

ORIGINAL ARTICLE

AIRWAY DISEASES

Stability of phenotypes defined by physiological variables and biomarkers in adults with asthma

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Abstract

Background: Although asthma is characterized by variable airways obstruction, most studies of asthma phenotypes are cross-sectional. The stability of phenotypes defined either by biomarkers or by physiological variables was assessed by repeated measures over 1 year in the Pan-European BIOAIR cohort of adult asthmatics.

Methods: A total of 169 patients, 93 with severe asthma (SA) and 76 with mild-to-moderate asthma (MA), were examined at six or more visits during 1 year. Asthma phenotype clusters were defined by physiological variables (lung function, reversibility and age of onset of the disease) or by biomarkers (eosinophils and neutrophils in induced sputum).

Results: After 1 year of follow-up, the allocation to clusters was changed in 23.6% of all asthma patients when defined by physiological phenotypes and, remarkably, in 42.3% of the patients when stratified according to sputum cellularity ($P = 0.034$). In the SA cohort, 30% and 48.6% of the patients changed allocation according to physiological and biomarker clustering, respectively. Variability of phenotypes was not influenced by change in oral or inhaled corticosteroid dose, nor by the number of exacerbations. Lower stability of single and repeated measure was found for all evaluated biomarkers (eosinophils, neutrophils and FeNO) in contrast to good stability of physiological variables (FEV₁), quality of life and asthma control.

Conclusion: Phenotypes determined by biomarkers are less stable than those defined by physiological variables, especially in severe asthmatics. The data also imply that definition of asthma phenotypes is improved by repeated measures to account for fluctuations in lung function, biomarkers and asthma control.

Initial attempts to define asthma phenotypes were based on the broad concepts of extrinsic (allergic) and intrinsic (nonallergic) asthma (1, 2). With increased understanding of pathobiology, classifications identifying distinct inflammatory phenotypes have been attempted (3, 4). Hastie et al. (5) proposed asthma phenotypes stratification using biomarkers of inflammation (namely eosinophils and neutrophils in induced

sputum), whereas Moore et al. (6) suggested another approach based only on physiological variables and medical history. Although these attempts represent important steps towards a mechanism-based classification of asthma, a significant limitation of the studies published to date is that they often derive from cross-sectional measurements taken at a single time point or during a short interval of time (7).

The Pan-European BIOAIR study of severe asthma includes repeated measures of many outcomes, including sputum data (8, 9). We therefore performed an analysis of the stability of phenotypes in adults with controlled and severe asthma using the BIOAIR cohort. The aim was to compare phenotypes defined either by biomarkers ('the Hastie approach') or by physiological variables ('the Moore approach') over 1 year of observations. Concerning inflammatory phenotypes defined by sputum analysis, there are conflicting data on its repeatability. Fleming et al. (10) found that sputum results were unstable in children with asthma, whereas previous studies in adults generally suggest reasonable stability and repeatability of sputum measurements (11–13).

Methods

Subjects

Patients aged 18–80 years were recruited for the study and diagnosed by pulmonary specialists according to standard criteria (14) at the 12 participating European centres. Inclusion in the severe asthma (SA) group required specialist treatment for at least 1 year and at least one exacerbation requiring oral steroid treatment in the past year, despite continuous treatment with high doses of inhaled corticosteroids (ICS; at least 1600 µg/day budesonide or beclomethasone, 800 µg/day fluticasone or equivalent), and long-acting β-agonists or oral theophylline for at least 1 year (8, 9, 15). Subjects with less heavy medications and no exacerbations in the past year were included in the mild-to-moderate asthma group (MA) as detailed in the online depository.

Study design

All patients after screening (V1) underwent 4 weeks of treatment optimization period (V2) followed by a 2-week double-blind placebo-controlled steroid intervention (V3). After the screening, patients were allocated to SA and mild-to-moderate asthma (MA) cohorts, and thereafter followed for 12 months with control visits at four-monthly intervals (V4–V6), as well as at additional visits in case of exacerbations (Vex). The study flow chart with time points for consecutive visits is presented in Fig. S1 (online repository). Information regarding lung functions, biomarkers [induced sputum (with exception of V5), peripheral blood, exhaled NO], atopy, medical history, asthma control and quality of life was collected at baseline and during consecutive visits. The study (clinicaltrials.gov NCT00555607) was approved by the ethics review boards in the twelve participating centres. More specifications concerning the BIOAIR study design and methodology are available in the online depository.

Definition of asthma phenotypes

Asthma phenotype based on clinical and physiological variables was defined by the use of the algorithm proposed by Moore et al. (6). Five clusters were identified by baseline lung function, reversibility and age of onset of the disease.

Asthma phenotype based on biomarkers was defined as proposed by Hastie et al. (5). Four subgroups were identified by percentage of eosinophils (<2% or ≥2%) and neutrophils (<40% or ≥40%) in induced sputum.

Statistical analysis

Data were entered into a central database through a web-based case record form (eCRF) system developed specifically for the BIOAIR study. Patient baseline characteristics are expressed as mean ± standard error of the mean (SEM). Continuous variables were analysed using Student's *t*-test or Mann–Whitney *U*-test for nonparametric values and categorical variables by chi-square test. Stability of eosinophil and neutrophil counts and lung function measurements was assessed using intraclass correlation coefficients (ICCs). An ICC was obtained for single measures (the stability of individual measurement) and average measures (the stability of a measurement relative to all measures in that individual). $P < 0.05$ was considered a statistically significant difference for all tests. The generalized estimating equations for logistic regression have been used to analyse stability of allocation to clusters (baseline *vs* at 1 year of follow-up and MA *vs* SA). All analyses were carried out using GraphPad Prism V.5 (GraphPad Software, Inc., La Jolla, CA, USA) or SPSS (V.19; IBM Corporation, Armonk, NY, USA).

Results

Baseline characteristics

A total of 169 asthmatic patients were included in the BIOAIR study, including 93 with severe and 76 with mild-to-moderate disease (Table 1). Of those, 138 patients (80 SA and 58 MA) completed 1 year of the follow-up study (Fig. S2 for flow chart and specifications).

Stability of a phenotype based on physiological variables

Data from 164 patients at baseline and 127 at 1 year of follow-up were complete and enabled allocation to clusters in line with an algorithm proposed by Moore et al. (6). At baseline, 17.7% of patients were allocated to cluster 1, 43.9% to cluster 2, 12.2% to cluster 3, 14% to cluster 4 and 12.2% to cluster 5 (Fig. 1A). After 1 year of follow-up, the allocation to particular clusters was stable in 76.4% and changed in 23.6% of the whole asthma population and was stable in 70% and changed in 30% of severe asthmatics (Fig. 2A).

Stability of a phenotype based on sputum eosinophils as biomarkers

One hundred and three patients produced evaluable sputum samples at baseline and 52 at 1 year of follow-up, which enabled allocation to inflammatory phenotypes in line with an algorithm proposed by Hastie et al. (5). At baseline, 24.3% of patients were allocated to cluster 1, 20.4% to cluster 2, 20.4% to cluster 3 and 35% to cluster 4 (Fig. 1B).

Table 1 Demographic data and baseline characteristics of the study cohort [mean values ($\pm$ SEM), unless stated differently]

	Severe asthma	Mild-to-moderate asthma	<i>P</i>
Number of patients in the BIOAIR cohort (<i>n</i>)	93	76	ND
Age (years, min–max)	50.0 $\pm$ 1.3 (18–72)	42.2 $\pm$ 1.5 (20–70)	0.001*
Females (%)	58	61	0.982†
FEV ₁ (% pred)	70.4 $\pm$ 2.1	88.7 $\pm$ 2.1	<0.0001*
FEV ₁ (l)	2.04 $\pm$ 0.08	2.79 $\pm$ 0.08	<0.0001*
FEV ₁ /FVC	0.67 $\pm$ 0.01	0.70 $\pm$ 0.01	0.093*
Reversibility	9.4 $\pm$ 0.8	10.6 $\pm$ 0.7	0.192*
ICS [median (mean $\pm$ SD)] beclomethasone eq.	1600 μ g* (2064 $\pm$ 939.7)	800 μ g (614 $\pm$ 218.6)	<0.0001*
OCS [median (mean $\pm$ SD; min–max)] prednisolone eq.	10 mg (14.15 $\pm$ 11.8; 2–50)	–	ND
BMI (kg/m ²)	28.5 $\pm$ 0.6	25.0 $\pm$ 0.4	<0.0001*
ACQ (Juniper)	2.03 $\pm$ 0.1	1.03 $\pm$ 0.7	<0.0001*
QoL (SGRQ)	45.9 $\pm$ 2.1	22.5 $\pm$ 2.0	<0.0001*
CRP (mg/l)	6.1 $\pm$ 0.9	3.5 $\pm$ 0.6	0.092*
Atopy (%)	43	48	0.642†
F _{ENO} (ppb)	46.3 $\pm$ 6.2	40.1 $\pm$ 4.1	0.962*
Sputum cells ($\times 10^6$)	3.34 $\pm$ 1.02*	1.83 $\pm$ 0.34	0.455*
Sputum eosinophils (%)	16.7 $\pm$ 3.49*	5.79 $\pm$ 1.71	0.018*
Sputum neutrophils (%)	42.2 $\pm$ 3.7	44.2 $\pm$ 4.4	0.338*

ACQ, Asthma Control Questionnaire; SGRQ, St George's Respiratory Questionnaire; OCS, oral corticosteroids; ICS, inhaled corticosteroids; CRP, serum C-reactive protein; atopy defined as at least one positive skin prick test (ND, not determined, *Mann–Whitney *U*-test, †chi-square test).

After 1 year of follow-up, the allocation to particular clusters was stable in 57.7% and changed in 42.3% of the whole asthma population and was stable only in 51.4% and changed in 48.6% of severe asthmatics (Fig. 2B).

Comparison between stability of phenotypes based on physiological parameters or biomarkers

Allocation to particular phenotypes defined by physiological parameters and biomarkers at baseline and after 1 year of follow-up is presented in Fig. 3. Significantly more patients changed allocation to phenotype based on biomarkers (sputum eosinophils and neutrophils) in the whole asthma population (42.3% *vs* 23.6 biomarker *vs* physiological variables clustering, *P* = 0.034) and in subgroups: severe asthma cohort (48.6% *vs* 30.0%, biomarker *vs* physiological variables clustering) and mild-to-moderate asthma cohort (32.8% *vs* 15.5%, biomarker *vs* physiological variables clustering). There was a tendency (*P* = 0.097) to lower stability of phenotypes in severe asthmatics both for physiological variables clustering (30.0% *vs* 15.5% patients changed allocation in SA *vs* MA, respectively) and for clustering based on eosinophils and neutrophils in induced sputum (48.6% *vs* 32.8% patients changed allocation in SA *vs* MA, respectively).

