

Expression of cPLA₂γ mRNA and protein differs the response of PBMC from severe and non-severe asthmatics to bacterial lipopolysaccharide and house dust mite allergen

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Abstract

Chronic inflammation in asthmatics is initiated/exacerbated by many environmental factors, such as bacterial lipopolysaccharide and allergens. Phospholipase A₂ and histone acetyltransferase/deacetylases are enzymes involved in inflammatory process, particularly in lipid inflammatory mediators production and control of transcription of many inflammatory genes, respectively. The aim of the study was to identify differences in the inflammatory process in patients with severe and non-severe asthma, taking as a criterion expression of two groups of enzymes: phospholipases A₂ and histone acetyltransferases/deacetylases. Thirty-two patients with severe, non-severe atopic to house dust mite asthmatics and 14 healthy volunteers were recruited. Peripheral blood mononuclear cells were stimulated with *Dermatophagoides pteronyssinus* allergen (nDer p1) and bacterial lipopolysaccharide (LPS). The expression of phospholipases A₂ and histone acetyltransferases and deacetylases were assessed using TaqMan Low Density Array Cards. The protein expression was analyzed with immunoblot. Increased expression of phospholipase A2 Group IVC (PLA2G4C) and cytosolic phospholipase A2 gamma (cPLA₂γ) protein was observed in peripheral blood mononuclear cells (PBMC) from severe asthmatics in response to LPS and nDer p1, compared to non-severe asthmatics. nDer p1-stimulated PBMC from severe asthmatics exhibit induced expression of *HDAC1* and similar trend was observed in protein concentration. Decreased expression of *EP300* occurred in PBMC of severe asthmatics. PBMC from non-severe asthmatics showed decreased expression of *HDAC2* and *PLA2G15* after LPS treatment. In conclusion, in response to LPS and dust mite allergen, PBMC from severe and non-severe asthmatics modulate expression of selected phospholipase A₂, histone acetyltransferases and deacetylases, while increased expression of cPLA₂γ characterizes PBMC response from severe asthmatics.

Keywords

allergens, asthma, cPLA₂, HAT, HDAC

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Background

Global Initiative for Asthma (GINA) defines asthma as a heterogeneous disease, usually characterized by chronic airway inflammation. The features of asthma involve a varied severity of clinical symptoms, different hallmarks of the inflammatory process and different responses to commonly used

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treatments. One of the approaches to classification of asthma phenotypes is research based on cluster analysis. It involves the assessment of a given population (in this case asthmatic patients) for many clinical, cellular and molecular features, and then the division of patients into relatively homogeneous groups (clusters) based on the similarities of the analyzed variables.¹ The principal advantage of performing classification numerically is objectivity. Furthermore, this method includes multiple variables that assume equal weighting, which helps minimize *a priori* bias.²

Phospholipases A₂ (PLA₂), present in cells involved in inflammation, participate in the production of lipid inflammatory mediators by releasing arachidonic acid from cell membranes.^{3,4} Both in vitro and in vivo studies indicate secretory phospholipase A₂ from group IIA, V, X, XII, and cytosolic PLA₂: α , β , γ are involved in asthma pathogenesis.^{5,6} Moreover, our previous studies showed that cytosolic phospholipase A₂ is involved in the pathogenesis of asthma.^{7,8} We have previously shown that microsatellite fragments (T)_n and (CA)_n in the promoter region of the cPLA₂ α gene (*PLA2G4A*), although highly polymorphic, are significantly shorter in patients with severe asthma than in healthy subjects.⁷

Bacteria and allergens are the factors that participate most in asthma pathogenesis and exacerbation.^{9–14} Proteins of house dust mite, and among them, Der p1 and Der p2, are major indoor allergens. Der p1 is potent immunomodulator, able to stimulate production of many cytokines in many cells types and might influence the cells phenotype.^{15,16}

Lipopolysaccharide (LPS) is a strong modulator of immunologic reactions – it induces the release of many pro-inflammatory cytokines and mediators responsible for the enhancement of inflammation.¹⁷ LPS also regulates the phospholipases expression and enzymatic activity and is also used as an adjuvant.^{18–20}

Gene expression regulation undergoes through control, involving many transcription factors but also by epigenetic mechanisms DNA methylation and histone acetylation. The acetylation of histones involves lysine residue modification in the tails of histone proteins. Histone acetyltransferases (HAT) are the enzymes that participate in the acetylation process. In turn, histone deacetylases (HDAC), act by removing acetyl residues, resulting in chromatin conformational change and

inhibition of transcription.²¹ An imbalance between HAT and HDAC activities was observed in patients with asthma.^{22,23} In relation to phospholipases, studies show that p300 acetyltransferase is involved in the regulation of *PLA2G4A* expression.^{24,25}

In bronchial biopsies from patients with asthma, an increased level of HAT is observed. Therefore, even a small reduction in HDAC activity, in comparison to normal airways, favors accelerated inflammatory gene expression.^{26,27} HDAC/HAT activities may contribute to glucocorticoid insensitivity which appears in patients with severe steroid-insensitive asthma.

There is evidence that the different HDACs act on different patterns of acetylation, thus regulating the expression of different types of genes. In biopsies from asthmatic patients, an increase in HAT activity and a reduction in HDAC activity occurred, favoring increased inflammatory gene expressions.^{27,28}

The aim of this study was to identify differences among severe, non-severe asthmatic and healthy subjects in the expression of two groups of enzymes – phospholipases A₂ and histone acetyltransferases and deacetylases.

Phospholipases A₂ play a crucial role in the generation of inflammatory lipid mediators, and a group of enzymes with acetyltransferase and deacetylase activity that regulate gene expression by modifying chromatin structure.

Materials and methods

Reagents

The LoTox natural *D. pteronyssinus* allergen 1 (nDerp1) was ordered from Indoor Biotechnologies (Cardiff, UK). The LPS from *Escherichia coli*, serotype R515, was purchased from Enzo Life Sciences (New York, NY). The TaqMan Low Density Array (TLDA) cards, High Capacity cDNA kit and AMI-V medium were obtained from Life Technologies (Carlsbad, CA) and the Histopaque 1077 – from Sigma Aldrich (Saint Louis, MO). The RNeasy Cell Mini Kit with QIAshredder and DNase set were purchased from Qiagen (Hilden, Germany). BCIP/NBT alkaline phosphatase substrate was purchased from Merck Millipore (Darmstadt, Germany). The BCA protein assay kit was purchased from Pierce Thermo Scientific (Rockford, Ill., USA). The rabbit anti-HDAC₁ (catalog number: sc-7872), anti-HDAC₂

