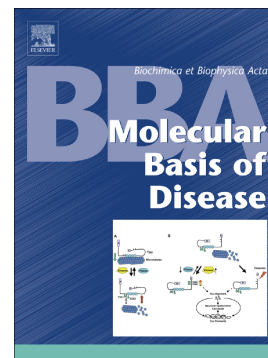


Plasma based targeted metabolomic analysis reveals alterations of phosphatidylcholines and oxidative stress markers in guinea pig model of allergic asthma

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Title: Plasma based targeted metabolomic analysis reveals alterations of phosphatidylcholines and oxidative stress markers in guinea pig model of allergic asthma

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- Targeted metabolomics analysis of plasma differentiates ovalbumin sensitised guinea pigs from naïve controls.
- Alterations in the phosphatidylcholines profiles, carnitine levels and metabolites involved in inflammation and oxidative stress were identified.
- The ROC curve analysis using selected metabolites showed good sensitivity and selectivity to detect eosinophilic inflammation in guinea pigs.

Abstract:

Bronchial asthma is one of the most common, chronic respiratory diseases, characterized by reversible airway obstruction, eosinophil and Th2 infiltration, airway hyperresponsiveness and airway remodelling; with many cells and mediators involved. Metabolomics is a relatively new field in “omics” sciences enabling the identification of metabolome for better diagnostics and studying of diseases phenotype.

The aim of this study was to investigate the role of targeted metabolomics study for better understanding of the bronchial asthma pathophysiology and finding potential biomarkers in experimental models of eosinophilic inflammation.

Plasma level of 185 metabolites was measured with the AbsoluteIDQ™ p180 kit in guinea pigs with experimentally-induced allergic inflammation (n= 15) compared to naïve non-sensitised and non-challenged controls (n= 18).

Of the 185 metabolites identified in plasma, 22 were significantly different and changed in ovalbumin sensitised animals. Plasma level of 13 phosphatidylcholines with saturated and unsaturated long-chain fatty acids, total phosphatidylcholines count, carnitine, symmetric dimethylarginine and its ratio to total unmodified arginine, and kynurenine to tryptophan ratio were found to be decreased, while total phosphatidylcholines, phospholipase A2 activity indicator, tryptophan, taurine and ratio of methionine sulfoxide to unmodified methionine were found to be increased in sensitised guinea pigs compared to naïve controls.

Targeted metabolomic analysis revealed significant differences in plasma metabolome of sensitised guinea pigs. Our observations point to the activation of inflammatory and immune pathways, as well as the involvement of oxidative stress.

Keywords: allergic inflammation, animal model, asthma, guinea pigs, plasma metabolome, targeted metabolomics.

The prevalence of allergic airway diseases is rapidly increasing worldwide in all age groups. Allergic asthma is a respiratory disease characterized by airway hyperresponsiveness, increased mucous secretion, oedema and airway remodelling, as well as by acute and chronic inflammation [1,2]. Management of asthma is principally directed to suppress airway inflammation with inhaled corticosteroids and to relieve bronchoconstriction using bronchodilators [3]. In spite of relatively effective management of the treatment, there is a crucial need to identify and better understand the underlying basis of asthma, understand the complex genetic and environmental influences, and develop appropriate treatment strategies. However, for obvious ethical reasons, the experiments required to distinguish accurately mechanisms that are involved at cellular or molecular level, are not [4] possible to do with human subjects. Thus, animal models of asthma have been used over decades [5]. There are many animal species which have been used in asthma models including *Drosophila*, mouse, rat, guinea pig, cat, dog, sheep, horse and primate [6–8]. Guinea pigs can be considered as an ideal model that mimics the physiological abnormalities of asthma in humans for many reasons: developed a well-characterized early and late asthmatic reactions, the response to the treatment similar to humans, possibility of sensitisation to allergen via inhalation and systemically, and involvement of both eosinophils and neutrophils during the late asthmatic reaction [9–11].

