Mechanisms of glucocorticoid insensitivity in asthma

Zijlstra, Jan

DOI:
10.33612/diss.136678943

Chapter 2

Characterization of glucocorticosteroid response in mild-to-moderate asthma

G. Jan Zijlstra, BSc; Franke Volbeda, MD; Dirkje S. Postma, MD, PhD; H. Marike Boezen, PhD; Monique E. Lodewijk, BSc; Machteld N. Hylkema, PhD; Wim Timens, MD, PhD; Nick H.T. ten Hacken, MD, PhD

Manuscript in preparation

Abstract

Background

Unresponsiveness to glucocorticosteroids (GC) in asthma has been associated with several clinical and inflammatory features, including smoking. It is largely unknown whether these features contribute independently to GC-unresponsiveness. We aimed to determine the independent role of airway inflammation markers predicting GC-unresponsiveness in relation to smoking.

Methods

Inflammation was assessed in peripheral blood, induced sputum, bronchial biopsies, and exhaled Nitric Oxide (eNO) from 60 mild-to-moderate asthmatics: 20 current-smokers, 41 females, 21 inhaled GC-users, median age 50 years, median FEV_1 96% predicted. GC-unresponsiveness was defined as no improvement in pre-bronchodilator FEV_1 after a 2-week course of 40 mg methylprednisolone/day.

Results

At baseline, GC-unresponsive asthmatics (n=33) were more often current-smokers, demonstrated significantly lower levels of bronchodilator reversibility and eNO than GC-responsive asthmatics (n=27), and had higher blood lymphocyte counts. GC-response correlated positively with sputum eosinophils and ECP ($r_{spearman}$=0.36 (p=0.019) and 0.54 (p<0.001) respectively) and both alveolar and bronchial eNO ($r_{spearman}$=0.46 (p=0.001) and 0.42 (p=0.003) respectively). Neutrophil-to-eosinophil ratios correlated negatively in blood sputum and bronchial biopsies . Multivariate regression showed that higher age (p=0.038), blood lymphocyte counts (p=0.008) and neutrophil/eosinophil ratios (p=0.050), lower alveolar eNO levels (p=0.003) and sputum eosinophil percentages (p=0.048) independently predicted lower GC-response (R^2=0.71, p<0.001). Cigarette smoking did not contribute independently to the model (p=0.197)

Conclusion

As current smoking did not affect GC-response independently, we suggest that smoking-induced inflammatory changes appear to be more important than direct effects of smoking itself.

Background

Asthma is characterized by inflammation of the central and peripheral airways involving many cell types and mediators. Glucocorticosteroids (GC) inhibit virtually all components of asthmatic airway inflammation and provide beneficial clinical effects in most patients. Unfortunately, some asthmatic patients are unresponsive to GC [1,2]. This GC-unresponsiveness has been associated with higher numbers of peripheral blood lymphocytes [3], higher neutrophil [4] and lower eosinophil sputum counts [5], and lower Nitric Oxide (NO) levels in exhaled air [5,6]. Several clinical studies in asthma have demonstrated that cigarette smoking increases GC-unresponsiveness [7-9]. Interestingly, smokers with asthma have increased neutrophil counts and decreased eosinophil counts in sputum [10] and bronchial biopsies [11], as well as decreased NO levels in exhaled air [11,12] compared to non–smoking asthmatics, leading to the hypothesis of a causal role of smoking. So far, the only study comparing bronchial biopsies between GC-sensitive and GC-insensitive subjects did not show any differences in inflammatory parameters [13]. However, it is largely unknown to what extent the inflammatory characteristics contribute to GC-unresponsiveness and whether smoking affects this relation independently.

We assessed inflammatory cells in peripheral blood, induced sputum, exhaled air (NO), and bronchial biopsies in smoking and non-smoking subjects with mild-to-moderate asthma. In addition, we measured Adenosine-5'-MonoPhosphate (AMP) responsiveness, which can also be considered as an inflammatory marker as it is associated with airway eosinophilia [14]. We aimed to determine the independent role of these markers of airway inflammation in predicting GC-unresponsiveness in relation to smoking.

Methods

Subjects

Participants were recruited from previous research cohorts and were well characterized asthma patients visiting our University Medical Center. Inclusion criteria were a doctor's diagnosis of asthma, a positive histamine provocation test in the past, and age >18 years. Main exclusion criteria were an FEV_1 <1.2 L, bronchiectases, upper respiratory tract infection (e.g. colds) and/or use of antibiotics or oral corticosteroids within 2 months, serious acute infections in the previous 3 months. Non-smokers did not currently smoke and had a maximum of 2 packyears. The medical ethics committee of the University Medical Center Groningen approved the study protocol

(Approval reference number METC2004.271). All subjects provided their written informed consent.

Design

Subjects visited our research facilities 4 times. If they used inhaled GC they first were asked to stop inhaled GC for four weeks prior to visit 1. At visit 1, they underwent lung function testing, blood collection and sputum induction. At least 1 week later we performed a challenge test with Adenosine-5'-MonoPhosphate (AMP) at visit 2. Exhaled NO was measured at least 1 week later prior to a bronchoscopy at visit 3. After a two-week course of oral methylprednisolone (40 mg/day), lung function was assessed at visit 4.