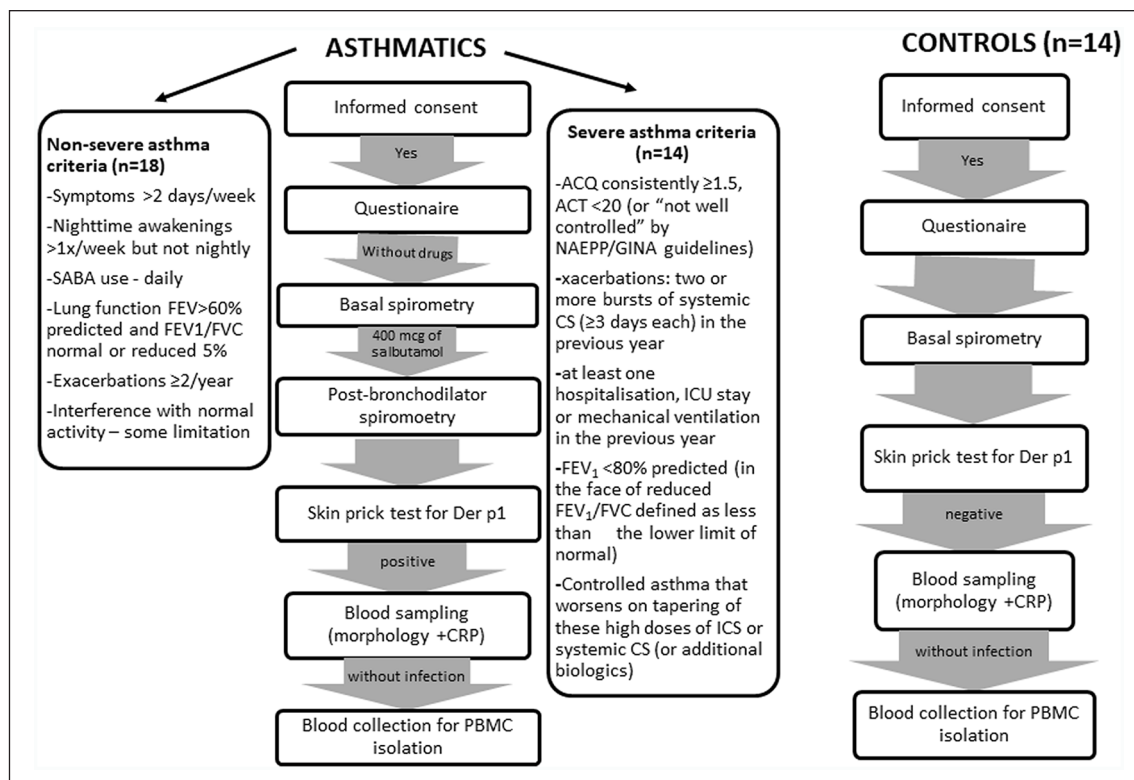


Figure 1. Scheme of subject enrollment procedure.

(catalog number: sc-7899) and anti-p300 (catalog number: sc-585) polyclonal antibodies were obtained from Santa Cruz Biotechnology (Dallas, Tex., USA); the rabbit anti-cPLA₂ polyclonal antibody (catalog number: HPA043083) from Sigma Aldrich (Saint Louis, MO); and the rabbit anti-β-actin monoclonal antibody (catalog number: 4970L) from Cell Signaling (Danvers, Mass., USA).

Patients

Thirty-two patients with severe and non-severe asthma of atopic origin, as well as 14 healthy volunteers, were enrolled into the study. The project, conducted from May 2014 to February 2015, was approved by the *Bioethical Committee* for Research Studies of the *Medical University of Lodz* (ethics approval number: RNN/102/11/KE) and informed written consent was obtained from all the subjects. The subjects were recruited from the Department of Internal Medicine, Asthma and Allergy of the Medical University of Lodz. Asthma had to be diagnosed at least 6 months prior to the study and meet the criteria of the Global Initiative for Asthma²⁹ and severe asthma was defined

according to American Thoracic Society (ATS) directions.³⁰ Figure 1 shows the scheme subject enrollment. Each of the participants were asked to fill a validated questionnaire, part of which was completed by the attending physician.^{31–33} Each of the volunteers had a resting spirometry, skin tests, morphology and CRP (all executed on the same day) and was evaluated by a doctor. All the patients were organized into the appropriate asthma cluster according to Moore's et al.³⁴ criteria. Patients were asked to abstain from systemic GC (glucocorticoids) – 1 month before the test, unless the patient was on stable low doses (up to 5 mg of prednisone) – 24 h without drugs before the test. AH1 (antihistamine drugs), LTRA (leukotriene receptor agonists), LABA (long acting β₂-agonists), LAMA (long acting antimuscarinic) and drugs in combination with inhaled GC – 24h before blood sampling. SABA, SAMA – 6 h before blood collection. Non-asthma medications – 2 weeks prior to the study, long-acting beta agonists hours and short-acting beta agonists before blood drawing. The period of non-administration of oral glucocorticosteroids by patients was determined with a specialist physician and it was determined by the clinical condition of patients with severe asthma.

Table 1. Parameters and characteristics of patients and controls.

	Severe asthmatics (SA)	Non-severe asthmatics (NSA)	Healthy subjects (HS)
(n)	14	18	14
Age	47	38	43
Gender F/M	8/6	10/8	8/6
Atopic to Der p1	14	18	0
FEV1 baseline (%)	47.7* [#]	91.6	107.1
FEV1 max (%)	66.4*	98.7	—
Cluster	4–5	1–2	—
WBC (g/l)	8.1	6.7	6.7
NE (%)	56.3	55.6	55.7
LIMF (%)	26.4	32.4	33.6
MO (%)	8.4	7.7	7.5
EOS (%)	7.9	3.6	2.6
BASO (%)	1	0.7	0.6
HGB (g/dl)	14.3	14.2	13.8
PLT (g/l)	275.9	260.8	262.7
CRP (mg/l)	4.3	2.5	2.2
Glucocorticoids use	High	low	n.a

FEV1: forced expiratory volume; WBC: white blood cells; NE: neutrophils; LIMF: lymphocytes; MO: monocytes; EOS: eosinophils; BASO: basophils; HGB: hemoglobin; PLT: platelets; CRP: C reactive protein.

* $P < 0.05$ in comparison to non-severe asthmatics.

[#] $P < 0.05$ in comparison to healthy subjects.

Moreover, the period of 24 h without the drug is the maximum time allowed by the Bioethics Committee. The issue of drug withdrawal is a common problem in trials involving patients with advanced disease. The wash-out time for a drug is a compromise between the desire to characterize severe asthma and not being able to turn off the treatment for a longer period of time. In the article, we included information on the doses of glucocorticosteroids taken by patients and the period of GCS deprivation to indicate the possibility of the influence of corticosteroids on the observed cell responses. It should be noted that systemic GCs are taken 1 or 2 times a day, so 24 h should be enough not to observe the effect of GCS on peripheral blood leukocytes.

To exclude atopy, the following skin tests were executed to healthy volunteers: mites: *D. peron*, *D. farinae*. Additionally, Artemisia, birch, hazel, alder, grass, cat hair, dog hair, *Cladosporium herba*, and *Alternaria tenuis*.

Detailed characteristics of the patients and controls are shown in Table 1.

