients

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