Lung Function

All measurements were performed by pulmonary technicians in the University Medical Center in Groningen using standardized protocols. FEV_1 was measured with a calibrated water-sealed spirometer according to guidelines [15]. Reversibility of FEV_1 (%predicted) was measured by inhaling 400 µg albuterol at visit 1 and 4. AMP provocation was performed using a two-minute inhalation procedure with a calibrated De Vilbiss nebulizer. FEV_1 was measured 30 and 90 seconds after each inhalation. Doubling concentrations of AMP, ranging from 0.04 to 320 mg/ml, were inhaled until a fall of 20% in FEV_1 (PC_{20} AMP) occurred or the highest dose of 320 mg/ml was reached. PC_{20} AMP was determined as described previously [14].

Sputum Induction and Sputum Processing

Sputum was induced by inhalation of nebulized hypertonic saline (5%) for 3 consecutive periods of 5 min. Whole sputum samples were processed as described previously [16]. May Grünwald Giemsa (MGG) staining was used to obtain cell differentials from in total 600 viable, non-squamous cells. Sputum was not scored if the percentage squamous cells was >80 percent or the total number of non-squamous cells was <600. Induced sputum was not obtained in 8 GC-unresponsive and 10 GC-responsive subjects, because they were unable to expectorate sufficient sputum meeting the standards of our protocol.

Blood

Blood differential counts were analysed by flow cytometry (Coulter-STKS; Beckman Coulter, Miami, FL, USA) in the routine hospital laboratory. Total serum IgE (IU/L) was measured by a solid-phase immunoassay (VIDAS total IgE kit, BioMérieux, Marcy l'Etoile, France). The Phadiatop screening test was used to determine atopic status and was performed on the ImmunoCap system according to the instructions of the

manufacturer (Phadia AB, Sweden). Results were presented as quotients (fluorescence of the serum of interest divided by the fluorescence of a control serum). Atopy was defined as patient serum/control serum >1.

Exhaled Nitric Oxide

Exhaled NO was measured on the Aerocrine NO system (Niox, Aerocrine AB, Stockholm, Sweden) in accordance with international guidelines [17]. Alveolar NO concentrations and bronchial NO fluxes were assessed according to Tsoukias & George [18] with some modifications [19]. Exhaled NO values of 3 GC-unresponsive and 4 GC-responsive subjects were not used because the calibration gas was not reliable during a short period of the study.

Collection, processing and immunohistochemical staining of bronchial biopsies

Bronchial biopsies were obtained under local anaesthesia using a flexible bronchoscope from segmental divisions of the main bronchi. The biopsies were fixed in 4% formalin, processed and embedded in paraffin. Bronchial biopsies were cut in 3µm thick sections.

The inflammatory profile was assessed with specific antibodies against eosinophilic peroxidase (EPX, laboratories of NA Lee and JJ Lee, Mayo Clinic, Scottsdale, USA), mast cell tryptase (AA1, DAKO, Glostrup, Denmark), macrophages (CD68, DAKO), neutrophil elastase (NP57, DAKO), T-lymphocytes (CD3 (DAKO), CD4 (Novocastra, Newcastle upon Tyne, UK) and CD8 (DAKO) and B-lymphocytes (CD20, DAKO). In short, sections were deparaffinised and after antigen retrieval, incubated with the primary antibodies. These antibodies were detected with Envision" Detection Kit (DAKO) followed by the chromogen NovaRED (Vector Labs, Burlingame, USA). EPX and CD8 were detected using biotinylated anti-mouse IgG1 (Southern Biotechnology, Birmingham AL, USA) and alkaline phosphatase- (DAKO) or peroxidase-labelled streptavidin conjugates (DAKO) followed by permanent Red (DAKO) and NovaRED chromogens, respectively. All stainings for inflammatory cell markers were performed in an automated system using the DAKO autostainer and were manually counterstained with methylgreen. All stainings were quantified by a blinded observer using computer-assisted image analysis at a magnification of 200x (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). Quantification was performed on the largest of three biopsy sections. Inflammatory cell numbers were quantified by counting the number of positively stained cells in the submucosal area 100µm under the basement membrane (BM), in a total area of 0.1mm^2 per biopsy sample. Epithelial layer integrity was assessed on HE-stained biopsy sections and expressed as the percentage of BM covered

with 1) normal, intact epithelium (basal and ciliated columnar epithelial cells) and 2) metaplastic epithelium (multi-layered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells). BM thickness was calculated based on computer-assisted measurements of BM surface area and BM length.

Statistics

Differences in continuous variables between groups were tested with Mann-Whitney U-test. Chi-square test was used to test differences in dichotomous variables between groups. To investigate which factors were associated with GC-unresponsiveness, we assigned subjects into two groups: GC-responsive and GC-unresponsive. Subjects with any increase in pre-bronchodilator FEV_1 (%predicted), i.e. >0% in response to a 2-week course of GC were assigned to the responder group and subjects without increase to the non-responder group. Correlations were described using Spearman's rank correlation coefficient. We performed linear regression analysis with the pre-bronchodilator FEV_1 response to GC as dependent variable and smoking status, age, gender, PC_{20} AMP, blood lymphocytes, neutrophil/eosinophil ratio in blood, sputum eosinophils and alveolar NO as independent variables entered simultaneously into the model. The model was corrected for FEV_1 at baseline. Since there existed a close relation between reversibility to albuterol and FEV_1 at baseline, reversibility to albuterol was excluded from the model. PC_{20} AMP, blood lymphocytes, sputum eosinophils, and NO values were transformed logarithmically to obtain normal distributions. Model assumptions were checked visually by inspection of the distribution of the residuals. P-values <0.05 were considered to be statistically significant (tested 2-sided). We used SPSS 14.0 for all statistical analyses.