Longitudinal data

Changes in lung function (individual and mean values of FEV₁% predicted) over the duration of the study for the severe and mild-to-moderate asthma cohorts are shown in Figs S5 and S6 (online repository). The ICC (95% CI) for FEV₁ (single measures) was 0.585 (0.506–0.661, *P* < 0.001) and for average measures was 0.849 (0.804–0.887, *P* < 0.001).

Changes in eosinophils and neutrophils over the duration of the study for the severe and mild-to-moderate asthma cohorts are shown in Figs S7 and S8 (online repository). The ICC (95% CI) for eosinophils (single measures) was 0.479 (0.386–0.573, *P* < 0.001) and for neutrophils 0.465 (0.297–0.638, *P* < 0.001). Intraclass correlation coefficient for eosinophils (average of replicate measures) was 0.786 (0.716–0.843, *P* < 0.001) and for neutrophils 0.777 (0.628–0.876, *P* < 0.001). These results consistently show improved reliability with repeated measures and that measurements of physiological variables (lung function) had better repeatability than measurements of cellular biomarkers (eosinophils and neutrophils in induced sputum).

The change in phenotype during 1 year of follow-up was compared with change in ICS dose during this time (Fig. S3). The phenotype change was not influenced by the change in the dose of inhaled corticosteroids used either in phenotypes defined by physiological variables (lung function, reversibility) and medical history (*P* = 0.275, Kruskal–Wallis test), or in phenotypes defined by biomarkers (eosinophils and neutrophils in induced sputum; *P* = 0.935; Fig. S9). Furthermore, 26 severe asthma patients were on oral corticosteroids (OCS) during the follow-up period. The phenotype change in this group was not influenced by the change in the dose of OCS (*P* = 0.563 for phenotypes defined by the physiological parameters and *P* = 0.479 for phenotypes defined by biomarkers, data not shown).

The change in phenotype during 1 year of follow-up was also compared with the number of exacerbations reported during this time (Fig. S4). The phenotype change was not influenced by the number of exacerbations either in phenotypes defined by physiological variables (lung function, reversibility) and by medical history (*P* = 0.55), or in phenotypes defined by

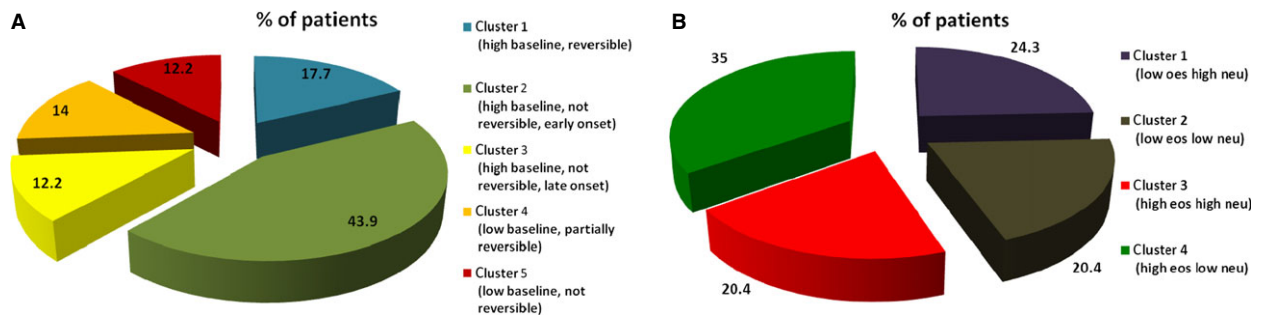


Figure 1 Allocation to particular asthma phenotypes at baseline (V1) based on physiological parameters (A) (baseline lung function and reversibility) and medical history (age of onset of the disease)

(11) and based on biomarkers (B) (eosinophils 'eos' and neutrophils 'neu' in induced sputum) (10).

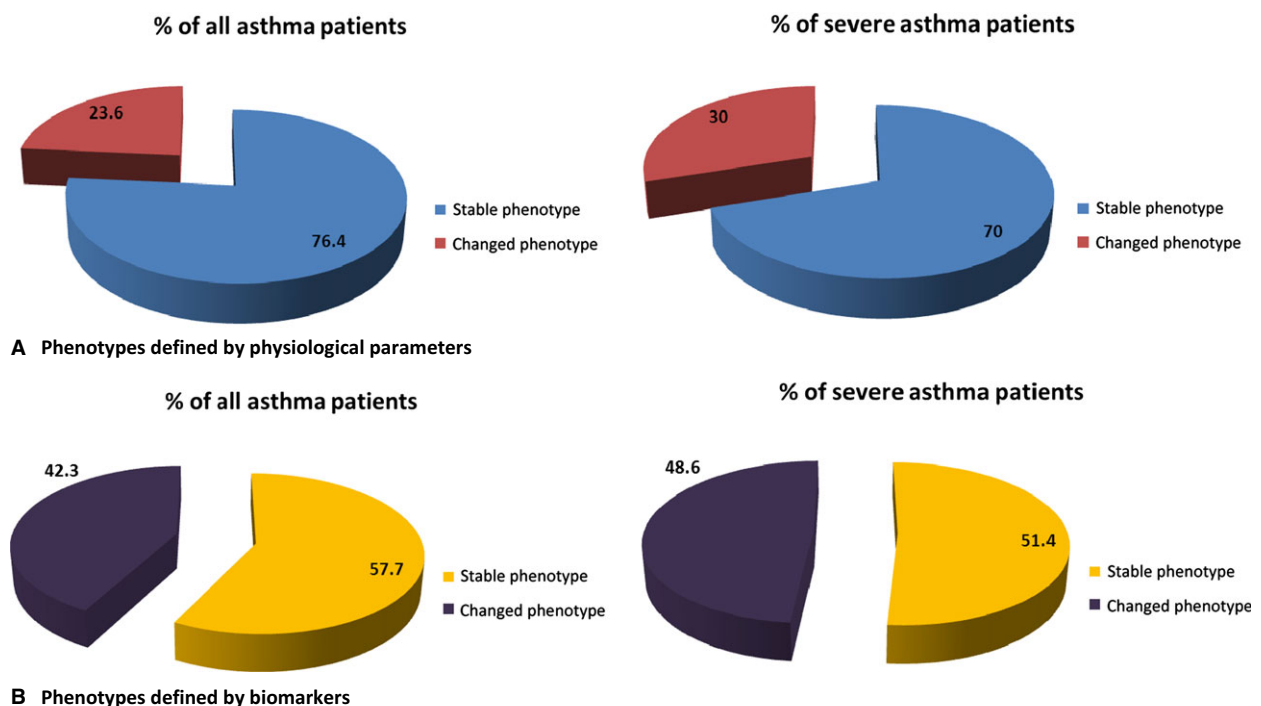


Figure 2 Stability of phenotypes defined by physiological parameters (A) and biomarkers (B) after 1 year of follow-up.

biomarkers (eosinophils and neutrophils in induced sputum; $P = 0.15$). Changes in the level of asthma control (defined with the use of Asthma Control Questionnaire), quality of life (defined with the use of St George's Respiratory Questionnaire) and exhaled NO (FeNO) are presented in Figs S10, S11 and S12, respectively, of the online supplement. Finally, only five patients changed allocation using both evaluated methods of defining phenotypes during the follow-up year.

Discussion

To our knowledge, this is the first study in adults with asthma that compares stability over 1 year of follow-up of phenotypes defined by physiological variables or sputum cells as biomarkers. We found that allocation to phenotypes

based on lung function and age of onset of the disease was more stable than allocation to phenotypes based on eosinophils and neutrophils in induced sputum. Moreover, phenotypes of mild-to-moderate asthmatics seem to be more stable than those of severe asthma patients. Although the proportion of subjects in the different groups remained similar after 1 year of follow-up, a significant number of patients shifted groups, meaning that variability in group allocation on the individual patient level was considerable. This is an important aspect if initial patient stratification should be used to allocate patients to a particular treatment. The variability in group assignment also raises concerns if patients should be studied for biomarkers and 'omics to define endotypes. We interpret our findings in this analysis as a strong indication that repeated measures are required

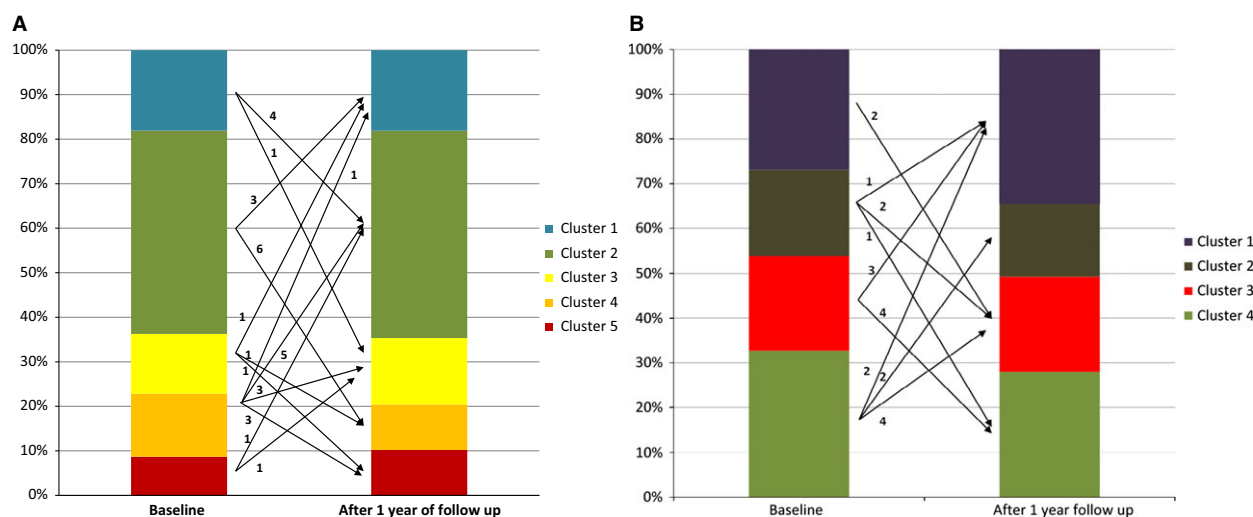


Figure 3 Allocation to particular phenotypes defined by physiological parameters (A) (11) and biomarkers (B) (10) at baseline and after 1 year of follow-up (percentage of the study group for those sub-

jects where all data points were available, $n = 127$ for physiological parameters and $n = 52$ for biomarkers). Migration of particular patients (number of patients) is shown with arrows.

to obtain reliable phenotyping data on subjects with asthma, in particular when using biomarkers.