PBMC isolation and stimulation

PBMCs were isolated by centrifugation on Histopaque 1077, a density gradient cell separation

medium according to the manufacturer's instructions. Cells were plated on a 24-well plate and cultured in AIM-V medium overnight. The next day, 6×10^6 /ml PBMCs were stimulated in vitro with LoTox nDer p1 (1 μ g/ml) or LPS from *E.coli* serotype R515 (500 ng/ml), or without vehicle (control) for 72 h.

RNA extraction and reverse transcription

Total RNA was isolated from PBMCs using the RNeasy Cell Mini Kit with QIA shredder according to the manufacturer's instructions. RNA was DNase treated, purified, eluted in 30 μ l of RNase-free water and stored at -80°C for further analysis. RNA was then reverse transcribed to cDNA using High Capacity cDNA kit.

TLDA cards

Each card of the low-density array contained eight separate loading ports that fed into 48 separate wells for a total of 384 wells per card. Each 2- μ l well contained a lyophilized TaqMan assay to enable a single gene to be detected. In this study, the TLDA card was configured into eight identical gene sets (*CREBBP*-Hs00231733_m1, *EP300*-Hs00914223_m1, *HDAC1*-Hs02621185_s1,

HDAC2-Hs00231032_m1, *NCOA1*-Hs00186661_m1, *NCOA2*-Hs00896106_m1, *PLA2G2A*-Hs00179898_m1, *PLA2G4A*-Hs00233352_m1, *PLA2G4C*-Hs01003743_m1, *PLA2G5*-Hs00173472_m1, *PLA2G10*-Hs00358567_m1, *PLA2G12A*-Hs00913513_m1, *PLA2G15*-Hs00952741_m1, *SIRT1*-Hs01009005_m1). Each set of 14 genes also contained two reference genes: *18S* (Hs99999901_s1) and *GAPDH* (Hs99999905_m1).

Real time PCR

qPCR was performed as described previously.¹⁰ Briefly, 300 ng of cDNA was used for each port of TLDA. The array was centrifuged for 1 min twice at $306 \times g$ to distribute the samples from the loading port into each well.

The card was then sealed and quantitative PCR (qPCR) was performed using an ABI Prism 7900HT (Applied Biosystems) sequence detection system. RQ Manager 1.2.1 software was used to analyze raw qPCR data. The results were analyzed in comparison to the expression of the reference gene, using Livak's method, and represented as the relative expression of mRNA in the form of $RQ = 2^{-\Delta\Delta C_q}$. The untreated sample was used as a calibrator.

The results are presented as relative expressions to the untreated control.

Immunoblotting

Total proteins from the PBMCs of patients with asthma and healthy controls were extracted in RIPA protein extraction buffer supplemented with protease inhibitor, and the protein concentrations were determined by the BCA protein assay kit. NuPAGE Bis-Tris Gels (Life Technologies) were used to separate proteins (200V, from 110 mA – start to 70 mA – end). Subsequently, the samples were transferred onto a nitrocellulose membrane using the eBlot Protein Transfer System. To block non-specific binding, 5% non-fat dry milk diluted in TBST (0.01% Tween 20 in TBS) was used. Then, membranes were washed three times with TBST and incubated for 12 h at 4°C with primary antibodies and subsequently for 1 h with secondary antibodies at room temperature. After incubation with secondary antibodies and triple washing in TBST once again, colorimetric detection of bands by means of BCIP/NBT Alkaline Phosphatase

Substrate (Millipore) was performed following the manufacturer's instructions.

Densitometric analysis of bands was performed with ImageJ 1.34s software (Wayne Rasband, National Institutes of Health, Bethesda, Md., USA) and the results are presented as fold change in optical density.

Statistical analyses

Data were analyzed using Statistica software (v. 10.0; StatSoft, Tulsa, OK). The distribution of data and the equality of variances were checked by Shapiro-Wilk and Levene's tests, respectively. Significant changes were determined by ANOVA with the appropriate post-hoc tests as multiple comparison procedure. Values of $P < 0.05$ were considered statistically significant.

Principal component analysis (PCA) was performed using the SigmaPlot 13.0. according to its technical manual, with using protein data. PCA is a statistical technique that analyzes a data set in which observations are described by several inter-correlated quantitative dependent variables. In this study, PCA analysis shows chosen gene expressions in relation to asthma severity.

Results

Blood and spirometry parameters

Complete blood count, spirometry and CRP analyses revealed significantly higher forced expiratory volume baseline in severe asthmatics, comparing to non-severe asthmatics healthy volunteers. Other parameters showed no significant differences (Table 1). Cell viability, assessed by trypan blue, was over 90% before stimulation and over 84% – after LPS stimulation.

Gene expression

House dust mite allergen and LPS modulated the expression of selected phospholipase A₂ genes. An increased expression of *PLA2G4C* and a decreased expression of *PLA2G12* was observed in nDer p1-stimulated PBMC from severe asthmatics ($P < 0.05$). Similarly, stimulation with LPS caused an increased expression of *PLA2G4C* gene in severe asthmatics ($P < 0.05$; Figure 2a and b).

Four histone acetyltransferase gene expressions was assessed in our study. We observed that the