Results

Demographic characteristics

The proportion of currently smoking subjects was significantly higher in the GC-unresponsive than in the GC-responsive group. Because of the distribution of smokers, the numbers of packyears and cigarettes/day were also significantly different between groups. Subjects who used inhaled GC prior to the study and had to stop this medication were equally distributed over both groups (Table 1).

Lung function

Overall, the median (range) pre-bronchodilator improvement in FEV_1% predicted after a 2-week course of methylprednisolone was –0.6 (-22.1-90.1) %. The median (range) value was: –3.5 (-22.1-0) % in the GC-unresponsive and +7.1 (0.5-90.1) % in the GC-responsive group. Table 1 demonstrates the differences between the GC-unresponsive

and GC-responsive subjects at baseline. Reversibility to albuterol (FEV_1 %predicted) was significantly lower in GC-unresponsive than in GC-responsive subjects.

Blood

GC-unresponsive subjects had more leukocytes in blood than GC-responsive subjects, which may be primarily due to higher numbers of both neutrophils and lymphocytes in this group (Table 2). Furthermore, a higher neutrophil/eosinophil ratio was significantly correlated with a lower response to GC ($r_{spearman}$=0.433, p<0.001) (Fig.2A).

Table 1. Subject characteristics at baseline

	GC-responsive (n=27)	**GC-unresponsive (n=33)**
Female, number (%)	19 (59)	22 (58)
Age, years	52 (22-67)	50 (19-70)
BMI, kg/m^2	26.8 (19.3-40.3)	27.0 (21.8-42.4)
Atopy, number (%)	21(78)	16 (53) p=0.054
Current-smoking, number (%)	**4 (15)**	**16 (49)***
Pack years of current-smokers	**15.7 (8.0-30.0)**	**23.5 (5.8-47.3)***
Cigarettes/day of current-smokers	**16 (5-20)**	**15 (7-25)***
Inhaled GC use prior to study	11 (41%)	10 (30%)
Dosis equivalent beclomethason	800 (28-2000)	900 (200-2000)
FEV_1 pre-bd, %pred	94.6 (53.5-110.1)	96.6 (67.4-118.0)
FEV_1/VC pre-bd, %	71.4 (40.5-94.4)	74.0 (50.0-89.6)
PEF pre-bd, L/s	7.02 (3.97-11.9)	7.96 (4.68-14.86)
MEF_{50} pre-bd, L/s	2.55 (0.96-5.03)	2.69 (1.00-5.55)
Reversibility, %pred	11.86 (-1.39-38.43)	7.56 (-2.23-23.27)*
PC_{20} AMP, mg/mL	32.27 (0.02-640)	155.79 (0.25-640)
Total IgE, IU/L	42.0 (0-1302.0)	43.0 (0-698.0)

Values are medians (ranges) or numbers (percentages), *: p≤0.05 vs. GC-responsive, bd: bronchodilator, AMP: Adenosine Mono-Phosphate, GC: glucocorticosteroid.

Table 2. Inflammation in blood, sputum and exhaled air

	GC-responsive	GC-unresponsive
Blood		
Leukocytes, 10^9/L	5.8 (3.5-9.7)	7.4 (4.1-14.2)*
Neutrophils, 10^9/L	3.14 (1.80-6.77)	4.28 (2.17-10.08)*
Eosinophils, 10^9/L	0.21 (0.08-1.16)	0.20 (0.01-0.51)
Basophils, 10^9/L	0.05 (0-0.22)	0.04 (0-0.90)
Lymphocytes, 10^9/L	1.82 (0.80-4.05)	2.48 (1.01-4.35)*
Monocytes, 10^9/L	0.28 (0-0.98)	0.24 (0-0.87)
Sputum		
Total cells, 10^5/mL	365.8 (188.6-1481.0)	450.8 (90.9-1800.0)
Neutrophils, %	44.3 (16.5-87.7)	58.2 (15.3-83.8)
Neutrophils, 10^3/mL	189.9 (35.2-734.7)	199.0 (18.3-1443.0)
Eosinophils, %	3.3 (0.2-65.8)	1.5 (0-16.7)
Eosinophils, 10^3/mL	12.4 (0.4-177.8)	4.7 (0-81.7)
Lymphocytes, %	0.7 (0-5.5)	0.3 (0-3.7)
Lymphocytes, 10^3/mL	2.8 (0-52.0)	1.0 (0-19.1)
Macrophages, %	30.5 (11.0-63.6)	35.2 (11.2-80.7)
Macrophages, 10^3/mL	106.6 (24.8-578.4)	114.1 (24.7-826.4)
ECP, ng/mL	50.9 (5.8-601.0)	21.0 (5.6-2467.0)*
Elastase, µg/mL	1.13 (0.23-5.0)	0.81 (0.19-5.0)
Exhaled air		
Alveolar NO, ppb	6.28 (0.88-51.7)	4.75 (2.33-18.34)*
Bronchial NO, nL/s	1.44 (0.19-10.38)	0.45 (0.06-3.17)*