Previous studies in adults with asthma have indicated reasonable stability of inflammatory biomarkers in sputum (reviewed in 7). The data in the literature are, however, conflicting. Many studies have been short term and in small cohorts of patients, with focus on eosinophils in induced sputum (11, 16). van Veen et al. (17) and Simpson et al. (18) thus reported that sputum eosinophilia is a consistent feature overtime despite treatment with high doses of corticosteroids, and they found inflammatory subtypes of asthma to be reasonably stable. However, several other inflammatory phenotypes may be identified by sputum analysis in asthmatics (19). Al-Samri et al. (20) analysed changes in sputum eosinophils and neutrophils over 1 year in 37 severe and 24 moderate adult asthmatics and found that sputum inflammatory profiles often changed independently of the severity of asthma. Fleming et al. (10) likewise found that inflammatory phenotype based on induced sputum measurements was unstable in children with asthma. McGrath et al. (13) reported on longitudinal sputum cytology data from 995 subjects with asthma enrolled in different clinical trials and found that a significant proportion of patients shifted groups. In addition, approximately half of mild-to-moderate asthmatics had persistent noneosinophilic inflammation, which may be associated with poor response to currently available anti-inflammatory therapy. Green et al. (21), in a study over 12 months, also found the noneosinophilic asthma phenotype to be relatively stable. Although the repeatability and reliability of sputum cellularity measurements in our study were reasonably high, it was nevertheless less reliable than physiological variables such as FEV₁. It is known that FEV₁ has lower variability than other lung function measures (22).

Several confounding factors, including change in medications, adherence to treatment, exacerbations, pollution and allergens' exposure, may have a potential influence on

phenotype stability in asthma patients. It seems unlikely that phenotype variability in our study was related to differences in the dose of inhaled or oral corticosteroids as there were no differences in the level of steroid treatment between the studied clusters (Fig. S3). Similarly, there were no differences due to the number of exacerbations during 1 year of follow-up (Fig. S4). One limitation the BIOAIR project shares with other observational studies of patient cohorts is that the compliance with the prescribed medication has not been rigorously evaluated. It has been estimated that the overall compliance with inhaled controller therapy in asthma is no more than 50% (21). In our study, an effort was, however, made to improve adherence by reminding the patients to take the inhaled corticosteroids every day when responding to the questions in the electronic daily card. Although we did not collect quantitative data on environmental triggers such as allergen exposure, infections or air pollution, it seems reasonable to assume that clinically important differences in environmental factors would be reflected as differences in the number of exacerbations between the clusters. However, the exacerbations were carefully monitored by daily recordings of lung function and symptoms (8), and there were no differences in exacerbations that could be associated with changes in phenotype cluster. As the dropout rate (18.3% of the included cohort, $n = 31$) was small, it was unlikely to influence the study results. Moreover, for the statistical analysis, only data from those subjects with complete data set were included. As data were expressed as percentage change, another possible limitation would be the smaller sample size for the inflammatory than for the physiological parameters ($n = 52$ vs $n = 127$), and this will need to be addressed in future studies.

We also evaluated whether it was the same subjects who changed allocation during 1 year of follow-up to phenotypes defined by physiological variables or by cellularity in induced sputum. However, only five patients consistently changed

allocation in both applied algorithms. In contrast, most patients who were unstable according to one method of clustering were stable using the other strategy. Taken together, the findings support the interpretation that biomarkers (in this case, eosinophils and neutrophils in induced sputum) and clinical/physiological variables such as lung function, the number of exacerbations and the level of required asthma treatment (23, 24) reflect different processes that cannot be assumed to correlate, even if being partly dependent variables.

In summary, our comparison of two published strategies for clustering of asthma found that the inflammatory phenotypes determined by the cellularity in induced sputum were less stable than those defined by physiological variables. This is yet another confirmation that asthma is a variable inflammatory disease, and therefore, phenotyping to understand mechanisms of the disease needs to be long term, build on repeated measures and should ideally integrate several different outcomes.

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Author contributions

Primary data aggregation and statistical analyses were performed by MK and SED, and who then discussed and interpreted with the other co-authors at face-to-face meetings. MK wrote the first draft of the manuscript. Revisions were made by MK together with SED and in an interactive process including all co-authors. All authors participated in the study design, conduction of the trial, collection and interpretation of data, and critical review of the draft versions of the report.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The design of the study (time points in weeks, V-visits, Vex – extra visits due to an exacerbation).

Figure S2. The study flow chart.

Figure S3. Change in dose of budesonide (eq; $\mu\text{g/day}$) for each phenotype change for phenotypes based on physiological variables (panel A) and for phenotypes based on biomarkers (panel B).

Figure S4. Number of exacerbations (median of events during 1 year of follow-up and range) for each phenotype change for phenotypes based on physiological variables (panel A) and for phenotypes based on biomarkers (panel B).

Figure S5. Changes in individual values of lung function measurements ($\text{FEV}_1\%$ of predicted) in severe (A) and mild-to-moderate (B) asthma cohorts.

Figure S6. Changes in lung function measurements ($\text{FEV}_1\%$ of predicted, mean $\pm$ SD) over the 1 year of follow-up in the severe asthma cohort (solid circles) and in the mild-to-moderate asthma cohort (empty squares).

Figure S7. Changes in individual values of eosinophils and neutrophils in induced sputum in severe (SA) and mild-to-moderate (MA) asthma cohorts.

Figure S8. Changes in sputum eosinophils (A) and neutrophils (B) (% of cells, mean $\pm$ SD) over the 1 year of follow-up in the severe asthma cohort (solid circles) and in the mild-to-moderate asthma cohort (empty squares).

Figure S9. Changes in median dose of ICS (budesonide eq) (A) in severe asthma (solid circles) and mild-to-moderate asthma (open squares). Changes in individual ICS dose (ACQ test) in severe asthma are presented in panel B and in mild-to-moderate asthma – in panel C.

Figure S10. Changes in mean values (A) of asthma control level (ACQ test) in severe asthma (solid circles) and mild-to-moderate asthma (open squares). Changes in individual values of asthma control (ACQ test) in severe asthma are presented in panel B and in mild-to-moderate asthma – in panel C.

Figure S11. Changes in mean values (A) of quality of life (SGRQ) in severe asthma (solid circles) and mild-to-moderate asthma (open squares). Changes in individual values of asthma control (SGRQ) in severe asthma are presented in panel B and in mild-to-moderate asthma – in panel C.

Figure S12. Changes in mean values (A) of exhaled nitric oxide (FeNO) in severe asthma (solid circles) and mild-to-moderate asthma (open squares). Changes in individual values of exhaled nitric oxide (FeNO) in severe asthma are presented in panel B and in mild-to-moderate asthma – in panel C.

Table S1. The fixed doses of medications used by patients during treatment optimization period (V1–V2a).

Table S2. Relation of biomarkers (eosinophils and neutrophils in induced sputum) to clusters defined by physiological parameters (lung function) and age of onset of the disease.

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Appendix 1

BIOAIR – Longitudinal Assessment of Clinical Course and BIOMarkers in Severe Chronic AIRway Disease Contributors to the BIOAIR study

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Stability of phenotypes defined by physiological variables and biomarkers in adults with asthma

The BIOAIR trial was a partly European Union supported project on biomarkers and clinical outcomes with a focus on severe asthma. The study had a two-group prospective design. Asthma patients were screened and allocated to severe (SA) or mild-to-moderate asthma (MA) cohort. After a four-week treatment optimisation period, patients were followed up for at least 1 year. Information regarding lung functions, biomarkers (induced sputum, peripheral blood, exhaled NO), atopy, medical history, asthma control and quality of life were collected at baseline and at particular time intervals during follow up. Moreover, lung functions (PEF and FEV₁), rescue medication use (SABA), day and night symptoms, and limitation of activities were recorded daily with the use of electronic diaries. This design enabled insight into stability of phenotypes, biomarkers and physiological parameters in line with the goal of our study. Methods of the cohort selection and baseline characteristics of the study subject are shared by all reports from the BIOAIR trial (E1, E2).

METHODS

Patients were screened during a half-day visit (V1) in one of participating clinics and then all subjects underwent a 4-week treatment optimization period (V1-V2). Fixed doses of medications used by patients during the optimization are presented in Table E1. The optimization period was followed by a 2-week double-blind placebo-controlled steroid intervention (V2-V3), consisting of a standard course of oral prednisolone of 0.5 mg/kg of body weight/day or placebo added to their regular treatment. Then, two cohorts were followed for 12 months with control visits at four-monthly intervals (V4-V6) and additional visits in case of exacerbations (Vex). Information regarding lung functions, biomarkers (induced sputum, peripheral blood, exhaled NO), atopy, medical history, asthma control (evaluated with the use of the Juniper Asthma Control Questionnaire) (E3) and quality of life (evaluated with the use of St Georges Respiratory Questionnaire) (E4) were collected at baseline and consecutive visits. Moreover, lung functions (PEF and FEV₁), rescue medication use (SABA), day and night symptoms, and limitation of activities were recorded daily with the use of electronic diaries (Vitalograph Electronic PEF/FEV₁ Diary – XM version, Vitalograph Ltd., Buckingham, UK).