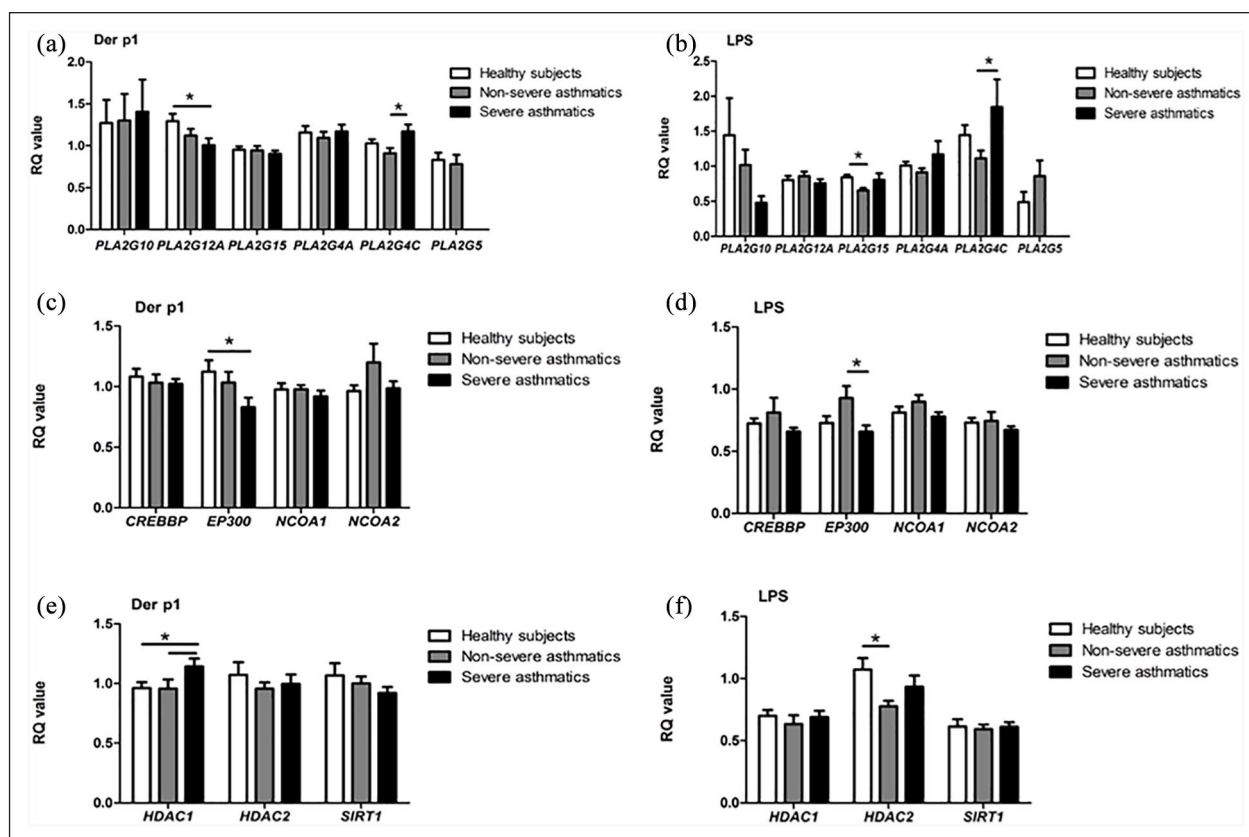


Figure 2. Results of qPCR. Expression of phospholipase A_2 (a, b) histone acetylase (c, d), histone deacetylase (e, f) genes in the PBMCs of healthy subjects (white bar, $n = 14$) and patients with non-severe (gray bar, $n = 18$), severe asthma (black bar, $n = 14$) after stimulation with nDer p1 and LPS have been assessed. Results are presented as the mean value of the RQ $\pm$ SEM. * $P < 0.05$.

stimulation of PBMC in severe asthmatics with nDer p1 – comparing to healthy subjects, and LPS – comparing to non-severe asthmatics, resulted in a decreased expression of *EP300* gene ($P < 0.05$; Figure 2c and d).

Among histone deacetylase genes, a difference in *HDAC1* gene expression was observed between severe asthmatics and other groups after PBMC incubation with nDer p1. Also, the expression of *HDAC2* significantly changed after LPS stimulation ($P < 0.05$; Figure 2e and f).

Protein expression

Based on the qPCR results, we evaluated the concentrations of the selected proteins. The immunoblotting analysis showed that among the phospholipases A_2 , cPLA $_2\gamma$ expression differed between the studied groups and reflected the changes observed on mRNA level (Figure 3a).

The immunoblotting analysis showed a similar trend in the p300 protein concentration (Figure

3d). However, histone deacetylase (*HDAC1* and *HDAC2*) protein concentrations did not differ significantly between the studied groups (Figures 2c and 3b).

Principal component analysis

Results of the PCA of the PBMCs after stimulation with nDer p1 for all subjects revealed that *EP300* variable is negatively correlated with the first component. Its factor loading informs that the correlation between the first principal component and the *EP300* expression equals -0.71 which constitutes 30.8% of the first component. *PLA2G12* has a smaller influence on the first component (25.4%) and is also negatively correlated with it (-0.68). *HDAC1* – similarly to the two previous variables but its influence on the first component is smaller. The correlation of the *PLA2G4* and the first component is the weakest, and the sign of that correlation is positive. The angle between the vectors illustrating the *PLA2G12* and *EP300* (similarly

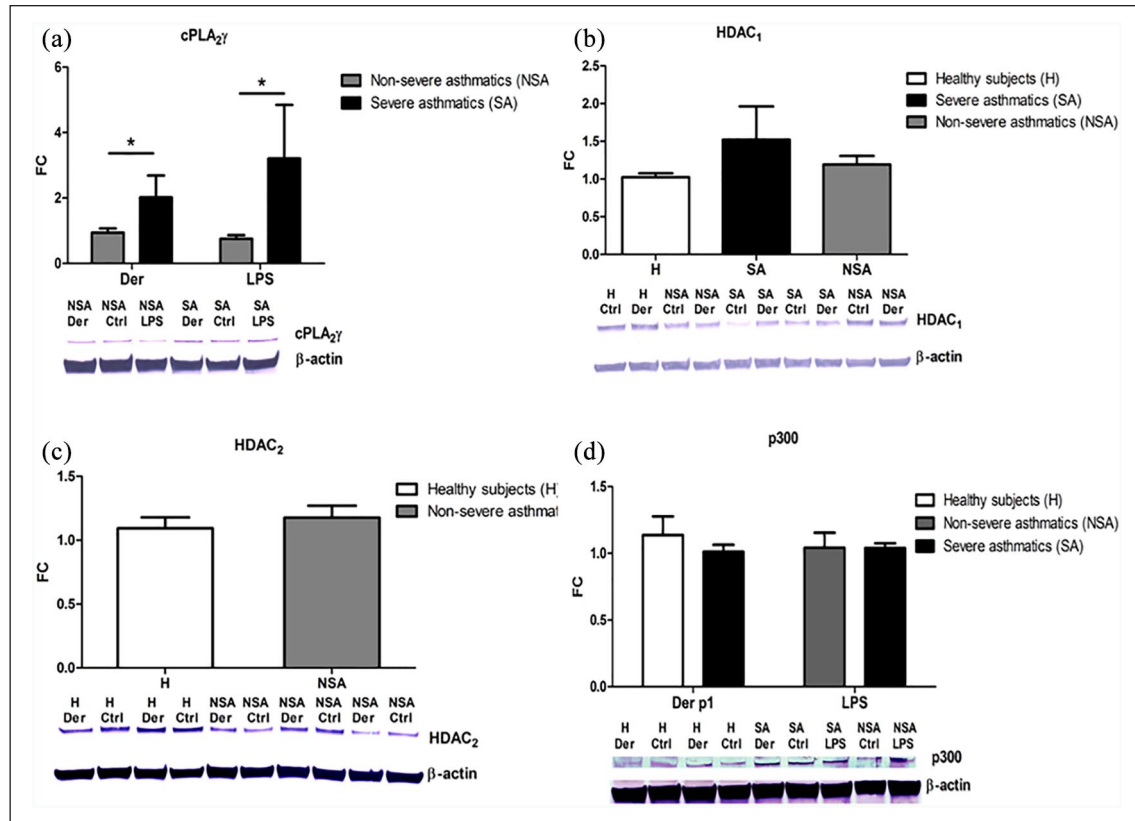


Figure 3. Relative cPLA₂γ (a), HDAC₁ (b), HDAC₂ (c), p300 (d) protein expression. PBMC from severe (black bar), non-severe asthmatics (gray bar) and healthy subjects (white bar) were in vitro stimulated with nDer p1 or LPS. Each sample of all participants was analyzed by Western Blot six times showing similar results, and the blots presented are representatives. The box-plots present densitometry results. Data presented as fold change in comparison to the vehicle-treated cells (control) both normalized to β-actin. Data represent the mean ± SEM.