Values are medians (ranges), *: p≤0.05 vs. GC-responsive, GC: glucocorticosteroid

Exhaled NO

Bronchial NO flux and alveolar NO concentration in exhaled air were both significantly lower in GC-unresponsive than GC-responsive subjects (Table 2). Lower GC-responses correlated significantly with lower bronchial NO fluxes and lower alveolar NO concentrations ($r_{spearman}$=0.42 (p=0.003) and 0.46 (p=0.001) respectively; Figure 1A & 1B, including stratification for smoking). Bronchial NO flux and alveolar NO concentration were both significantly lower in smoking than in non-smoking subjects: (median (range)) 0.26 (0.06-0.59) nL/s vs 1.33 (0.32-10.38) nL/s (p<0.001), and 3.44 (2.33-6.24) ppb vs 6.73 (3.57-51.72) ppb (p<0.001), respectively.

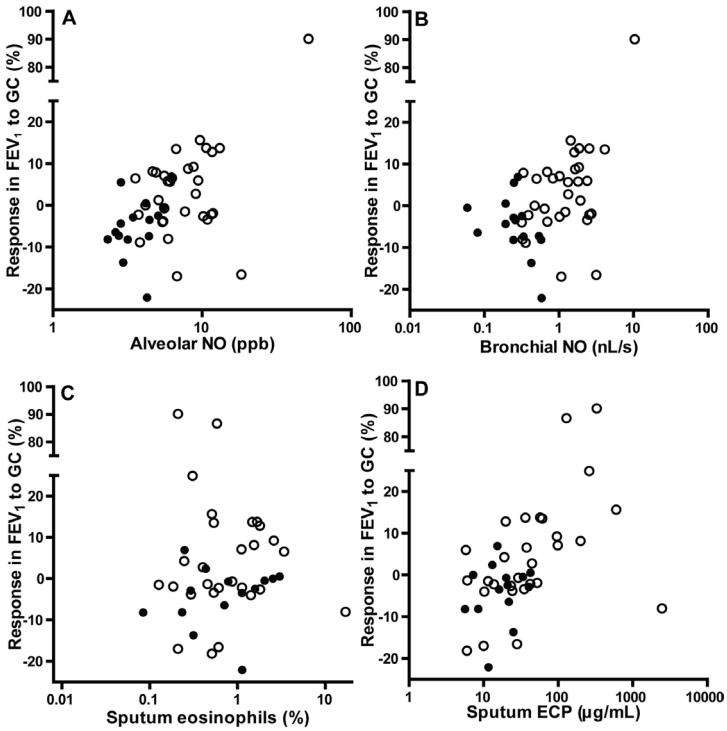

Figure 1. Scatter plots of GC-response (Y-axis) in relation to (A) alveolar NO concentration (ppb), (B) bronchial NO flux (nL/s), (C) sputum eosinophils (%), (D) sputum ECP concentration (ng/mL). Closed circles are smokers, open circles are non-smokers. GC response (%) correlated significantly with alveolar NO ($r_{spearman}$ = 0.46, p=0.001), bronchial NO ($r_{spearman}$ = 0.42, p=0.003), sputum eosinophils ($r_{spearman}$ = 0.36, p=0.019), sputum ECP ($r_{spearman}$ = 0.54, p<0.001). GC= Glucocorticosteroid.

Sputum

The total numbers and the proportions of the various inflammatory cells in sputum were not different between the two groups. However, the concentration of ECP was significantly lower in GC-unresponsive subjects than in GC-responsive subjects (Table 2). Lower GC-responses correlated significantly with lower percentages of sputum eosinophils and lower concentrations of ECP: $r_{spearman}$=0.36 (p=0.019) and $r_{spearman}$ = 0.54 (p<0.001) respectively (Figure 1C & 1D, including identification of smokers). A higher neutrophil/eosinophil ratio was significantly correlated with a lower response to GC ($r_{spearman}$=-0.33, p=0.041) (Fig.2B), as was the elastase-to-ECP ratio ($r_{spearman}$ =-0.42, p=0.005). Sputum eosinophil numbers were comparable in smokers and non-smokers.

Biopsies

Biopsies were obtained from 23 GC-responsive subjects and 20 GC-unresponsive subjects. No significant differences could be observed between GC-unresponsive and GC-responsive subjects. However, a trend was observed for a higher neutrophil/eosinophil ratio in GC-unresponsive subjects (p=0.071) (Table 3). Furthermore, a higher neutrophil/eosinophil ratio was significantly correlated with a lower response to GC: $r_{spearman}$ = -0.37 (p=0.01) (Fig.2D).