Subjects

Asthma was diagnosed by pulmonary specialists according to published criteria (E5, E6, E7, E8). Diagnosis of asthma had to be confirmed by at least one of the four criteria for reversible airway obstruction as documented during the last 5 years before the study or at screening visit, namely:

1) an increase in FEV₁ (Forced Expiratory Volume in 1 second) $\geq$ 9% of predicted (or improvement by 200 ml) after administration of four puffs of 100 µg salbutamol dose-aerosol inhaled via a spacer, or after additional

inhalation of four puffs of 20 µg ipratropium bromide administered through a large volume spacer;

2) mean diurnal variation in Peak Expiratory Flow (PEF) of $\geq 15\%$ on ≥ 4 days/week for at least 2 weeks, as calculated by the following equation: $(\text{highest PEF} - \text{lowest PEF})/\text{mean PEF}$;

3) an increase in FEV1 of at least 400 ml after a course of prednisolone 0.5 mg/kg/day for 14 days;

4) in patients with a FEV1 $\geq 70\%$ predicted, demonstrated bronchial hyperresponsiveness to histamine, methacholine, isocapnic hyperventilation, exercise or other indirect challenges (according to established local methods). Patients included in the severe asthma (SA) group had been under specialist treatment for at least one year and had experienced at least one exacerbation in the past year. Exacerbations were defined either by the initiation of a course of oral steroid (OCS) therapy in those individuals on regular inhaled corticosteroid (ICS) treatment, or for those on regular OCS therapy, a significant temporary increase in their dose of oral steroids for an acute deterioration in their disease control. Severe asthmatics had to require continuous treatment with high doses of inhaled steroids (at least 1600 µg/day budesonide or beclomethasone, 800 µg/day fluticasone or equivalent). For those taking oral steroids, the dose of inhaled steroids had to be at least 800 µg/day budesonide or beclomethasone, 400 µg/day fluticasone or equivalent. In addition, the patients had to require continuous treatment with long-acting β agonists or oral theophylline, as documented for at least one year. Patients in the mild-to-moderate asthma (MA) group had stable disease and received daily treatment with a maximum of 800 µg/day budesonide or beclomethasone, 500 µg/day fluticasone or equivalent. The MA patients used short-acting β -agonists as needed but did not require treatment with long-acting β -agonists and had had neither exacerbations nor hospitalizations in the past year. Asthma patients who had smoked for more than 5 pack years were excluded. Patients were excluded if they were pregnant, had a history of alcohol or illicit drug abuse, had other acute or chronic pulmonary disorders or had clinically relevant psychiatric disease. They were not allowed to be receiving immunosuppressants other than corticosteroids, or undergoing immunotherapy. All exacerbations and adverse events during the study were recorded in the study case report form for further analysis. In the definition of adverse events, asthma exacerbations were excluded as they were study end-points for the follow-up study.

The allocation to cluster as proposed by Moore et al has been based on an algorithm included in the original publication by Moore et al (E9). In short, allocation to particular clusters was based on baseline lung function (FEV1 $\geq 68\%$ of predicted vs $<68\%$, with a bronchodilator withhold), max FEV1 (after albuterol, $\geq 108\%$ vs $<108\%$ for clusters 1,2,3 or $\geq 65\%$ vs $<65\%$ for clusters 4 and 5) and age of onset of the disease (<40 years vs ≥ 40 years). It has been found by Moore et al (E9) that the performance of the above described algorithm is acceptable and results in correct allocation of 80% of subjects.

Measurements

Pulmonary function

Lung function, including forced expiratory volume in 1 second (FEV₁), forced vital capacity (FVC), and forced inspiratory volume in 1 second (FIV₁) were measured at each visit with daily calibrated spirometers according to previously published guidelines (E10). Patients were asked to withhold the use of short- and long-acting β -agonists for 6-10 hours before the measurements. The highest value of three technically satisfactory attempts was recorded, and predicted values were calculated according to established formulas (E10). An extended reversibility test was performed at screening visit. After FEV₁ measurement, the patient inhaled four puffs of 100 μ g salbutamol (MDI via spacer) and after 15 min FEV₁ was measured again and reversibility as percent predicted was calculated. If an improvement of 9% was not achieved, four puffs of 20 μ g ipratropium bromide MDI via a large volume spacer were administered, FEV₁ was measured after 15 min and reversibility was calculated.

Atopy and inflammatory markers

In addition to a complete physical examination, skin prick tests to common aeroallergens were performed in all patients at the screening visit. Patients were tested with six standard allergens (Dermatophagoides pteronyssinus, mixed grasses, cat, dog, Alternaria and Aspergillus fumigates) and two regional allergens (Southern Europe: parietaria and olive, Ferrara: parietaria and birch, Northern Europe: birch and mugwort, hazel or alder). Allergens were compared to a positive control (histamine) and a negative control (allergen diluent). ALK Soluprick (SoluprickTM, ALK lab, Copenhagen, Denmark) solutions were used and the results were expressed as a mean diameter in millimetres of two right angle measurements. A wash-out period of 3 days for short-acting and 5 days for long-acting anti-histamines was required prior to the skin prick test. A positive response was taken as a wheal $\geq$ 3 mm in comparison to the negative control. Patients were considered atopic when at least one allergen showed a positive result. Total wheal size was calculated in mm as the total of the 8 allergens.

At each visit, venous blood samples were taken for routine blood chemistry and haematology, according to the normal routine at each clinic. Total and differential white blood cell count (WBC) was measured, as well as serum C-reactive protein (CRP). Induced sputum was obtained at all centers according to recommendations of the ERS (European Respiratory Society) task force on induced sputum (E11). Patients with a postbronchodilator FEV₁ percent of predicted < 60%, a peak flow (PEF) variability of > 30% during the four days preceding the sputum induction, or who had experienced severe bronchoconstriction or other adverse reactions at previous attempts, were excluded from sputum induction. The procedure was stopped if the patient displayed more than a 15% fall in FEV₁ at 2 minutes after inhalation of saline (0,9% NaCl) during induction of sputum. Differential cell counts were expressed as percentage of non-squamous cells. Samples with more than 20% squamous cells or with a viability of less than 40% were excluded.

Exhaled NO

Exhaled nitric oxide (eNO) was measured with the NIOX analyzer (Aerocrine AB, Solna, Sweden) according to

ATS guidelines (E12,E13) at a flow rate of exhaled air of 50 mL/second. For each patient at least three correctly executed exhalations with the device were required and the mean value was recorded.

The design of the study with time points for consecutive visits is presented on Figure E1.

RESULTS

The study flow chart is presented on Figure E2. From a cohort of 15 000 to 20 000 asthma patients who were under follow up in the 12 centers of the BIOAIR study close to 800 were invited and 202 gave their written consent to be included in the trial. Of those 33 were screening failures (due to exclusion criteria: current smoking or a history of smoking more than 5 years, pregnancy, history of alcohol or drug abuse, other chronic pulmonary disorders, use of other then corticosteroids immunosuppressants) and 169 were included into the optimization phase. This group was used for further statistical analysis. Twenty three subjects dropped out during the optimization phase mainly due to lack of compliance with study procedures and withdrawn consent thus 146 patients were in 1 year follow up. This phase was concluded by 138 subjects (81.7% of the included cohort). Eight drop outs at this phase were mainly due to lack of follow up. The total dropout rate was 31 patients (18.3% of the included cohort). There were no significant differences in regards of baseline lung function, distribution of severity, age, sex, BMI, atopy, ACQ score, SGRQ score, FeNO, eosinophils, neutrophils and total cell number in induced sputum ($p>0.05$ for all outcomes) between patients with 1 year of follow up and those with lost of follow up.

The change in phenotype during one year of follow up was also compared to the change in ICS dose (Figure E3) and to the number of exacerbations reported during this time (Figure E4). Changes in individual and mean values of FEV1 in severe and mild-to-moderate asthma cohorts are presented on Figures E5 and E6. Changes in individual and mean values of sputum eosinophils and neutrophils in severe and mild-to-moderate asthma cohorts are presented on Figures E7 and E8. Changes in the median and individual doses of ICS (budesonide eq) are presented in Figure E9. Changes in the mean and individual values of asthma control (defined with the use of ACQ), quality of life (defined with the use of SGRQ) and exhaled NO (FeNO) are presented in Figures E10, E11 and E12, respectively. The ICC (95% CI) for asthma control (single measures) was 0.759 (0.700 to 0.813, $p<0.001$) and for average measures was 0.940 (0.921 to 0.956, $p<0.001$). The ICC (95% CI) for quality of life (single measures) was 0.905 (0.870 to 0.934, $p<0.001$) and for average measures was 0.980 (0.971 to 0.986, $p<0.001$). The ICC (95% CI) for exhaled NO (single measures) was 0.624 (0.480 to 0.760, $p<0.001$) and for average measures was 0.892 (0.822 to 0.940, $p<0.001$). These results indicate good reliability of asthma control and quality of life determinations with applied questionnaires. The reliability of FeNO, similarly to other evaluated biomarkers (eosinophils and neutrophils in induced sputum), is significantly lower both for single and repeated measures.

There were in total 70 SA in the physiological phenotype cluster group (55.1% of the group) including 7 SA

(30.4%) in cluster 1 (baseline FEV₁≥68%, maxFEV₁≥108%), 27 SA (46.5%) in cluster 2 (baseline FEV₁≥68%, maxFEV₁<108%, age of onset <40 years), 12 SA (70.6%) in cluster 3 (baseline FEV₁≥68%, maxFEV₁<108%, age of onset ≥40 years), 14 SA (77.8%) in cluster 4 (baseline FEV₁<68%, maxFEV₁≥65%) and 11 SA (100%) in cluster 5 (baseline FEV₁<68%, maxFEV₁<65%). There were in total 35 SA in the “inflammatory phenotype” group (67.3% of the group) including 4 SA (28.6%) in cluster 1 (eos<2%, neu≥40%), 7 SA (70%) in cluster 2 (eos<2%, neu<40%), 9 SA (81.8%) in cluster 3 (eos≥2% and neu ≥40%) and 15 SA (88.2%) in cluster 4 (eos≥2% and neu<40%). In general distribution of clusters based on biomarkers seems to be balanced among clusters based by physiological parameters with exception of cluster 5 (defined by baseline FEV₁<68%, maxFEV₁<65%) where majority of patients (81.9%) are characterized by high levels (≥2%) of eosinophils in induced sputum (Table E2).

Characteristics of the persistently non-eosinophilic patients

Those asthmatic patients that remain persistently non-eosinophilic (defined as less than 2% of eosinophils in induced sputum at baseline and after one year of follow up, cluster 1 and 2 according to Hastie et al) include a sub-group of 29 patients, 19 SA and 20 MA, 17 females (58.6%), with mean age 44.6 years and 26.56 BMI. There are 15 (51.7%) atopic subjects in this cohort (defined as at least 1 positive skin prick test) and 5 past-smokers (17.2%). Those patients are characterized by reasonably good lung function (FEV₁ 88.3%, FVC 73.9%, reversibility 9.74%), by definition low eosinophils in induced sputum (0.47%), 47.97% neutrophils, total cell number 2.74 x 10⁶ cells, low blood eosinophils level (0.28 x 10⁹/L), 4.53 x 10⁹/L neutrophils, 7.45 x 10⁹/L WBC and 27.69 ppb of exhaled NO.