*P < 0.05 as comparison between studied groups.

EP300 and *HADCI*) is small, which means those variables are strongly correlated (Figure 4).

The results of PCA in the PBMCs after stimulation with LPS for all subjects showed that *EP300* variable is negatively correlated with the first component. Its factor loading informs that the correlation between the first principal component and the *EP300* equals −0.26 which constitutes 3.99% of the first component. *PLA2G12* and *PLA2G4* constitutes 47% of the first component. The second component represents chiefly the original variable – *HDAC1* (51.1%) and *EP300* (42.7%) the remaining original variables are reflected in it to a slight degree. The vectors representing original values almost reach the rim of the unit circle which means they are all well represented by the two initial principal components which form the coordinate system. *PLA2G4* and *PLA2G12* vectors are located close to each other, which suggests that their expression is highly correlated. The vector of the

EP300 and *HDAC1* point to an entirely different direction. They are only slightly correlated with the remaining original values, which are shown by the inclination angle with respect to the remaining original values (Figure 5).

Discussion

In present study we assessed how bacterial lipopolysaccharide and house dust mite allergen influence expression of selected phospholipase A₂ and histone acetyltransferase/deacetylase genes and proteins in studied groups. PBMC from severe asthmatics turned out to be more sensitive to stimuli – in this group, allergen treatment resulted in more explicit modulation, comparing to control group. This might be explained by the fact that on the surface of PBMC, there are PAR receptors, which are involved in Der p1-signal transduction.³⁵ We therefore hypothesized that observed changes

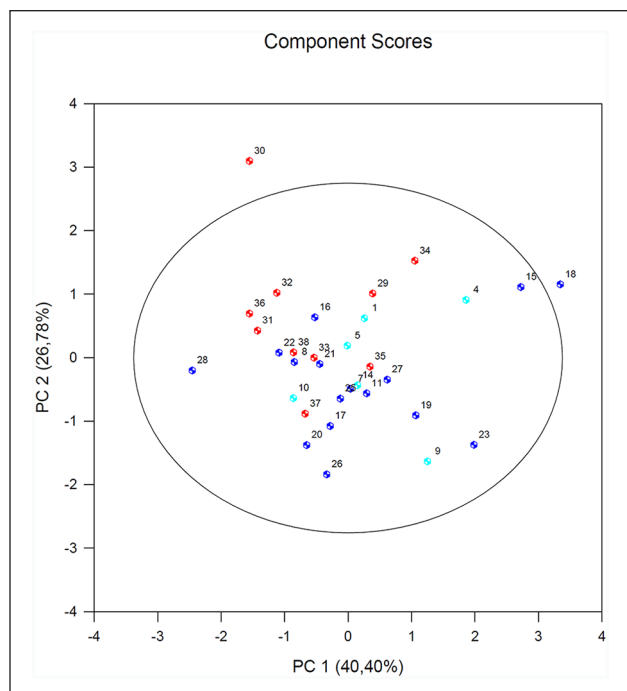


Figure 4. Results of PCA in the PBMCs after stimulation with nDER p1 for all subjects. A biplot of individual subjects according to their scores of the first (PC1) and second (PC2) principal component (with prediction ellipse 95%). The HA group (healthy subjects; 1–13) is marked in turquoise; the NSA group (non-severe asthmatics; 14–28) is marked in navy blue; and the SA group (severe asthmatics; 29–39) is marked in red.

are specific to this allergen action. However further studies with specific PAR inhibitors or siRNA may be needed to understand the mechanism of observed changes.

LPS and allergen-stimulated PBMC of patients with phenotype of severe asthma characterized increased cPLA₂γ gene and protein expression in comparison to non-severe asthmatics. Human cPLA₂γ shares 30% homology and the same amino acids in the catalytic center with cPLA₂α. cPLA₂γ, unlike cPLA₂α, lacks the regulatory phosphorylation sites, thus, the C2 domain is Ca²⁺ –independent and is constantly bound to the cell membrane.³⁶ The enzyme possesses phospholipase A₂ and lysophospholipase/transacylase activities.³⁷ The mechanism of this phenomenon might be connected to phospholipids remodeling, which cPLA₂γ contributes.³⁸ Bickford et al.³⁹ proved that TNF-α induces the cPLA₂γ mRNA and protein expression. This mechanism is important – it highlights the significant role of TNF-α in the pathophysiology of severe asthma and its increased presence in PBMCs and the airway of severe asthmatics.^{39,40}

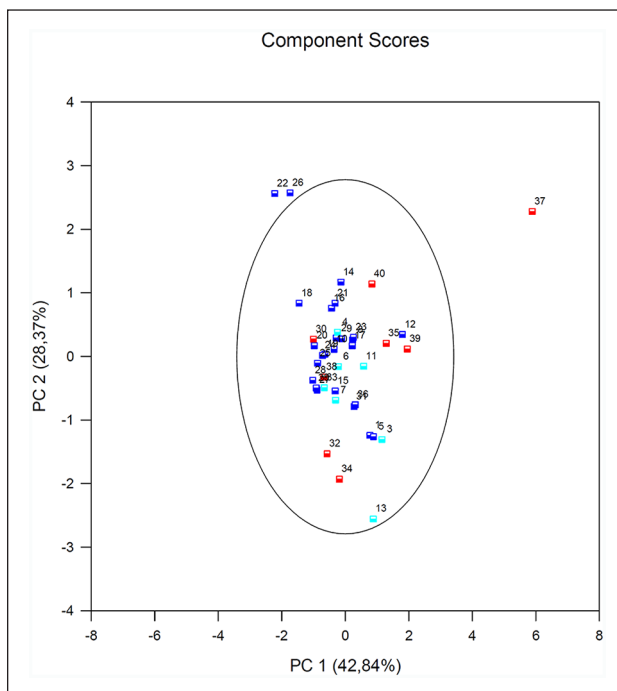


Figure 5. Results of PCA in the PBMCs after stimulation with LPS for all subjects. A biplot of individual subjects according to their scores of the first (PC1) and second (PC2) principal component (with prediction ellipse 95%). The HA group (healthy subjects; 1–13) is marked in turquoise; the NSA group (non-severe asthmatics; 14–28) is marked in navy blue; and the SA group (severe asthmatics; 29–40) is marked in red.