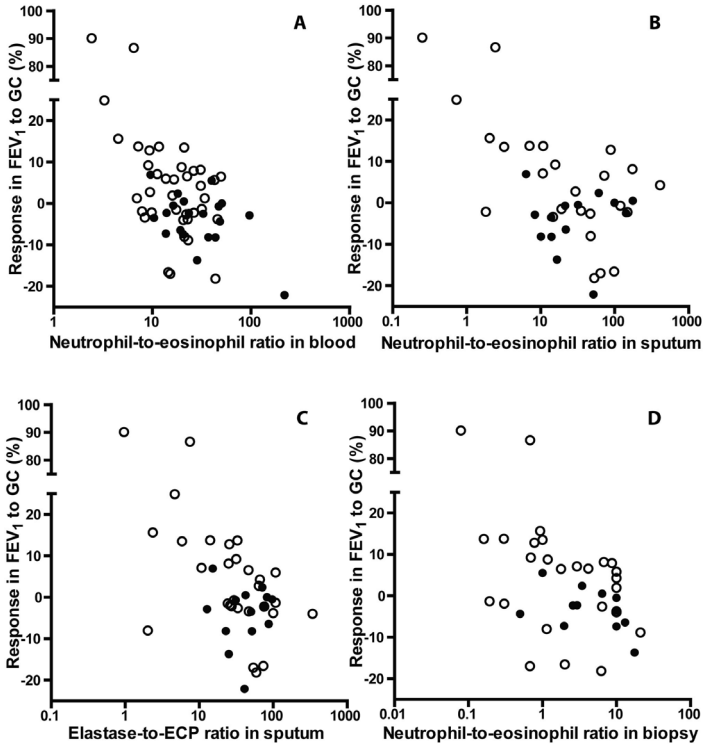

Figure 2. Scatter plots of GC-response (Y-axis) in relation to (A) neutrophil/eosinophil ratio in blood, (B) neutrophil/eosinophil ratio in sputum, (C) elastase-to-ECP ratio in sputum, (D) neutrophil/eosinophil ratio in biopsies. Closed circles are smokers, open circles are non-smokers. GC response (%) correlated significantly with neutrophil/eosinophil ratio in blood ($r_{spearman}$=-0.43, p=0.001), neutrophil/eosinophil ratio in sputum ($r_{spearman}$=-0.33, p=0.041), elastase-to-ECP ratio in sputum ($r_{spearman}$=-0.42, p=0.005), neutrophil/eosinophil ratio in biopsies ($r_{spearman}$=-0.37, p=0.014). GC= Glucocorticosteroid.

Multivariate regression analyses

Multiple linear regression analysis on GC-response provided a model with an R^2 of 0.71 ($p<0.001$). Lower alveolar NO, lower percentage of sputum eosinophils, older age, higher neutrophil/eosinophil ratio and numbers of lymphocytes in blood were all independently and significantly associated with a lower GC-response (Table 4). Current smoking and PC_{20} AMP did not affect the GC-response significantly in our model.

Table 3. biopsy data

	GC-responsive	GC-unresponsive
Inflammatory cells		
NP57$^+$ Neutrophils, cells/0.1mm^2	4.45 (0-19.81)	7.46 (0-46.02)
EPX$^+$ Eosinophils, cells/0.1mm^2	2.5 (0-25.6)	2.0 (0-25.8)
AAI$^+$ Mast cells, cells/0.1mm^2	7.46 (0-18.71)	11.14 (0-19.08)
CD3$^+$ T-Lymphocytes, cells/0.1mm^2	75.26 (21.31-216.19)	67.45 (18.02-294.20)
CD8$^+$ T-Lymphocytes, cells/0.1mm^2	18.33 (1.02-91.95)	24.09 (1.94-103.17)
CD68$^+$ Macrophages, cells/0.1mm^2	13.05 (0.31-57.01)	12.05 (0-37.15)
Neutrophil/eosinophil ratio	0.85 (0-8.67)	2.01 (0-21.12)*
Remodelling		
Goblet, n	35.16 (3.68-97.37)	36.36 (7.08-92.23)
Mucus, %	8.60 (1.49-26.44)	8.07 (0.94-32.67)
Epithelial Thickness, µm	18.85 (8.13-48.13)	20.15 (7.92-36.76)
Ki67$^+$, %intact	3.20 (0.97-13.61)	5.42 (0-15.50)
Ki67$^+$, %basal	3.43 (0.57-19.34)	5.45 (0.66-25.47)
Normal epithelium, %	3.87 (0-29.09)	10.14 (0-65.27)
Basal membrane thickness, µm	5.67 (3.06-12.55)	6.24 (3.80-9.04)

Values are medians (ranges), GC: glucocorticosteroid, *: p=0.071 vs GC-responsive asthma.

Table 4. Multivariate linear regression analysis on GC-response

	B	Standard Error	p-value
Log alveolar NO	17.987	5.513	**0.003**
Log blood lymphocytes	-13.808	4.498	**0.008**
Age	-0.431	0.196	**0.038**
Log % sputum eosinophils	4.855	2.327	**0.048**
Neutrophil/eosinophil ratio in blood	-0.143	0.069	**0.050**
Log PC_{20} AMP	2.089	1.184	0.091
Current-smoking	-9.414	7.079	0.197

R-squared: 0.71, $p<0.001$. Corrected for gender and FEV_1 %predicted at baseline.

Discussion

This study demonstrates that lower exhaled NO levels, lower sputum eosinophil numbers, higher neutrophil/eosinophil ratios in blood, higher blood lymphocyte numbers and older age were independent predictors of a poorer FEV_1 response to a 2-week course of oral glucocorticosteroids in subjects with mild-to-moderate asthma. Current smoking did not contribute independently to GC-unresponsiveness, in contrast with findings in the literature [8,20].