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Table S1. The fixed doses of medications used by patients during treatment optimisation period (V1-V2a). In case the severe asthma patient was taking oral corticosteroid -the dose was unchanged.

	Severe asthma	Mild-to-moderate asthma
inhaled steroids	1600 µg budesonide or 1500 µg beclomethasone, or 1000 µg fluticasone	800 µg budesonide or beclomethasone, or 500 µg fluticasone
LABA	formoterol 9-24 µg b.i.d. or salmeterol 50 µg b.i.d	formoterol 9-24 µg b.i.d. or salmeterol 50 µg b.i.d.
other	short-acting βagonists as needed	short-acting βagonists as needed

Table S2. Relation of biomarkers (eosinophils and neutrophils in induced sputum) to clusters defined by physiological parameters (lung function) and age of onset of the disease.

Allocation to Moore's clusters based on physiological variables	Allocation to Hastie's clusters based on biomarkers (eosinophils and neutrophils in induced sputum)	% of patients	"eos" subgroups	% of patients
Cluster 1 (baseline FEV ₁ ≥68%, maxFEV ₁ ≥108%)	Cluster 1 (low eos, high neu)	25.0%	Low eos (<2%)	41.7%
	Cluster 2 (low eos, low neu)	16.7%		
	Cluster 3 (high eos, high neu)	8.3%	High eos (≥2%)	58.3%
	Cluster 4 (high eos, low neu)	50.0%		
Cluster 2 (baseline FEV ₁ ≥68%, maxFEV ₁ <108%, age of onset <40 years)	Cluster 1 (low eos, high neu)	26.7%	Low eos (<2%)	48.9%
	Cluster 2 (low eos, low neu)	22.2%		
	Cluster 3 (high eos, high neu)	17.7%	High eos (≥2%)	51.0%
	Cluster 4 (high eos, low neu)	33.3%		
Cluster 3 (baseline FEV ₁ ≥68%, maxFEV ₁ <108%, age of onset ≥40 years)	Cluster 1 (low eos, high neu)	9.1%	Low eos (<2%)	45.5%
	Cluster 2 (low eos, low neu)	36.4%		
	Cluster 3 (high eos, high neu)	27.3%	High eos (≥2%)	54.6%
	Cluster 4 (high eos, low neu)	27.3%		
Cluster 4 (baseline FEV ₁ <68%, maxFEV ₁ ≥65%)	Cluster 1 (low eos, high neu)	33.3%	Low eos (<2%)	53.3%
	Cluster 2 (low eos, low neu)	20.0%		
	Cluster 3 (high eos, high neu)	13.3%	High eos (≥2%)	46.6%
	Cluster 4 (high eos, low neu)	33.3%		
Cluster 5 (baseline FEV ₁ <68%, maxFEV ₁ <65%)	Cluster 1 (low eos, high neu)	9.1%	Low eos (<2%)	18.2%
	Cluster 2 (low eos, low neu)	9.1%		
	Cluster 3 (high eos, high neu)	45.5%	High eos (≥2%)	81.9%
	Cluster 4 (high eos, low neu)	36.4%		

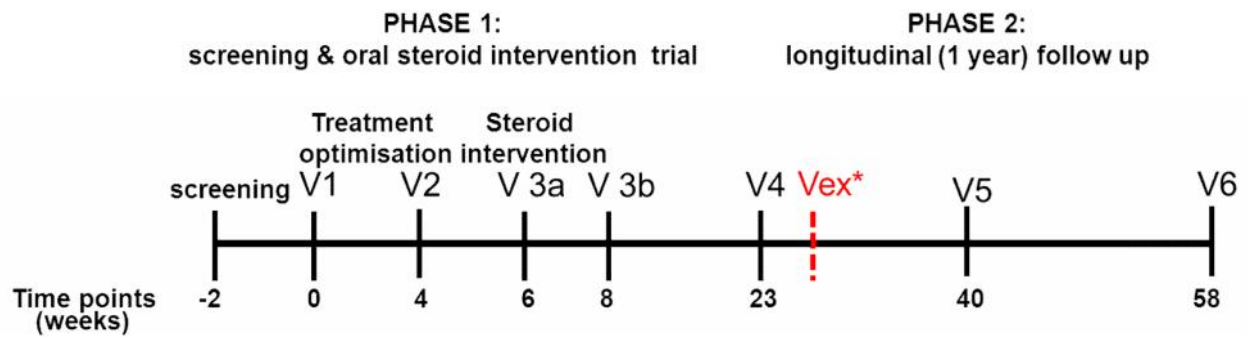


Figure S1. The design of the study (time points in weeks, V-visits, Vex – extra visits due to an exacerbation).

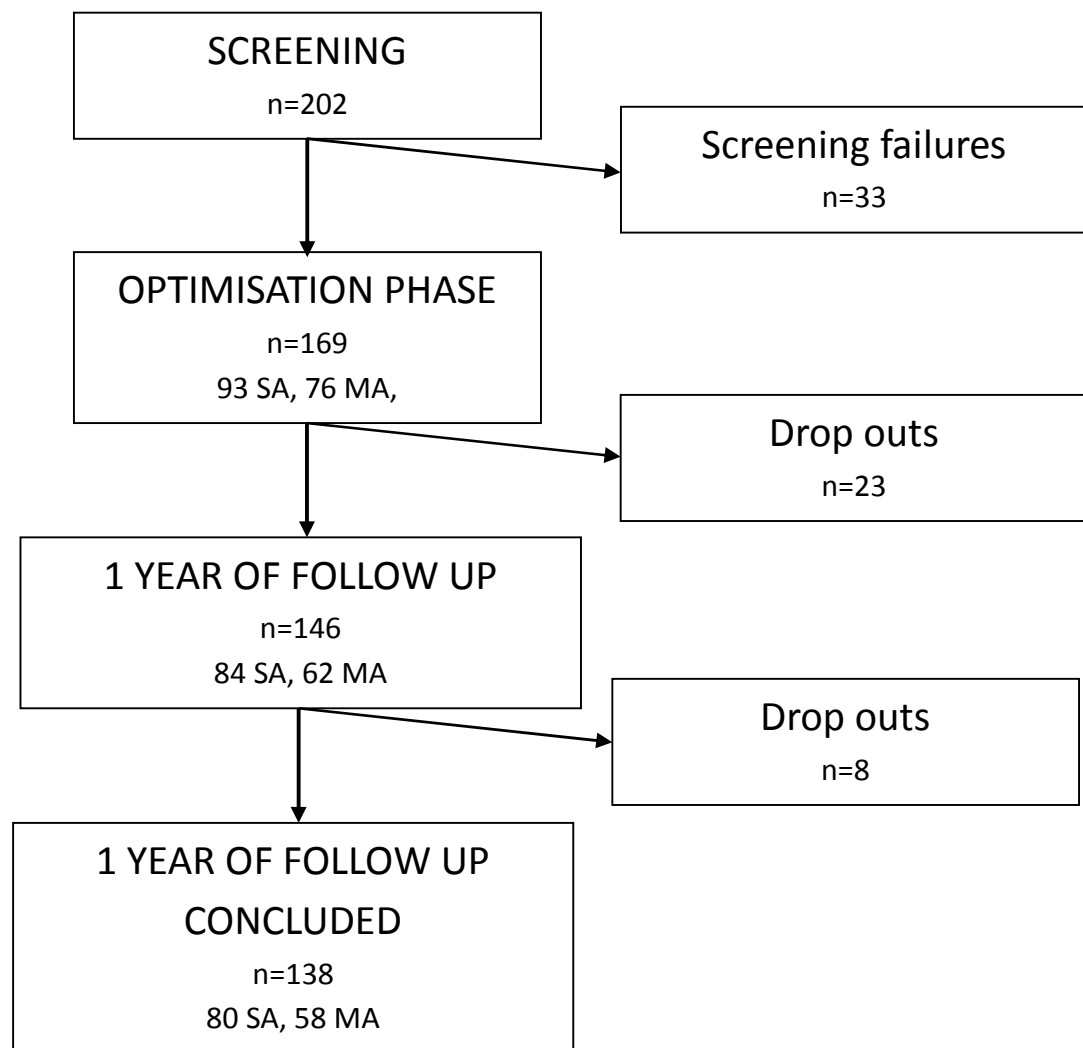
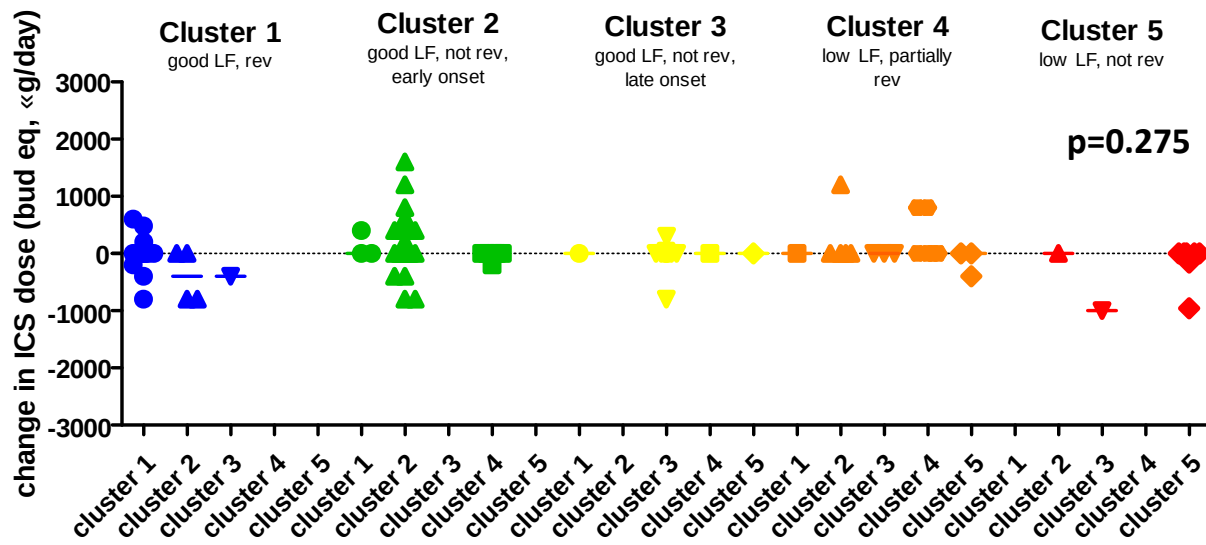


Figure S2. The study flow chart.