They also³⁹ revealed an increased expression of cPLA₂γ in allergic inflammation in a mouse model of asthma. In our study, the increased expression of cPLA₂γ in severe asthmatics may appear by a mechanism of pro-inflammatory cytokines such as TNF or NF-κB, which may be induced by Der p1/LPS and involved in further cellular mechanism.⁴¹

House dust mite allergen increased expression of the *HDAC1* gene in patients with severe asthma. This might be through the mechanism involving NF-κB, strongly connected with asthma development.⁴² Decreased activity of the HDAC enzyme is associated with increased intensity of the inflammatory process, thus rendering the histone deacetylase as a potential anti-inflammatory drug. In our study, Der p1 induced expression of *HDAC1* in PBMCs. Such action may promote increased airway hyper-responsiveness and bronchi constriction. As severe asthmatics are administrated corticosteroids, which are known to act through HDAC genes, the increased expression of *HDAC1* could be partially due to their action.⁴³ Treatment of animal asthma models with HDAC inhibitors

helped to reduce airway hypersensitivity and remodeling with debatable impact on airway inflammation.⁴⁴ These differences are probably due to the action of different histone deacetylase enzymes.

Both LPS and Der p1 decreased the expression of the *EP300* gene in severe asthmatics. The mechanism of LPS impact on the expression of histone acetyltransferases may be cell-specific and dose-dependent. It is difficult to pinpoint the result of Der p1 and LPS-EP300 interaction because the significant changes were observed only on the mRNA, not protein, level. However, in THP-1 human promonocytes, HAT enzymes were decreased by LPS and acetylated lysine modification was dose-dependent.⁴⁵ Gunawardhana et al.⁴⁶ showed that there were no differences in EP300 and HDACs 1 and 2 expressions in blood monocytes from subjects with asthma.⁴⁷ However, since the authors did not specify the severity of asthma in their study, it is possible that the dissimilarities result from different study group designs. Moreover, these differences in expression were not reflected in activity; thus factors other than HAT or HDAC transcription may be important in this context. An analysis of a larger panel of HAT and HDAC expression might be useful in future studies, as the selection of HATs, HDACs, and their importance in airway inflammation, was mainly based on existing literature.

The PCA revealed that the *PLA2G12* and *EP300* (similarly *EP300* and *HADC1*) variables are strongly correlated, whereas the *PLA2G4* and the *EP300*, *PLA2G12* are only slightly correlated, in the PBMCs after stimulation with nDer p1; the *PLA2G12* and *PLA2G4* variables are strongly correlated and the *EP300* and *HADC1* are only slightly correlated in the PBMCs after stimulation with LPS. PCA showed differences in allergen and LPS action in relation to different PLA₂ isoforms. nDer p1 is more potent in the stimulation of *PLA2G4C* expression in severe asthmatics, whereas LPS induces *PLA2G4C* as well as *PLA2G12* in similar manners. Data obtained from protein expression in PBMCs from severe, non-severe asthma, and healthy individuals were used for PCA analysis. For this, the expression data of four of the thirteen genes tested was used due to the fact that only four genes showed the expression significantly different between groups. Protein data was used, due to the fact that from the biological point of view, it is more important and additionally, the mRNA data is

not always confirmed on the protein level. Despite the observed correlations, studies including more participants are necessary to verify the indicated relationships.

In our study, some data from gene and protein expression is contradictory. The first study indicating the possibility of a correlation between the concentration of mRNA (easy to determine) and protein concentration (difficult to determine) appeared in 2004. However, only approx. 40% of the protein concentration corresponded to the amount of mRNA.⁴⁸ The lack of correlation between mRNA and protein expression may be a result of the time difference of transcription and translation. Furthermore, the process of gene expression is a multistep process, and each of the stages might be responsible for the observed differences in the expression of mRNA and protein (control of transport of mRNA, mRNA stability, translation process control, control of protein degradation). Our study is limited because it did not separately analyze protein concentration in the nucleus and cytoplasm. As HAT and HDAC proteins are more abundant in the nucleus, this type of analysis may be more valuable in the assessment of mRNA and protein correlation. Another limitation is lack of the statistical power calculation, however the results obtained by statistical analyzes performed, allow us to consider that the size of the groups were sufficient.

Asthma is characterized by the presence of persistent inflammation (higher CRP level), hence the inflammatory cells undergo natural priming. The patients undertake specific therapy to manage this. Since we cannot exclude these aspects during study design, they should be taken into consideration in critical analysis of results from molecular studies. Three strategies were used to decrease the negative impact of these factors in our study: first, we carefully selected patients (without exacerbation, without high doses of GC, drug deprivation); second, after the isolation of PBMCs, we incubated them overnight before stimulation with Der p1; and third, we decided to validate the results from gene expression on the protein level. Only for cPLA₂γ, we reported both the elevated gene and protein expression in severe asthmatics. Current literature does not indicate other diseases with cPLA₂γ involvement, except for breast and colon cancer. However, more sophisticated studies are needed to confirm specificity of observed changes to severe asthma phenotype.

In the present study, we used PBMC as an experimental model. Though these cells might only partially reflect the changes occurring in targeted organs, the presence of systemic inflammation in chronic diseases also influences the function of PBMC. Additionally, the changes in the PBMC are easily observable, and they might be easily employed for possible therapeutic solutions.

Conclusion

In conclusion, this project provided differences observed in gene expression of selected phospholipases A₂ in PBMCs of patients with severe and non-severe asthma, compared to a control of healthy volunteers. Enzymes responsible for epigenetic control of gene expression in the pathogenesis of asthma were also evaluated. This project constituted a complex analysis of LPS/Der p1 and phospholipases A₂/histone acetyltransferases/deacetylases interactions and helps to verify existing literature data. This study aimed to explore the molecular responses of bacterial factor (lipopolysaccharide) and house dust mite allergen exposure in those with varying degrees of asthma. The results of the study show that cytosolic phospholipase A₂ gamma (cPLA₂γ) may be the molecule that differentiates the responses seen between different asthmatics.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethics approval

Ethical approval for this study was obtained from Bioethical Committee for Research Studies of the Medical University of Lodz (ethics approval number: RNN/102/11/KE).

Informed consent

Written informed consent was obtained from all subjects before the study.

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Supplemental material

Supplemental material for this article is available online.

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PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

Please indicate the correct answer by putting ✓ or x in the correct ☐ or write the correct answer in the dotted box

White fields are filled in by a volunteer, gray fields are filled in by a doctor

Date of completing the questionnaire			Form no (filled in by the doctor)	
First name		Last name Birth date		
Contact phone number:				
Contact address.....				
Sex W ☐ /M ☐	Age	sex W ☐ /M ☐	Age	BMI.....
Do you smoke cigarettes or other tobacco? ☐ YES, from years, around cigarettes a day ☐ I have smoked in the past (period of abstinence minimum 6 months) years, around cigarettes a day ☐ NO				Pack Years
Has anyone in your family (parents, siblings, grandparents) suffered from asthma or other allergic diseases, if so who? ☐ allergic rhinitis ☐ has diagnosed nasal polyps ☐ allergic conjunctivitis Atopic dermatitis who Other allergic diseases, such as who?				
Thank you, the rest of the section is to be completed only by the doctor				
Performed tests (completed by the doctor):			☐ a sample for genetic tests (3 tubes and 9 ml for EDTA)	
☐ morphology	☐ CRP	☐ morphology	☐ CRP	☐ morphology
Study group (to be completed by a doctor):				
☐ Severe asthma	☐ Severe asthma OMA	☐ Severe asthma	☐ Severe asthma OMA	
FEV1 resting	☐ ≥65% ☐ <65%	FEV1 resting	☐ ≥65% ☐ <65%	
Asthma beginning	☐ <40 y	Asthma beginning	☐ <40 y	