In recent decades, the “omics” disciplines including genomics, epigenomics, transcriptomics and proteomics have emerged as important tools for better understanding of pathophysiological mechanisms in many diseases [12]. One of the youngest siblings in the “omics family” is metabolomics – a comprehensive analysis of small molecule metabolites (<1 kDa) which are intermediates or products of metabolism, e.g. amino acids, carbohydrates, fatty acids, biogenic amines and many other classes of compounds [13]. There are two main approaches in metabolomics; untargeted metabolomics, which is intended for a comprehensive analysis of the all measurable analytes in a sample including unknown chemicals, and targeted metabolomics, i.e.

annotated metabolites [14]. For both types of analyses, two major techniques are used to gather metabolomics data, mass spectrometry (MS) coupled with liquid chromatography (LC) or gas chromatography (GC), and nuclear magnetic resonance (NMR; [15]. Metabolomics studies generate large amount of data; therefore, various types of data analysing and data processing approaches are used. Most of metabolomics studies are interpreted using various multivariate statistical methods to analyse and interpret metabolomics data [16]. However, for biomarker analysis it is important to use different approaches, based especially on “machine learning” methods [17].

The rising popularity of metabolomics and its applicability is observed in almost every field of biomedical research and investigation of asthma is not an exception. In recent years, there has been an increasing number of studies aimed at the identification of metabolic disturbances of asthma in experimental conditions using various animal models, as well as in human studies [18]. The results of asthma metabolomic studies performed in humans should be carefully considered because metabolome could be influenced by many intrinsic and extrinsic factors [19]. Thus, for a better understanding of pathophysiology, and eventually for a deeper understanding of drug actions on metabolome, the best choice is to study metabolome in asthma animal models. Recent animal metabolomic studies have mainly used asthma murine models, and LC-MS employed approached for untargeted analysis of bronchoalveolar lavage fluid (BAL; [20], serum [21], plasma [20,22], and lung tissue [23,24].

To date, few metabolomic studies mentioned above have been performed with asthma or asthma model, but the vast majority of them utilized targeted approach or used the limited classes of metabolites. In this study, targeted metabolomic analysis of plasma samples from ovalbumin-sensitised guinea pigs (animal model of allergic asthma) and non-sensitised naïve guinea pigs was used to identify metabolomic changes in the model of experimental allergic asthma. The targeted metabolomic approach used in our study covers six classes of metabolites, which have a potential

results of our study could be interpolated in asthma patients and after further testing could be used for better diagnostics.

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Animals

The study protocol of experiments was approved by the local Ethics Committee of the Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovakia and by the National Veterinary Board, Slovakia. All experiments were performed in accordance with guidelines for the protection of animals used for scientific-educational purposes.

Male, four weeks old guinea pigs of TRIK-strain, weighing 250–350 g, purchased from the Department of Experimental Pharmacology of the Slovak Academy of Sciences in Dobra Voda (Slovakia), were used in the study. Guinea pigs were kept in an animal house to undergo a 7-days process of acclimation and quarantine. Animals had unlimited access to adequate food and water *ad libitum* during the acclimation process.

Animals were randomly divided into 2 groups: control group (n=18; naïve, non-sensitised guinea pigs) and model group (n=18; sensitised group).

Ovalbumin-induced airway hyperresponsiveness

The sensitisation of animals by ovalbumin was performed according to our previously described method [25]. Briefly: ovalbumin (Sigma Aldrich, Germany) was dissolved in *aqua pro injectione* at 1% concentration and administered on the 1st day of sensitisation intraperitoneally (0.5 mL) and subcutaneously (0.5 mL), and on the 3rd day intraperitoneally (1.0 mL). The sensitisation was followed by inhalation challenge by 1% ovalbumin dissolved in saline on the 14th and 21st day. During sensitisation and allergen challenge three guinea pigs died due to anaphylactic reaction. Thus, the final number of animals in the model group for further testing was 15. The same procedure was done with animals in the control group but only vehicle (*aqua pro injectione* and saline) was administered.