A Clusters based on physiological variables (lung function – LF, reversibility – rev) and medical history (age of onset)



B Clusters based on biomarkers (eosinophils – eos and neutrophils – neu in induced sputum)

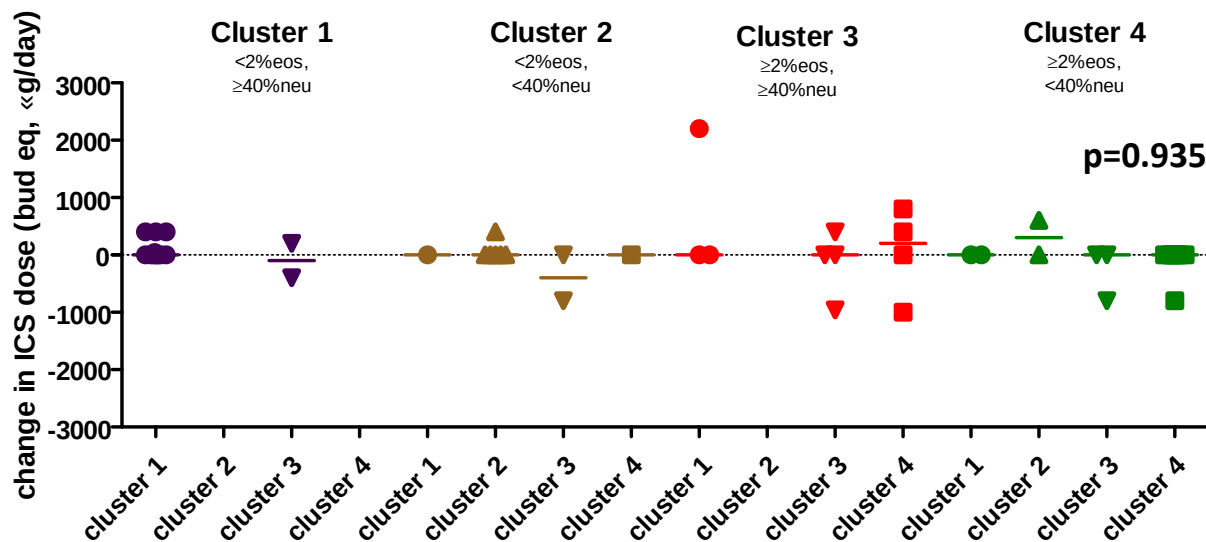
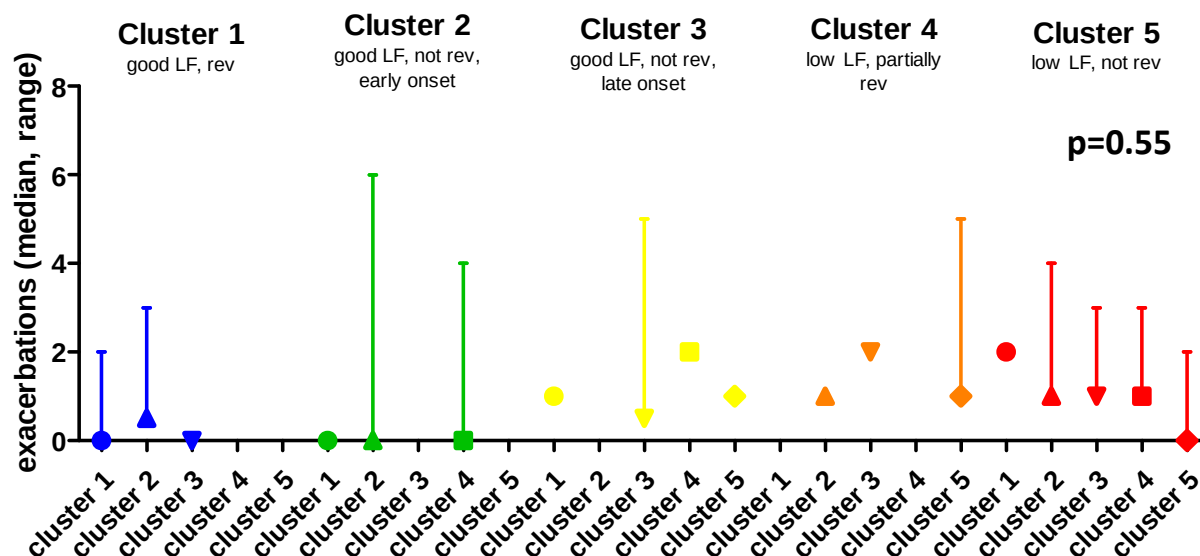


Figure S3. Change in dose of budesonide (eq)(µg/day) for each phenotype change for phenotypes based on physiological variables (panel A) and for phenotypes based on biomarkers (panel B). The phenotype observed at baseline is shown above and the phenotype observed after 1 year of follow up is shown on the x axis. Comparison between groups was made using the Kruskal-Wallis test. There was no significant difference between the groups.

A Clusters based on physiological variables (lung function – LF, reversibility – rev) and medical history (age of onset)



B Clusters based on biomarkers (eosinophils – eos and neutrophils – neu in induced sputum)

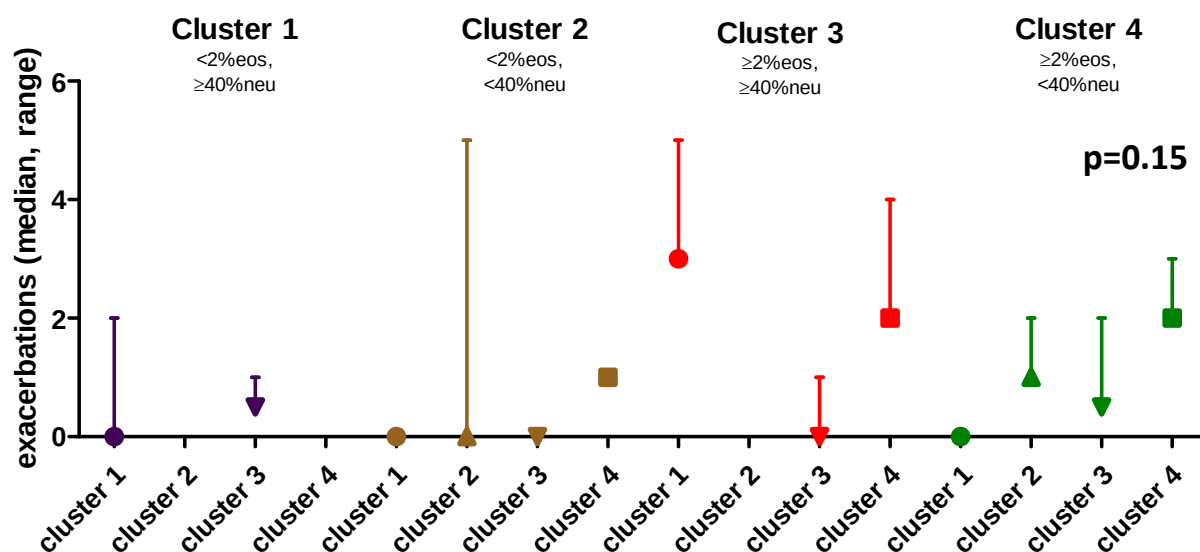
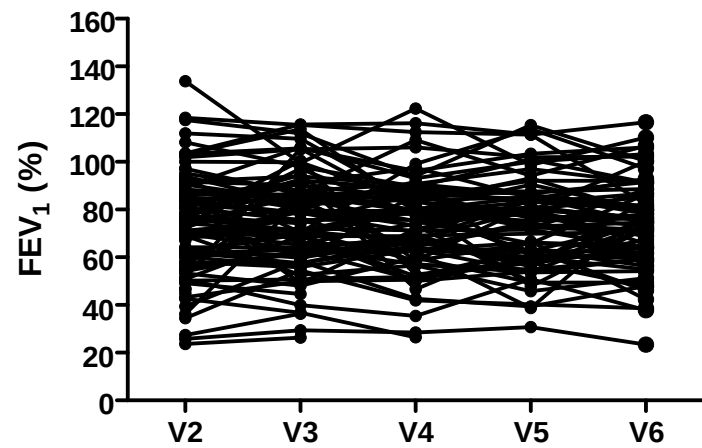


Figure S4. Number of exacerbations (median of events during one year of follow up and range) for each phenotype change for phenotypes based on physiological variables (panel A) and for phenotypes based on biomarkers (panel B). The phenotype observed at baseline is shown above and the phenotype observed after 1 year of follow up is shown on the x axis. Comparison between groups was made using the Kruskal-Wallis test. There was no significant difference between the groups.

A SA FEV₁ % of predicted



B MA FEV₁ % of predicted

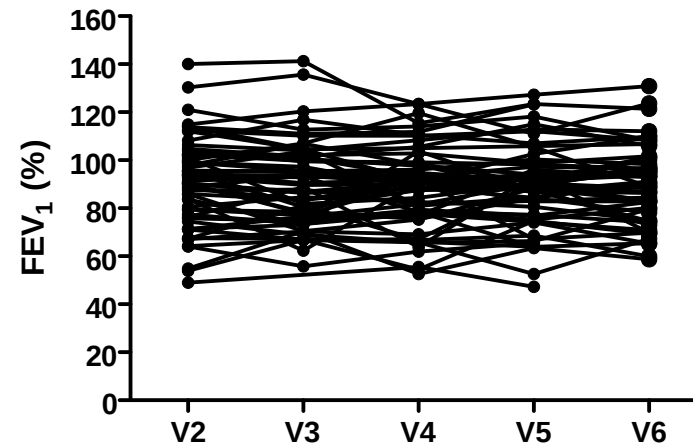


Figure S5. Changes in individual values of lung function measurements (FEV₁ % of predicted) in severe (A) and mild-to-moderate (B) asthma cohorts.