To be completed only by asthma patients

PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

1.	At what age did asthma symptoms appear (wheezing, shortness of breath, cough)		
	☐ <3 y ☐ 3-7 y ☐ 7-16 y ☐ 16-40 y ☐ > 40 y		
2.	The year of asthma recognition (by the doctor):.....		
3.	Since when asthma is severe (persistent symptoms despite treatment, frequent exacerbations, oral or intravenous steroids, hospitalization) year..... ☐ n/a		
4.	In the last 4 weeks:	YES	NO
1)	Have asthma symptoms cough, shortness of breath, wheezing, tightness in the chest (at least one symptom) more than 2 weeks?	☐	☐
2)	Was there any restriction in daily activities due to asthma?	☐	☐
3)	Did you experience asthma symptoms at night: cough, shortness of breath, wheezing, chest tightness (at least one of the symptoms)?	☐	☐
4)	Due to asthma symptoms did the patient use a reliever medication (SABA) more than twice a week?	☐	☐
5)	is the PEF or FEV1 value <80% of the predicted or maximum personal best score?	☐	☐
Asthma control according to GINA			
☐ controlled		☐ partly controlled	☐ uncontrolled

PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

5	Have there been severe exacerbations in the last year (requiring the use of oral or intravenous steroids, ambulance service, help at the Emergency Department or Emergency Room or hospitalization)? ☐ There hasn't been in the last year ☐ There was 1 ☐ There were 2 ☐ There have been 3 or more in the last year He is now																					
<i>If there were severe exacerbations:</i>																						
5a.	How many times during the last year was emergency medical help required (PR, A&E, IP, visit to the clinic with intravenous / intramuscular / nebulized drugs, hospitalization)? (give me a number):.....																					
5b.	Number of additional unplanned medical visits in the last year (please enter the number):																					
5c.	Number of hospitalizations in the last year (provide the number):																					
5d.	Have you had a life-threatening asthmatic incident in the past (need for intubation, adrenaline use, loss of consciousness during an asthma attack due to breathlessness)? ☐ NO ☐ YES																					
.5.	Please list all your CURRENT asthma medications - inhaled and oral <table border="0"> <thead> <tr> <th>Drug name</th><th>Dose</th><th>Times a day</th></tr> </thead> <tbody> <tr> <td>1)</td><td>..... mcg/mg/g/amp</td><td>.....</td></tr> <tr> <td>2)</td><td>..... mcg/mg/g/amp</td><td>.....</td></tr> <tr> <td>3)</td><td>..... mcg/mg/g/amp</td><td>.....</td></tr> <tr> <td>4)</td><td>..... mcg/mg/g/amp</td><td>.....</td></tr> <tr> <td>5)</td><td>..... mcg/mg/g/amp</td><td>.....</td></tr> <tr> <td>6)</td><td>..... mcg/mg/g/amp</td><td>.....</td></tr> </tbody> </table>	Drug name	Dose	Times a day	1)	 mcg/mg/g/amp		2)	 mcg/mg/g/amp		3)	 mcg/mg/g/amp		4)	 mcg/mg/g/amp		5)	 mcg/mg/g/amp		6)	 mcg/mg/g/amp	
Drug name	Dose	Times a day																				
1)	 mcg/mg/g/amp																					
2)	 mcg/mg/g/amp																					
3)	 mcg/mg/g/amp																					
4)	 mcg/mg/g/amp																					
5)	 mcg/mg/g/amp																					
6)	 mcg/mg/g/amp																					
6.	Has the patient been using systemic glucocorticosteroids chronically in the last 3 months (or was it necessary to use them for most days in the period in question)? ☐ NO ☐ YES, <i>dose of prednisone calculaiton</i> mg																					

PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

7.	Does the patient take inhaled glucocorticoids as a single component or a combined drug for maintenance treatment? ☐ NO ☐ YES, <i>a dose of BDP-CFC calculaiton/day mcg/mg</i>					
8	Is the patient taking LABA (ultraLABA) maintenance therapy as a single component or a combination drug? ☐ NO ☐ YES ☐ formoterol ☐ salmeterol ☐ other					
9	Is the patient taking SAMA or LAMA? ☐ NO ☐ YES, ☐ ipratropium ☐ tiotropium ☐ other					
10	Is the patient taking anti-leukotriene drugs? ☐ NO ☐ YES ☐ montelukast ☐ zafirlukast ☐ other					
11.	Is the patient taking anti IgE? ☐ NO ☐ YES <i>the month dose mg</i>					
Treatment intensity according to GINA						
☐ 1 degree		☐ 2 degree		☐ 3 degree		
☐ 4 degree		☐ 5 degree				
12.	Do you suffer from a) for allergic rhinitis b) has nasal polyps c) allergic conjunctivitis d) atopic dermatitis e) other allergic diseases <i>others.....</i>				YES ☐ ☐ ☐ ☐ ☐	NO ☐ ☐ ☐ ☐ ☐

PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

13.	Have you ever experienced any side effects after taking painkillers / anti-inflammatory / antipyretic drugs, e.g. aspirin? ☐ NO ☐ YES, ☐ I am not sure <i>if yes:</i> a) after which drug:..... b) what symptoms..... c) after what time of drug..... d) how many times..... e) when the last time year f) did the doctor diagnosed hypersensitivity to non-steroidal anti-inflammatory drugs ☐ NO ☐ YES, ☐ I am not sure g) Was a provocation with this drug performed by a doctor? ☐ NO ☐ YES, ☐ I am not sure
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PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

Asthma Control Test

1) 1) Over the past 4 weeks, how often did your asthma stop you from performing your usual activities at work, at school, university or at home?

☐ Always (1)

☐ Very common (2)

☐ Sometimes (3)

☐ Rare (4)

☐ No

2) 2) How often in the last 4 weeks have you felt short of breath?

☐ More than once a day (1)

☐ Once a day (2)

☐ 3 to 6 times a week (3)

☐ Once or twice a week (4)

☐ No (5)

3) How often in the last 4 weeks have you been waking up earlier than usual during the night or morning due to asthma-related symptoms (such as wheezing, coughing, shortness of breath, chest tightness or pain) ?

☐ 4 nights a week or more (1)

☐ 2 to 3 nights a week (2)

☐ Once a week (3)

☐ One or two (4)

☐ No (5) 4)

4) How often have you used your fast-acting 'reliever inhaler' in the last 4 weeks?

☐ 3 times a day or more (1)

☐ 1 or 2 times a day (2)

☐ 2 or 3 times a week (3)

☐ Once a week or less (4)

☐ No (5)

5) How would you rate your asthma control over the past 4 weeks?