The specific airway resistance (sRaw) was considered as a marker of airway reactivity in *in vivo* conditions and was measured using double-chamber whole-body plethysmograph for small laboratory animals (HSE type 855; Hugo Sachs Elektronik, Germany). The method is based on a measurement of the time delay between the pneumotachometer signals from thoracic and nasal chambers, whereas the volume changes in the nasal and thoracic chambers were independent. This method was previously described by Pennock et al. [26]. The specific airway resistance was calculated from a phase displacement between the two chambers and was evaluated after 2 min inhalation of histamine (Sigma Aldrich, Germany) at the concentration of 10^{-6} mol/L in saline, which served as a bronchoconstriction agent on the 14th and 21st day of sensitisation 5 hours after the challenge with ovalbumin. The specific airway resistance measured after saline inhalation was used as a benchmark. During the intervals between inhalations, fresh air was insufflated into the nasal chamber of plethysmograph. The results of a specific airway resistance were evaluated by special software (HSE Pulmodyn Pennock, Hugo Sachs Elektronik, Germany). Contribution of mucosal oedema and changes in epithelial secretion to specific airway resistance could lead to misinterpretation of these data; therefore, tissue samples from lungs and trachea were analysed in *in vitro* conditions.

***In vitro* airway reactivity evaluation**

After sensitisation and challenge by ovalbumin, on the 22nd day of sensitisation, the animals were overdosed by intraperitoneal administration of the combination of following anesthetics: tiletamine and zolazepam (ZOLETIL, at the dose of 20 mg/kg b.w.), and xylazine (XYLARIEM, at the dose of 20 mg/kg b.w.). Subsequently, the animals were killed by exsanguination, and trachea with lungs was excised immediately. The *in vitro* airway reactivity was measured in organ baths using multi-chamber tissue bath systems (Experimetria-ISO-09-TSZ8, Hungary). Tracheal strips (approximately 15 mm) were cut on the opposite side of the smooth muscle. Lung tissue strips ($2 \times 2 \times 15$ mm) were prepared from the margin of upper lobes of the left lungs. The strips

buffer (NaCl 110.00 mmol/L, KCl 4.80 mmol/L, CaCl₂ 2.35 mmol/L, Mg₂SO₄ 1.20 mmol/L, KH₂PO₄ 1.20 mmol/L, NaHCO₃ 25.00 mmol/L, and glucose 10.00 mmol/L; in distilled water). The temperature of buffer was maintained at $37.0 \pm 0.5^{\circ}\text{C}$ and aerated continuously with a mixture of 95% O₂ and 5% CO₂ to maintain pH 7.5 ± 0.1 . One of the hooks was connected to a force transducer (EXP, Experimetria, Hungary) and an amplifier (EXP CLSG-4, Experimetria, Hungary), and the tension changes were recorded continuously using specialized computer software (SPEL Advanced Iso Sys v3.2, Experimetria, Hungary). The initial tension of tissue strips was set at 4 grams for 30 min (loading phase). Afterwards, the tension of each strip was readjusted to a baseline value of 2 grams for next 30 min (adaptation phase). The tissue strips were washed at 10 min intervals with the pre-heated Krebs-Henseleit buffer during both periods. The contractile response (in grams) to cumulative doses (10^{-8} – 10^{-3} mol/L) of histamine was considered as a marker of *in vitro* airway smooth muscle reactivity after the adaptation phase had finished.

Assessment of haematological parameters and selected interleukins

Samples of the blood were taken to EDTA K₂ tubes directly from the heart after overdosing the animals with anaesthetics during the exsanguination phase. The blood samples were aliquoted and one aliquot was used for haematological parameters assessment by veterinary haematologic analyser Sysmex XT-2000i (Sysmex, Sweden). Rest of aliquots were centrifuged at $2500 \times g$ for 10 min at 4°C and plasma were stored as 100 μL aliquots at -80°C until metabolomic analysis. Bronchoalveolar lavage (BAL) fluid of the right lung was performed twice using instillation and immediate suction of pre-heated saline at 37°C at a ratio 0.01 mL/g of body weight. The total leukocyte count and differential leukocytes counts in the BAL fluid were evaluated same as the blood samples. Standard ELISA method with commercially available kits for guinea pigs (Antibodies-online Inc., USA, Cusabio Life Sciences, USCN Life Sciences Inc., Blue Gene Biotech) was used for determination of plasma concentrations of selected cytokines involved in

differentiation (IL-4).