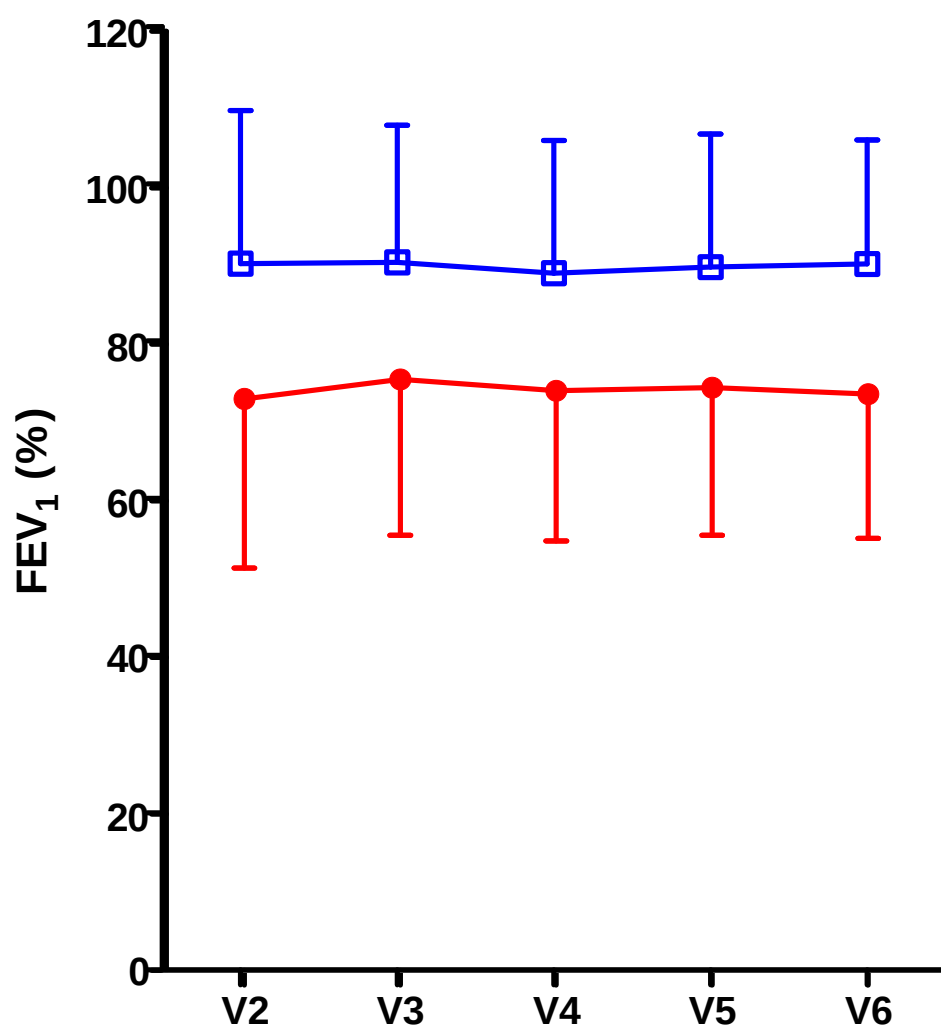
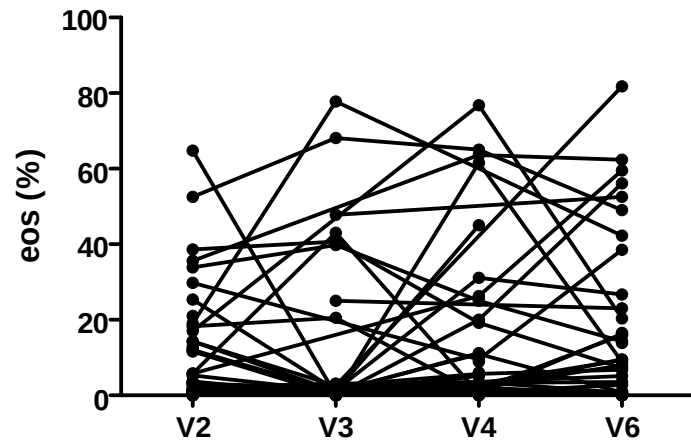
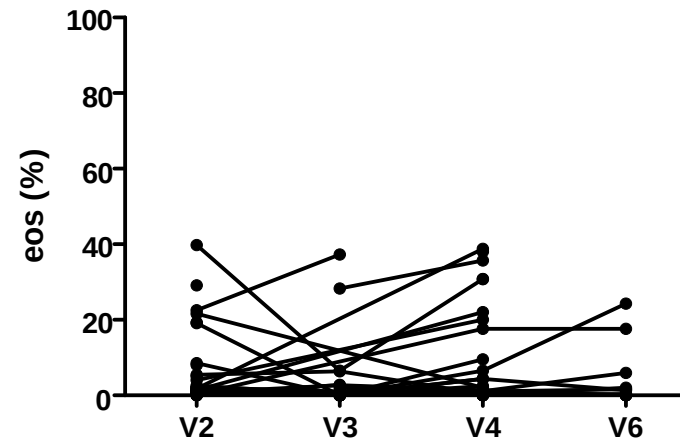


Figure S6. Changes in lung function measurements (FEV₁ % of predicted, mean $\pm$ SD) over the 1 year of follow up in the severe asthma cohort (solid circles) and in the mild-to-moderate asthma cohort (empty squares).

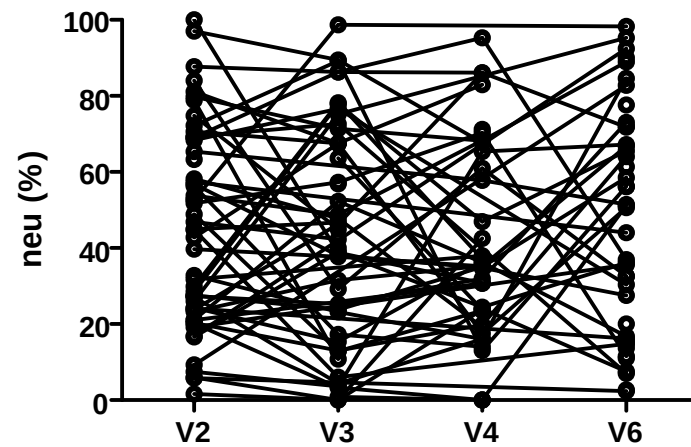
A SA eosinophils



B MA eosinophils



C SA neutrophils



D MA neutrophils

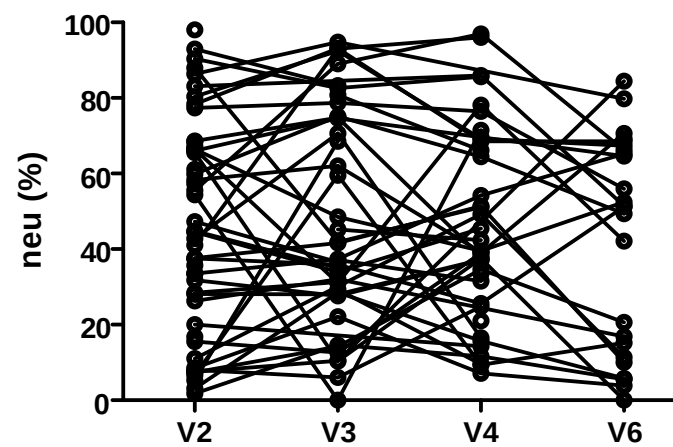


Figure S7. Changes in individual values of eosinophils and neutrophils in induced sputum in severe (SA) and mild-to-moderate (MA) asthma cohorts. Sputum induction was not performed on V5.

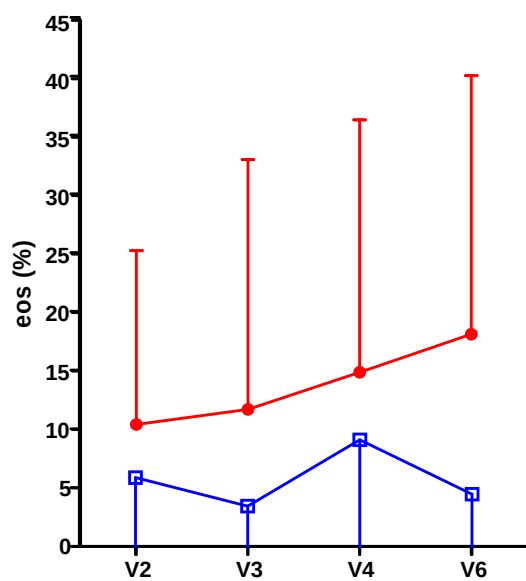
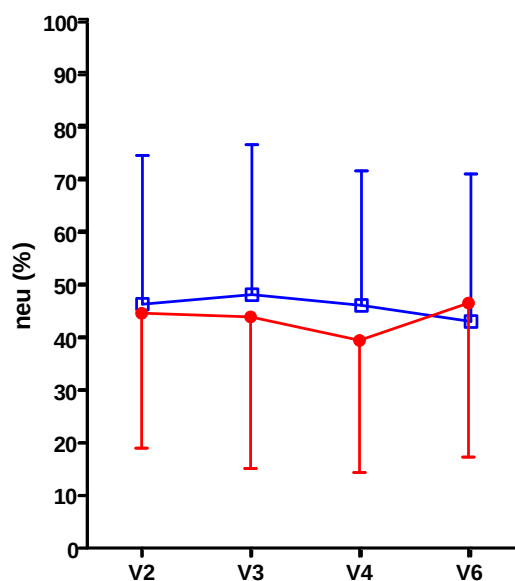
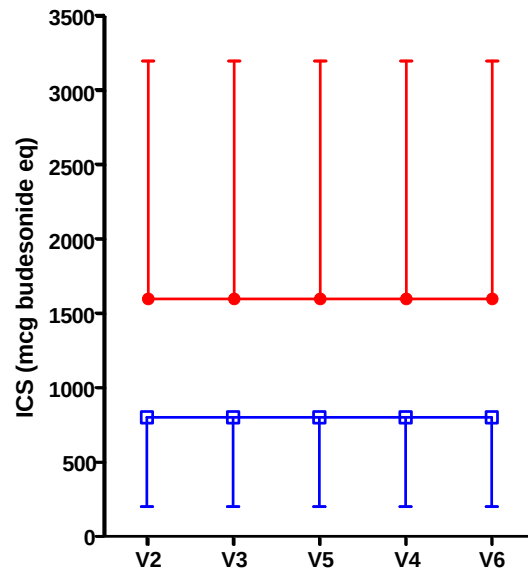
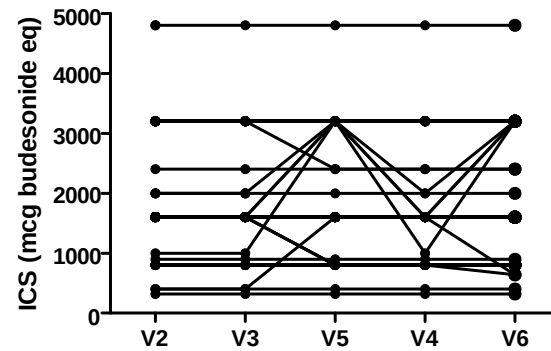
A**B**

Figure S8. Changes in sputum eosinophils (A) and neutrophils (B) (% of cells, mean $\pm$ SD) over the 1 year of follow up in the severe asthma cohort (solid circles) and in the mild-to-moderate asthma cohort (empty squares). Sputum induction was not performed on V5.