That we found lower exhaled NO values to be associated with GC-unresponsiveness can be due to a variety of mechanisms. First, lower exhaled NO values may reflect a lower degree of lung inflammation [21], thereby giving less room for improvement with anti-inflammatory treatment. Second, lower exhaled NO values may be present in a subset of patients with a different *type* of lung inflammation, i.e. in this case less response to the anti-inflammatory effects of methylprednisolone.

Generally, the neutrophil is seen as the least GC-sensitive and the eosinophil is seen as the most GC-sensitive inflammatory cell type. In our study, a higher percentage of eosinophils in sputum was significantly correlated with a better GC-response. Little et al [5] did not find a correlation between sputum eosinophils and GC response but demonstrated that sputum eosinophilia (≥4%) significantly predicts an increase in FEV_1 ≥15%. In contrast to our study, Little *et al* only included *non*-smoking asthma patients with a lower mean FEV_1 %predicted of 76%, whereas sputum was processed using the plug method [16,22]. Our results are more in line with the study of Meijer et al [23], who included both smoking and non-smoking subjects and demonstrated higher numbers of sputum eosinophils to be associated with GC response. A drawback of their study was that effects of inhaled GC and oral prednisolone were analysed together. Overall, we conclude that our data is in line with the literature showing that a low percentage of eosinophils in sputum associates with a low response to GC.

We observed a significant correlation between a higher neutrophil/eosinophil ratio in biopsies and decreased GC-response. In another biopsy study Chakir *et a*l [13] did not observe baseline differences between oral GC-sensitive and insensitive subjects, similar to our study. However, they did not determine the ratio of neutrophils to eosinophils. Furthermore, the studies are not easy to compare because Chakir investigated a lower number of subjects, who had moderate-to-severe asthma while our subjects had mild-to-moderate asthma. A strong point of our study is that association of a higher neutrophil/eosinophil ratio with a lower GC-response was also present in both sputum and blood. We regard this as evidence of a shift away from the GC-responsive eosinophilic inflammation to the more GC-unresponsive neutrophilic inflammation.

Our results show that a higher number of blood lymphocytes is associated with a lower response to GC. Unfortunately, we do not know which lymphocyte subtype is responsible for this higher number of blood lymphocytes in our study population. Based on findings in the literature we can speculate about this. First, CD8+ T-lymphocytes tend to respond less to GC than CD4+ T-lymphocytes [24,25]. Second, within CD4+ T-lymphocyte subsets Th1 and Th17 are associated with a lower response to GC [26,27], whereas Th2 lymphocytes respond well to GC [28]. Therefore, we postulate that the association between higher blood lymphocyte counts and lower response to GC can be due to an increase in CD8+, Th1, or Th17-lymphocytes, but not an increase in Th2-lymphocytes.

Interestingly, older age was also associated with GC-unresponsiveness. Older asthmatics generally have a higher prevalence of irreversible airway obstruction, which may be due to airway remodelling and parenchymal changes [29]. Such processes may reflect accumulation of on-going airway inflammation and repetitive exposures to e.g. cigarette smoke, occupational irritants and microbial agents. In our study epithelial airway remodelling parameters did not differ between GC-sensitive and GC-insensitive asthmatics. However, other parts of the airway wall and surrounding lung tissue may contribute more extensively to airway wall stiffness. Another explanation for the observation that older age is associated with GC-unresponsiveness may be that aging has profound effects on the immune system, e.g. elderly individuals have increased neutrophil counts in broncho-alveolar lavage fluid, cells known to be less responsive to GC [30]. In our study, older age was not associated with changes in inflammatory cell types in blood, sputum or biopsies. Finally, older subjects are more likely to have co-morbidities, e.g. subclinical heart failure, which can limit expiratory flow and does not likely respond to GC [31].

To our surprise, current smoking was not independently associated with GC-unresponsiveness when tested in the multivariate regression analysis. Nevertheless, this does not rule out that smoking affects GC-responsiveness. First, the number of current-smokers was significantly higher in the GC-unresponsive group in the univariate analysis, in line with earlier reports [7,8]. Second, cigarette smoking has been associated with lower numbers of sputum eosinophils and exhaled NO levels [10,12,21,32,33]. In the present study lower sputum eosinophil counts and exhaled NO levels were significantly associated with less GC-responsiveness. Consequently, smoking subjects have a low GC-response, low exhaled NO and low sputum eosinophils. Together these data suggest that smoking changes the inflammatory profile into one that is not responsive to GCs, and is not directly responsible for the observed GC-unresponsiveness itself.

A potential drawback of our study is the inclusion of relatively mild-to-moderate asthmatic subjects. GC-responsiveness may not be easy to measure in this subgroup of asthmatics due to a low degree of airway inflammation and a ceiling effect for GC-responsiveness in airway patency. However, we believe it is important to study GC-unresponsiveness, particularly in mild-to-moderate asthmatics because of the high prevalence of this disease stage and its relatively high GC use [34]. In the past a frequently used cut-off value for GC responsiveness in (severe) asthma was a 15% improvement in $FEV_{1;}$ but as only 4 subjects in our study showed a response larger than 15%, we decided to present our results on basis of a more realistic cut-off value (0%). Furthermore, we decided to perform linear regression instead of logistic regression analysis, in order to take the presence of some "high"-responders on GC into account. Indeed, GC-unresponsiveness is generally assumed to be a characteristic of severe asthma, but our study shows that this may also occur in milder disease states and with mild airway inflammation. A second potential drawback of our study is the lack of a placebo control in our study. We did not deem the placebo control essential in our study, since we aimed to describe the predictive value of inflammatory parameters on GC-responsiveness. We feel safe to investigate this in a non- placebo controlled study, because we only investigated objective variables as both dependent and independent variables.