Targeted metabolomic analysis

For the determination of plasma metabolites, a targeted metabolomics approach with the AbsoluteIDQ™ p180 kit (BIOCRATES Life Science AG, Austria) according to the manufacturer's instructions was used. This approach allowed the simultaneous quantification of a total of 185 metabolites covering the following compound classes: 42 amino acids and biogenic amines, 40 acylcarnitines, 88 glycerophospholipids (consisted from lysophosphatidylcholines-LPCs and phosphatidylcholines-PCs), 14 sphingolipids and a sum of hexoses (including glucose). The analytical system consisted of liquid chromatography ACQUITY UPLC™ I-Class (Waters, Czech Republic), composed of flow-through-needle sample manager (FTN-SM), binary solvent manager (BSM) pump and column manager (CM) coupled with XEVO TQ-S triple quadrupole mass spectrometer (Waters, Czech Republic).

Briefly, after the addition of 10 µL of supplied internal standard solution to each well on filter spot of the 96-well extraction plate, 10 µL of each plasma sample, quality control (QC) samples or calibration standard were added to appropriate wells. The plate was then dried under a gentle stream of nitrogen. Subsequently, amino acid and biogenic amines were derivatized with phenyl isothiocyanate (Sigma Aldrich, Germany), and dried again. Metabolites extraction was performed with 5 mM ammonium acetate in methanol (Sigma Aldrich, Germany). Final extracts were analysed after appropriate dilution by LC-MS/MS using the ACQUITY UPLC™ BEH C18 2.1 mm x 75 mm 1.7 µm column fitted with an ACQUITY UPLC™ BEH C18 1.7 µm VanGuard pre-column (column and pre-column were purchased from Waters, Czech Republic) for analysis of amino acids and biogenic amines, and the flow injection analysis (FIA-MS/MS) for analysis of acylcarnitines, glycerophospholipids, sphingolipids and a sum of hexoses.

standardized by applying spiked-in isotopically labelled standards (44 in total) in the positive ionisation mode. For calibration, a calibrator mix consisting of seven different concentrations was used. Quality controls (QCs) derived from lyophilized human plasma samples were included for 3 different concentration levels. The intra-plate correction was achieved through QC normalisation using the QC2 sample, which was analysed in four technical replicates. The MS/MS signals were integrated using MassLynx software version 4.1 (Waters, Czech Republic) and metabolite final concentrations were automatically calculated in μmol using MetIDQTM software version Carbon (BIOCRATES Life Science AG, Austria). Lastly, glycerophospholipids are differentiated according to the presence of ester and ether bonds in the glycerol moiety. Double letters (aa = diacyl, ae = acyl-alkyl) indicate that two glycerol positions are bound to a fatty acid residue, while a single letter (a = acyl or e = alkyl) indicates a bond with only one fatty acid residue. Lipid side-chain composition is abbreviated as C $x : y$, where x denotes the number of carbons in the side chain and y denotes the number of double bonds. The assay details and the metabolite nomenclature for AbsoluteIDQTM p180 kit have been previously described [27].

Statistical and metabolomic data analysis

Non-metabolomic data (*in vivo*, *in vitro*, haematological parameters and interleukins) were processed as follows: Shapiro-Wilk test of normality and quantile-quantile (Q-Q) plot with the 95% bootstrap confidence interval were used to assess normality of a random variable. As the vast majority of the variables was not Gaussian, the Wilcoxon rank-sum test with continuity correction was used to compare medians of the variables in the animals from control and sensitised groups. Distribution of sRaw, as *in vivo* marker before sensitisation, on the 14th day and 21st day was Gaussian, therefore; the ordinary ANOVA with Tukey HSD *post-hoc* test was used to compare the means of sRaw at the three-time points.