☐ Not controlled at all (1)

☐ Lately controlled (2)

☐ Moderately controlled (3)

☐ Well controlled (4)

☐ Fully controlled (5)

PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

15.	Result of the test: ☐ 25 points - asthma is fully controlled ☐ 20 - 24 points - asthma is well controlled ☐ ≤ 19 points - inadequately controlled asthma
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PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

Thank you, the rest of the section is to be completed only by the doctor

Wyniki badań dodatkowych alergologicznych				
16.	Skin prick tests: ☐ the study was performed during the visit. ☐ the historical result ☐ positive, including house dust mite from RodzDer.p ☐ positive but negative for house dust mites from RodzDer.p ☐ negative result			
17.	Detailed test results:	positive	negative	not done
	House dust mites Der.p	☐	☐	☐
	House dust mites Der.f	☐	☐	☐
	Pantry mites	☐	☐	☐
	Grasses	☐	☐	☐
	Rye	☐	☐	☐
	Birch	☐	☐	☐
	Hazel	☐	☐	☐
	Alder	☐	☐	☐
	Mugwort	☐	☐	☐
	Cat	☐	☐	☐
	Dog	☐	☐	☐
	<i>Cladosporium</i>	☐	☐	☐
	<i>Alternaria</i>	☐	☐	☐
	Others.....	☐	☐	☐
		☐	☐	☐

PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

18.	Resting spirometry ☐ the examination was performed at the visit ☐ historical result, date..... Was the recommended wash-out times of the drugs observed? ☐ YES ☐ NO, comment..... FEV1 [L]..... FVC [L]..... FEV1 [% wn]..... FVC [%wn] FEV1/FVC..... Diastolic test ☐ the examination was performed at the visit ☐ historical result, date..... FEV1 [L]..... FVC [L]..... FEV1 [% wn]..... FVC [%wn]..... Δ FEV1[%]..... Δ FVC [%]..... Δ FEV1 [ml]..... Δ FVC [ml]..... FEV1/FVC..... Reversible obstruction ☐ YES ☐ NO Diastolic test positive ☐ YES ☐ NO
<i>Dalsza część tylko dla pacjentów chorych na astmę</i>	
19.	Has the patient had a total serum IgE test measured? ☐ NO ☐ YES IU/ml Has the patient been tested for Der.p. specific IgE? ☐ NO ☐ YES IU/ml <i>Class</i>

PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

20.	Diagnosis of bronchial asthma: year of diagnosis Recognized on the basis of (select all items that were relevant in this case) ☐ an interview ☐ a pharmacological test ☐ diastolic test ☐ diurnal variability of PEF ☐ bronchial hyperresponsiveness test ☐ other methods, such as
ATS diagnosis criteria for severe asthma (at least one large and two small)	

PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

1) Therapy with permanent or almost permanent ($\geq 50\%$ of the year) use of oral glucocorticosteroids (high criterion according to ATS 2000)

☐ YES ☐ NO

2) Necessary use of high doses of GCS [$> 1200 \mu\text{g} / \text{d}$ of budesonide or an equivalent dose of another GCS]; (large criterion according to ATS 2000)

☐ YES ☐ NO

a) Need for additional daily treatment with a controlling medication (e.g. LABA, theophylline or LTRA)

☐ YES ☐ NO

b) Asthma symptoms requiring SABA use every day or almost every day

☐ YES ☐ NO

c) Persistent airway obstruction ($\text{FEV}_1 < 80\%$ predicted, daily variability in $\text{PEF} > 20\%$)

☐ YES ☐ NO

d) ≥ 1 visit to the HED / emergency room / emergency room due to asthma

☐ YES ☐ NO

e) ≥ 3 per year of oral GCS insertions

☐ YES ☐ NO

f) Immediate deterioration after $\leq 25\%$ dose reduction of oral or inhaled glucocorticoids

☐ YES ☐ NO

g) A history of a life-threatening asthma attack

☐ YES ☐ NO

21. Does the patient's asthma meet the ATS criteria for severe asthma?

☐ YES ☐ NO

The end

PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

Please indicate the correct answer by putting $\surd$ or x in the correct ☐ or write the correct answer in the dotted box

White fields are filled in by a volunteer, gray fields are filled in by a doctor

Date of completing the questionnaire			Form no (filled in by the doctor)	
First name	Last name.....		Birth date.	
Contact phone number:				
Contact address.....				
sex W ☐ /M ☐	Age	Weight (kg)	Height (cm)	BMI
Do you smoke cigarettes or other tobacco? ☐ YES, from years, around cigarettes a day ☐ I have smoked in the past (period of abstinence minimum 6 months) years, around cigarettes a day ☐ NO				Pack Years
Has anyone in your family (parents, siblings, grandparents) suffered from asthma or other allergic diseases, if so who? ☐ allergic rhinitis ☐ has diagnosed nosakto polyps ☐ allergic conjunctivitis Atopic dermatitis who Other allergic diseases, such as who?				
Thank you, the rest of the section is to be completed only by the doctor				
Performed tests (completed by the doctor):			☐ a sample for genetic tests (3 tubes and 9 ml for EDTA)	
☐ morphology	☐ CRP	☐ resting spirometry	☐ diastolic	☐ skin tests
Study group (to be completed by a doctor):				
☐ Severe asthma	☐ Severe asthma OMA	☐ Mild asthma	☐ healthy volunteer	
FEV1 resting	☐ $\geq 65\%$ ☐ $< 65\%$	FEV1 after diastole	☐ $\geq 108\%$ ☐ $< 108\%$ ☐ $\geq 65\%$ ☐ $< 65\%$	
Asthma beginning	☐ < 40 y	☐ ≥ 40 y	☐ n/a	

PROJECT: Environmental factors as modifiers of the expression of genes involved in the pathogenesis of bronchial asthma

Results of additional allergological tests				
16.	Skin prick tests: ☐ positive, including house dust mite from RodzDer.p ☐ positive but negative for house dust mites from RodzDer.p ☐ negative result			
17.	Detailed test results:	positive	negative	not done
	House dust mites Der.p	☐	☐	☐
	House dust mites Der.f	☐	☐	☐
	Pantry mites	☐	☐	☐
	Grasses	☐	☐	☐
	Rye	☐	☐	☐
	Birch	☐	☐	☐
	Hazel	☐	☐	☐
	Alder	☐	☐	☐
	Mugwort	☐	☐	☐
	Cat	☐	☐	☐
	Dog	☐	☐	☐
	<i>Cladosporium</i>	☐	☐	☐
	<i>Alternaria</i>	☐	☐	☐
	Others.....	☐	☐	☐
		☐	☐	☐
18.	Resting spirometry ☐ the examination was performed at the visit ☐ historical result, date..... Was the recommended wash-out times of the drugs observed? ☐ YES ☐ NO, comment..... FEV1 [L]..... FVC [L]..... FEV1 [% wn]..... FVC [%wn] FEV1/FVC.....			
<i>The end</i>				