Do our results imply that our group of GC-unresponsive asthmatics should not receive inhaled or oral GC at all? We believe this is not necessarily the case. Even if FEV_1 does not improve, GC may have beneficial effects on other parameters relevant to asthma like airway wall inflammation, remodelling and bronchial hyperresponsiveness [35-38]. Symptoms may change when treated with GC even without a change in FEV_1 and exacerbations can be prevented through treatment with GC. Treatment of asthmatics should not solely be based on FEV_1 but on other important clinical outcome variables as well [39].

Conclusion

We conclude that GC-unresponsiveness in mild-to-moderate asthmatics is associated with lower exhaled NO values, lower sputum eosinophil numbers, higher neutrophil/eosinophil ratios in blood, higher blood lymphocytes, and higher age. To our surprise, smoking did not affect GC-responsiveness independently, but our data suggests that smoking appears to inhibit GC-responsiveness by changing the type of inflammation.

Acknowledgements

The authors would like to thank all participants of the study and thank the lung function department of Beatrixoord in Haren for their help in collecting all lung function and sputum data. This study was funded by the Dutch Asthma foundation (Grant AF 3.2.00.38).

References

1. Schwartz HJ, Lowell FC, Melby JC. Steroid resistance in bronchial asthma. *Ann Intern Med* 1968; 69: 493-499.
2. Carmichael J, Paterson IC, Diaz P, Crompton GK, Kay AB, Grant IW. Corticosteroid resistance in chronic asthma. *Br Med J (Clin Res Ed)* 1981; 282: 1419-1422.
3. Corrigan CJ, Brown PH, Barnes NC, Szefler SJ, Tsai JJ, Frew AJ, Kay AB. Glucocorticoid resistance in chronic asthma. Glucocorticoid pharmacokinetics, glucocorticoid receptor characteristics, and inhibition of peripheral blood T cell proliferation by glucocorticoids in vitro. *Am Rev Respir Dis* 1991; 144: 1016-1025.
4. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 2002; 57: 875-879.
5. Little SA, Chalmers GW, MacLeod KJ, McSharry C, Thomson NC. Non-invasive markers of airway inflammation as predictors of oral steroid responsiveness in asthma. *Thorax* 2000; 55: 232-234.
6. Smith AD, Cowan JO, Brassett KP, Filsell S, McLachlan C, Monti-Sheehan G, Peter HG, Robin TD. Exhaled nitric oxide: a predictor of steroid response. *Am J Respir Crit Care Med* 2005; 172: 453-459.
7. Chalmers GW, MacLeod KJ, Little SA, Thomson LJ, McSharry CP, Thomson NC. Influence of cigarette smoking on inhaled corticosteroid treatment in mild asthma. *Thorax* 2002; 57: 226-230.
8. Chaudhuri R, Livingston E, McMahon AD, Thomson L, Borland W, Thomson NC. Cigarette smoking impairs the therapeutic response to oral corticosteroids in chronic asthma. *Am J Respir Crit Care Med* 2003; 168: 1308-1311.
9. Lazarus SC, Chinchilli VM, Rollings NJ, Boushey HA, Cherniack R, Craig TJ, Deykin A, DiMango E, Fish JE, Ford JG, Israel E, Kiley J, Kraft M, Lemanske RF,Jr., Leone FT, Martin RJ, Pesola GR, Peters SP, Sorkness CA, Szefler SJ, Wechsler ME, Fahy JV. Smoking affects response to inhaled corticosteroids or leukotriene receptor antagonists in asthma. *Am J Respir Crit Care Med* 2007; 175: 783-790.
10. Chalmers GW, MacLeod KJ, Thomson L, Little SA, McSharry C, Thomson NC. Smoking and airway inflammation in patients with mild asthma. *Chest* 2001; 120: 1917-1922.
11. Broekema M, ten Hacken NH, Volbeda F, Lodewijk ME, Hylkema MN, Postma DS, Timens W. Airway epithelial changes in smokers but not in ex-smokers with asthma. *Am J Respir Crit Care Med* 2009; 180: 1170-1178.
12. Verleden GM, Dupont LJ, Verpeut AC, Demedts MG. The effect of cigarette smoking on exhaled nitric oxide in mild steroid-naive asthmatics. *Chest* 1999; 116: 59-64.
13. Chakir J, Hamid Q, Bosse M, Boulet LP, Laviolette M. Bronchial inflammation in corticosteroid-sensitive and corticosteroid-resistant asthma at baseline and on oral corticosteroid treatment. *Clin Exp Allergy* 2002; 32: 578-582.
14. van den Berge M, Meijer RJ, Kerstjens HA, de Reus DM, Koeter GH, Kauffman HF, Postma DS. PC(20) adenosine 5'-monophosphate is more closely associated with airway inflammation in asthma than PC(20) methacholine. *Am J Respir Crit Care Med* 2001; 163: 1546-1550.
15. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993; 16: 5-40.
16. van den Berge M, Kerstjens HA, Meijer RJ, de Reus DM, Koeter GH, Kauffman HF, Postma DS. Corticosteroid-induced improvement in the PC20 of adenosine monophosphate is more closely associated with reduction in airway inflammation than improvement in the PC20 of methacholine. *Am J Respir Crit Care Med* 2001; 164: 1127-1132.
17. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. *Am J Respir Crit Care Med* 2005; 171: 912-930.