Using targeted metabolomic analysis, every plasma sample was measured in duplicate, and concentrations of metabolites were averaged over the duplicates. The QC2 sample was measured

considered as a marker of the reliability of metabolites concentrations (metabolites with a coefficient of variation larger than 0.3 were excluded). Limit of detection (LOD) for each metabolite was another criterion of the reliability, and metabolites with more than 30% of values under the LOD in each group were excluded. The data were summarized by the mean, standard deviation, median and interquartile range (IQR), separately for control and sensitised animals. For each metabolite, fold change (FC) of each metabolite in the sensitised group relative to those in control group was calculated from the means. Q-Q plot with the 95% bootstrap confidence interval was employed to assess normality of metabolite concentrations. Subsequently, the p-values in the univariate analysis were calculated using the Wilcoxon test and followed by Benjamini-Hochberg correction. The Volcano plot (combination of FCs and q-values) using threshold 0.1 and 1.3 for adjusted false discovery rate (FDR) p-values and FCs, respectively, was selected as a conventional statistical method. Different prioritization of the metabolites was obtained by the nested cross-validation feature selection in the Random Forest algorithm (machine learning method). The minimal depth criterion was used, together with the medium degree of conservativeness in the feature selection. The variable hunting was done with the 5-fold cross-validation and variable importance plot was used to visualize the ranked metabolites. The final set of metabolites was obtained as an intersection of metabolites selected by Volcano plot and Random Forest. To obtain a realistic estimate of the predictive ability of the selected metabolites, these were fed into the Random Forest to obtain the Out-of-Bag based receiver operating characteristic (ROC). The optimal cut-off was selected to minimize the misclassification error. Finally, partial least squares discriminant analysis (PLS-DA) plot was generated to confirm if they produce visually distinct clusters of samples that correspond to studied groups of animals (the concentrations were log-transformed and Pareto scaled). A custom program written in R software version 3.5.2, applying the various libraries was used for statistical analyses and prioritization of metabolites (Supplemental file). The MetaboAnalyst software was used for performing Volcano plot and PLS-

for Windows (GraphPad Software, San Diego, California USA; www.graphpad.com).

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As a marker of *in vivo* airway responsiveness, sRaw measured after inhalation of histamine at the concentration of 10^{-6} mol in the whole body double chamber plethysmograph was used. After the sensitisation and challenge of animals by allergen (ovalbumin) in the sensitised group, a significant increase of sRaw on the 14th as well as on the 21st day was observed. There were no changes of sRaw during both measured days in the control group (Fig. 1).