A ICS (median±range)



B ICS individual doses SA



C ICS individual doses MA

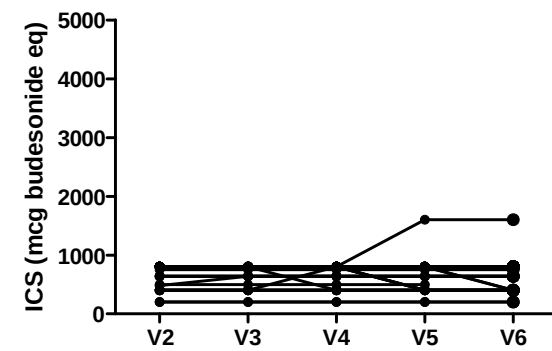


Figure S9. Changes in median dose of ICS (budesonide eq) (A) in severe asthma (solid circles) and mild-to-moderate asthma (open squares). Changes in individual ICS dose (ACQ test) in severe asthma are presented in panel B and in mild-to-moderate asthma – in panel C.

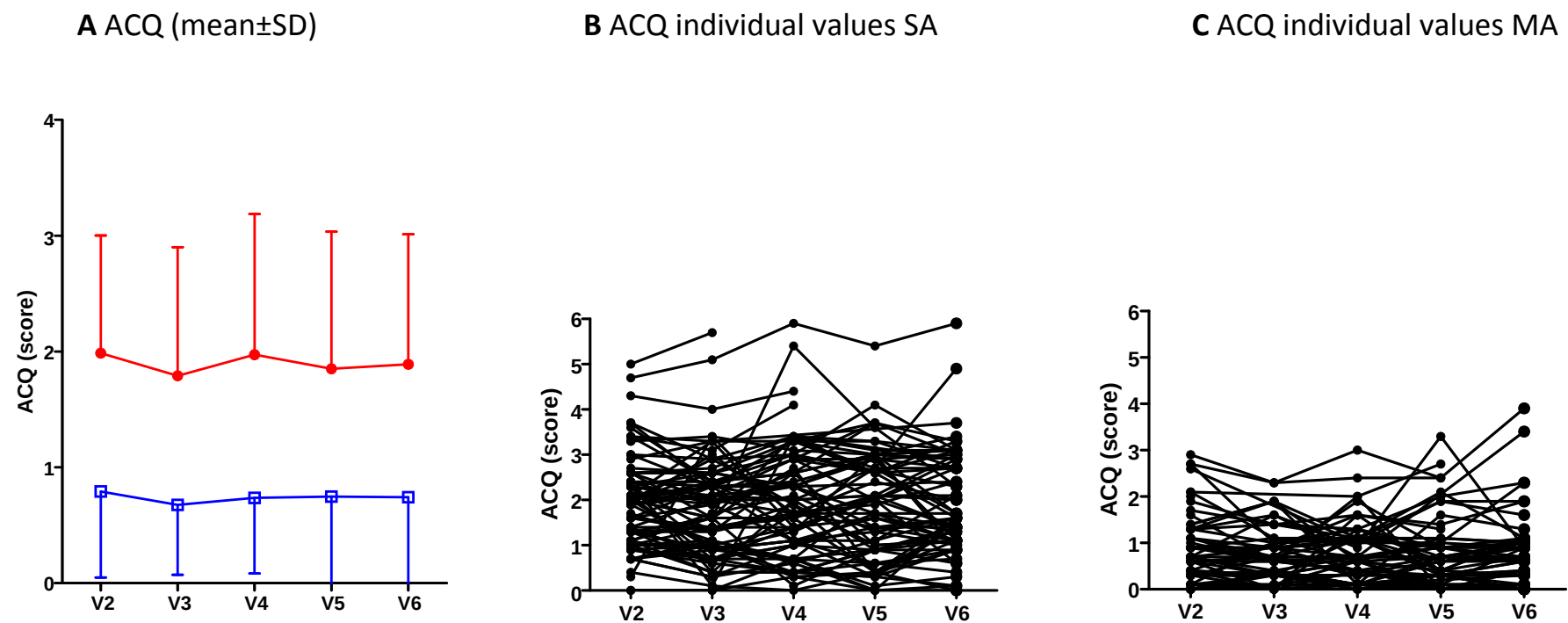
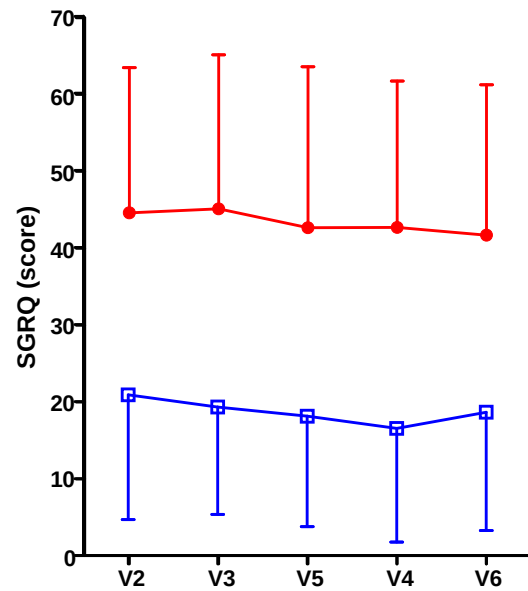
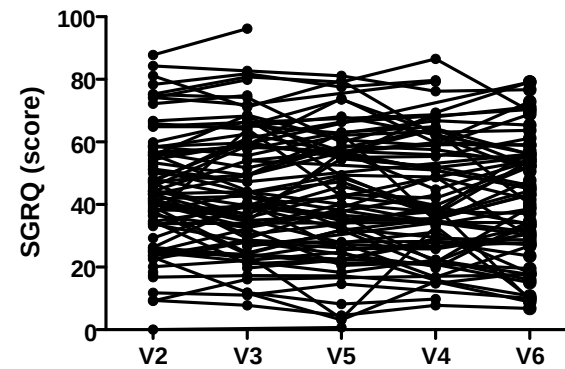


Figure S10. Changes in mean values (A) of asthma control level (ACQ test) in severe asthma (solid circles) and mild-to-moderate asthma (open squares). Changes in individual values of asthma control (ACQ test) in severe asthma are presented in panel B and in mild-to-moderate asthma – in panel C.

A SGRQ (mean $\pm$ SD)



B SGRQ individual values SA



C SGRQ individual values MA

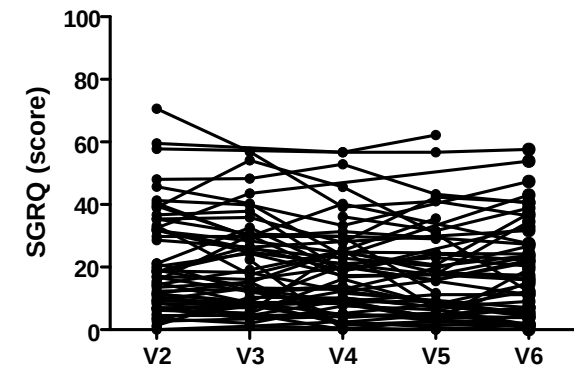
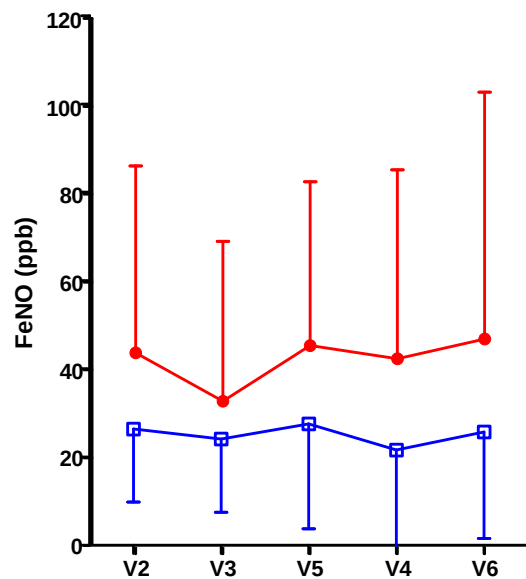
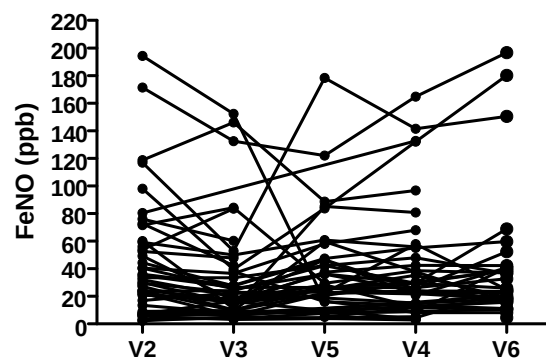


Figure S11. Changes in mean values (A) of quality of life (SGRQ) in severe asthma (solid circles) and mild-to-moderate asthma (open squares). Changes in individual values of asthma control (SGRQ) in severe asthma are presented in panel B and in mild-to-moderate asthma – in panel C.

A FeNO (mean $\pm$ SD)



B FeNO individual values SA



C FeNO individual values MA

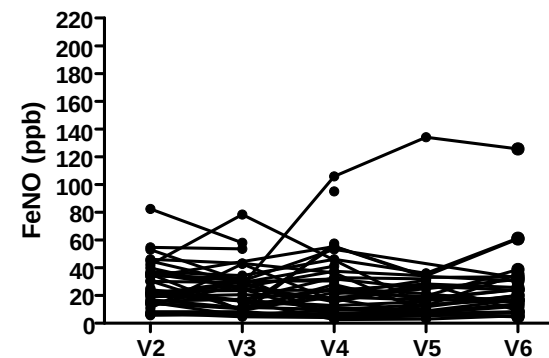


Figure S12. Changes in mean values (A) of exhaled nitric oxide (FeNO) in severe asthma (solid circles) and mild-to-moderate asthma (open squares). Changes in individual values of exhaled nitric oxide (FeNO) in severe asthma are presented in panel B and in mild-to-moderate asthma – in panel C.