18. Tsoukias NM, George SC. A two-compartment model of pulmonary nitric oxide exchange dynamics. *J Appl Physiol* 1998; 85: 653-666.

19. Rottier BL, Cohen J, van der Mark TW, Douma WR, Duiverman EJ, ten Hacken NH. A different analysis applied to a mathematical model on output of exhaled nitric oxide. *J Appl Physiol* 2005; 99: 378-379.

20. Livingston E, Chaudhuri R, McMahon AD, Fraser I, McSharry CP, Thomson NC. Systemic sensitivity to corticosteroids in smokers with asthma. *Eur Respir J* 2007; 29: 64-71.

21. Persson MG, Zetterstrom O, Agrenius V, Ihre E, Gustafsson LE. Single-breath nitric oxide measurements in asthmatic patients and smokers. *Lancet* 1994; 343: 146-147.

22. Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, Dolovich J. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992; 47: 25-29.

23. Meijer RJ, Kerstjens HA, Arends LR, Kauffman HF, Koeter GH, Postma DS. Effects of inhaled fluticasone and oral prednisolone on clinical and inflammatory parameters in patients with asthma. *Thorax* 1999; 54: 894-899.

24. Mastruzzo C, Greco LR, Nakano K, Nakano A, Palermo F, Pistorio MP, Salinaro ET, Jordana M, Dolovich J, Crimi +, Vancheri C. Impact of intranasal budesonide on immune inflammatory responses and epithelial remodeling in chronic upper airway inflammation. *Journal of Allergy and Clinical Immunology* 2003; 112: 37-37.

25. Syed F, Bingham B, Johnson M, Markham AF, Morrison JF. The CD4+ T lymphocyte is a site of steroid resistance in asthma. *QJM* 1998; 91: 567-572.

26. Yang M, Kumar RK, Foster PS. Pathogenesis of steroid-resistant airway hyperresponsiveness: interaction between IFN-gamma and TLR4/MyD88 pathways. *J Immunol* 2009; 182: 5107-5115.

27. McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, Henry A, Irvin CG, Piganelli JD, Ray A, Kolls JK. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J Immunol* 2008; 181: 4089-4097.

28. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, Arron JR, Koth LL, Fahy JV. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med* 2009; 180: 388-395.

29. Vignola AM, Scichilone N, Bousquet J, Bonsignore G, Bellia V. Aging and asthma: pathophysiological mechanisms. *Allergy* 2003; 58: 165-175.

30. Meyer KC, Rosenthal NS, Soergel P, Peterson K. Neutrophils and low-grade inflammation in the seemingly normal aging human lung. *Mech Ageing Dev* 1998; 104: 169-181.

31. Heaney LG, Conway E, Kelly C, Johnston BT, English C, Stevenson M, Gamble J. Predictors of therapy resistant asthma: outcome of a systematic evaluation protocol. *Thorax* 2003; 58: 561-566.

32. Kharitonov SA, Robbins RA, Yates D, Keatings V, Barnes PJ. Acute and chronic effects of cigarette smoking on exhaled nitric oxide. *Am J Respir Crit Care Med* 1995; 152: 609-612.

33. Malinovschi A, Janson C, Holmkvist T, Norback D, Merilainen P, Hogman M. Effect of smoking on exhaled nitric oxide and flow-independent nitric oxide exchange parameters. *Eur Respir J* 2006; 28: 339-345.

34. Dusser D, Montani D, Chanez P, de Blic J, Delacourt C, Deschildre A, Devillier P, Didier A, Leroyer C, Marguet C, Martinat Y, Piquet J, Raherison C, Serrier P, Tillie-Leblond I, Tonnel AB, Tunon de Lara M, Humbert M. Mild asthma: an expert review on epidemiology, clinical characteristics and treatment recommendations. *Allergy* 2007; 62: 591-604.

35. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, Wardlaw AJ, Pavord ID. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002; 360: 1715-1721.

36. Kerstjens HA, Brand PL, Quanjer PH, van der Bruggen-Bogaarts BA, Koeter GH, Postma DS. Variability of bronchodilator response and effects of inhaled corticosteroid treatment in obstructive airways disease. Dutch CNSLD Study Group. *Thorax* 1993; 48: 722-729.

37. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. *Am J Respir Crit Care Med* 1999; 159: 1043-1051.

38. Ward C, Pais M, Bish R, Reid D, Feltis B, Johns D, Walters EH. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. *Thorax* 2002; 57: 309-316.

39. Bateman ED, Clark TJ, Frith L, Bousquet J, Busse WW, Pedersen SE. Rate of response of individual asthma control measures varies and may overestimate asthma control: an analysis of the goal study. *J Asthma* 2007; 44: 667-673.