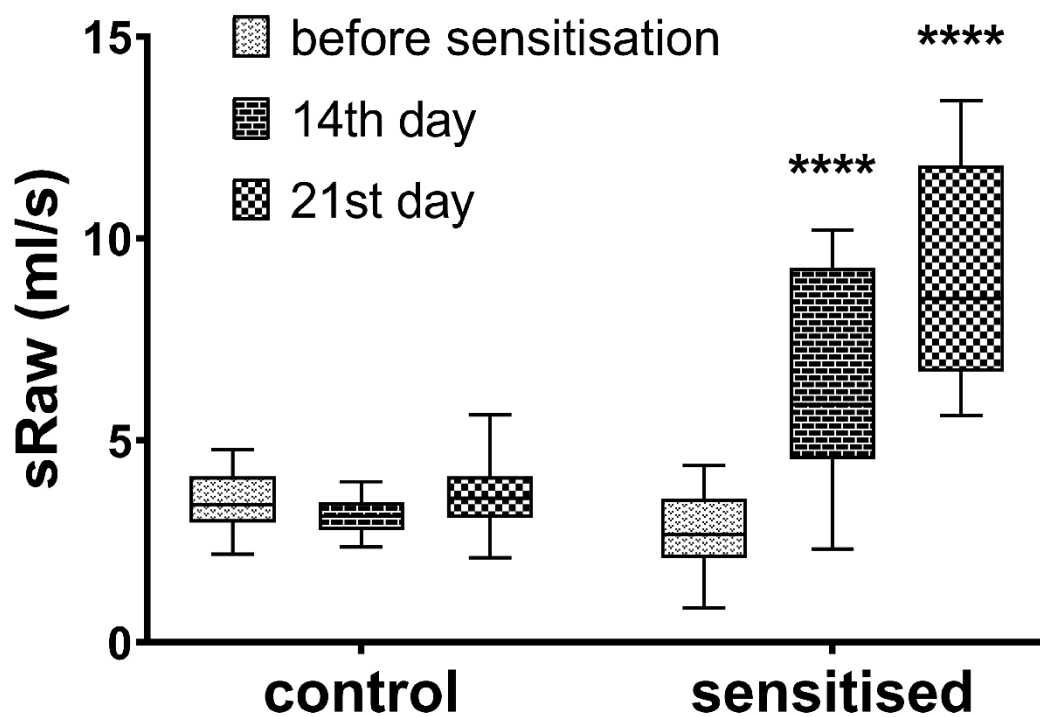


Figure 1 Specific airway resistance (sRaw) measured after inhalation of an aerosol of histamine (10^{-6} mol/L; 2 minutes) measured before, on the 14th and 21st day of sensitisation. Data are expressed as a box and whisker plots. Ordinary ANOVA with Tukey HSD post-hoc test was used to compare the mean of sRaw at the three-time points. ****P<0.0001.

from the sensitised group are more prone to constriction after cumulative doses of histamine compared to the control group, and increased contractility was observed mainly at lower concentrations of histamine (10^{-8} – 10^{-6} mol/L) (Fig. 2).

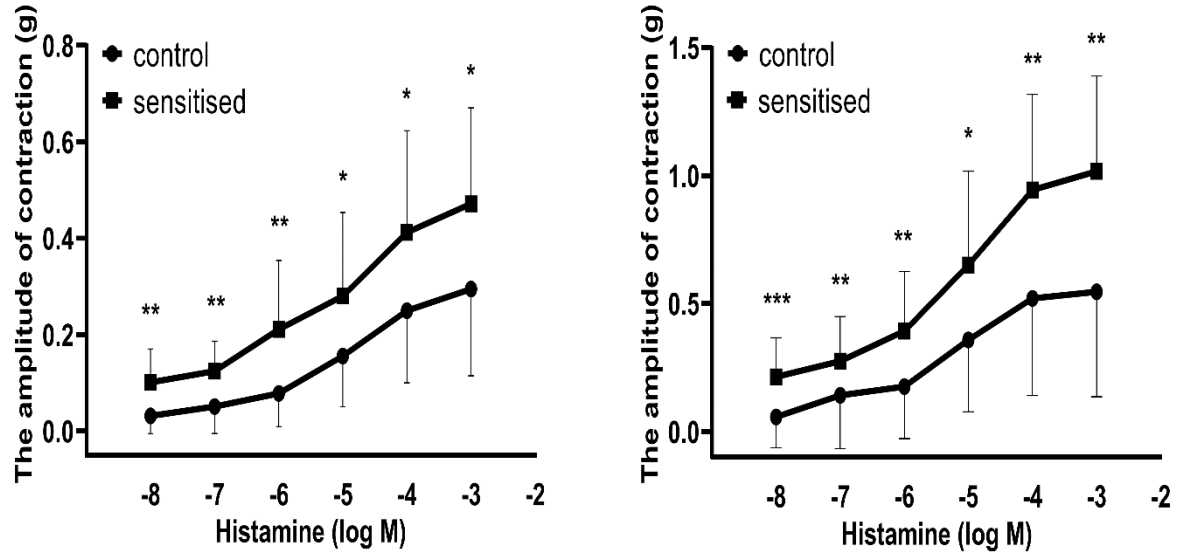


Figure 2: In vitro airway reactivity of tracheal (left panel) and lung (right panel) tissue strips to cumulative concentrations of histamine in control and ovalbumin sensitised guinea pigs. Data are expressed as means; error bars express SD. Wilcoxon rank-sum test with continuity correction was used. *P<0.05, **P<0.01, ***P<0.001.

increase in total leukocyte count in the blood and in differential counts of eosinophils in the blood and BAL fluid, as well as elevated plasma concentrations of IL-4 and IL-5 compared with the naïve control group (Table 1).

	Blood							BAL		
	Leu ($\times 10^9$ /L)	Lym (%)	Neu (%)	Mon (%)	Eos (%)	IL-4 (pg/mL)	IL-5 (pg/mL)	Mon- Mac (%)	Neu (%)	Eos (%)
Control group	2.0 (0.39)	58.2 (7.0)	37.2 (4.7)	3.4 (2.3)	1.2 (0.8)	115.3 (30.2)	183.5 (69.2)	95.1 (1.5)	3.67 (1.0)	1.3 (0.7)
Sensitised group	3.1 (1.21)	52.7 (14.1)	40.9 (12.4)	4.3 (3.8)	2.0 (0.9)	196.1 (25.4)	822.6 (66.5)	58.4 (6.4)	7.8 (3.8)	33.7 (6.6)
	***	ns	ns	ns	**	**	****	****	***	****

Table 1 Absolute and relative counts of leukocytes in the blood and bronchoalveolar lavage (BAL) fluid in control and sensitised group. Leu, absolute number of leukocytes; Lym, lymphocytes; Neu, neutrophils; Mon, monocytes; Eos, eosinophils; IL-4, interleukin 4; IL-5, interleukin 5; Mon-Mac, monocytes-macrophages; BAL, bronchoalveolar lavage fluid. Data are expressed as means (SD). Wilcoxon rank-sum test with continuity correction was used. ^{ns}P>0.05, ^{}P<0.01, ^{***}P<0.001, ^{****}P<0.0001**

Plasma concentration of 185 metabolites of each guinea pig was measured using the Biocrates AbsoluteIDQ™ p180 kit. As a result, 127 out of 185 metabolites were used for further statistical evaluation based on QCs and LODs criteria. In addition to the information gained from individual metabolites, 17 ratios and sums were selected for their capacity to provide detailed insight into biological functions and nutritional information. Finally, 144 features (127 metabolites, 17 ratios and sums) were used for statistical evaluation (Supplemental file - Table S1).

For comparison of the metabolites concentrations in naïve control and sensitised groups, the Wilcoxon rank test with Benjamini-Hochberg correction was used. Using FDR-adjusted p values

values and FCs, respectively, and only 40 metabolites met the threshold requirements. Another set of 38 metabolites was selected by Random forest algorithm. For hypothesis-generating we used metabolites obtained by intersection of above-mentioned methods. As a result, 22 metabolites were selected as an important; four out of them were increased, on the contrary 18 were decreased in sensitised group (Table 2 and Fig. 3).

Metabolite	q-value (FDR) [$\times 10^{-2}$]	FC	Depth of tree	Production change
Carnitine	1.55	0.74	2.161	Down
Kynurenine/Trp	7.46	0.74	2.302	Down
Met-SO/Met	9.14	1.41	2.287	Up
PC aa C34:1	4.85	0.75	2.304	Down
PC aa C34:2	1.42	0.66	2.230	Down
PC aa C34:3	2.73	0.59	2.233	Down
PC aa C34:4	1.92	0.59	2.221	Down
PC aa C36:2	1.42	0.68	2.277	Down
PC aa C36:6	1.42	0.61	2.159	Down
PC aa C38:6	0.80	0.62	2.171	Down
PC aa C40:6	1.42	0.74	2.169	Down
PC ae C36:0	1.92	0.73	2.211	Down
PC ae C38:0	2.29	0.75	2.297	Down
PC ae C38:3	2.63	0.71	2.280	Down
PC ae C38:6	3.11	0.57	2.301	Down
PC ae C40:1	1.92	0.66	2.295	Down
PLA2 activity	0.48	1.34	2.078	Up
SDMA	8.97	0.65	2.294	Down
SMDA/Arg	5.22	0.54	2.253	Down
Taurine	6.95	1.35	2.232	Up
Total PC	2.29	0.71	2.314	Down
Trp	5.22	1.31	2.289	Up

Table 2 List of metabolites with significantly changed concentrations in a sensitised group of guinea pigs. Trp, tryptophan; Met-SO, methionine sulfoxide; Met, methionine; PC, phosphatidylcholine; PLA2, phospholipase A2; SDMA, symmetric dimethylarginine; Arg, arginine; Trp, tryptophan; FC, fold change (ratio of means in sensitised group relative to control group). The Wilcoxon test followed by Benjamini-Hochberg correction (q -value) was used.

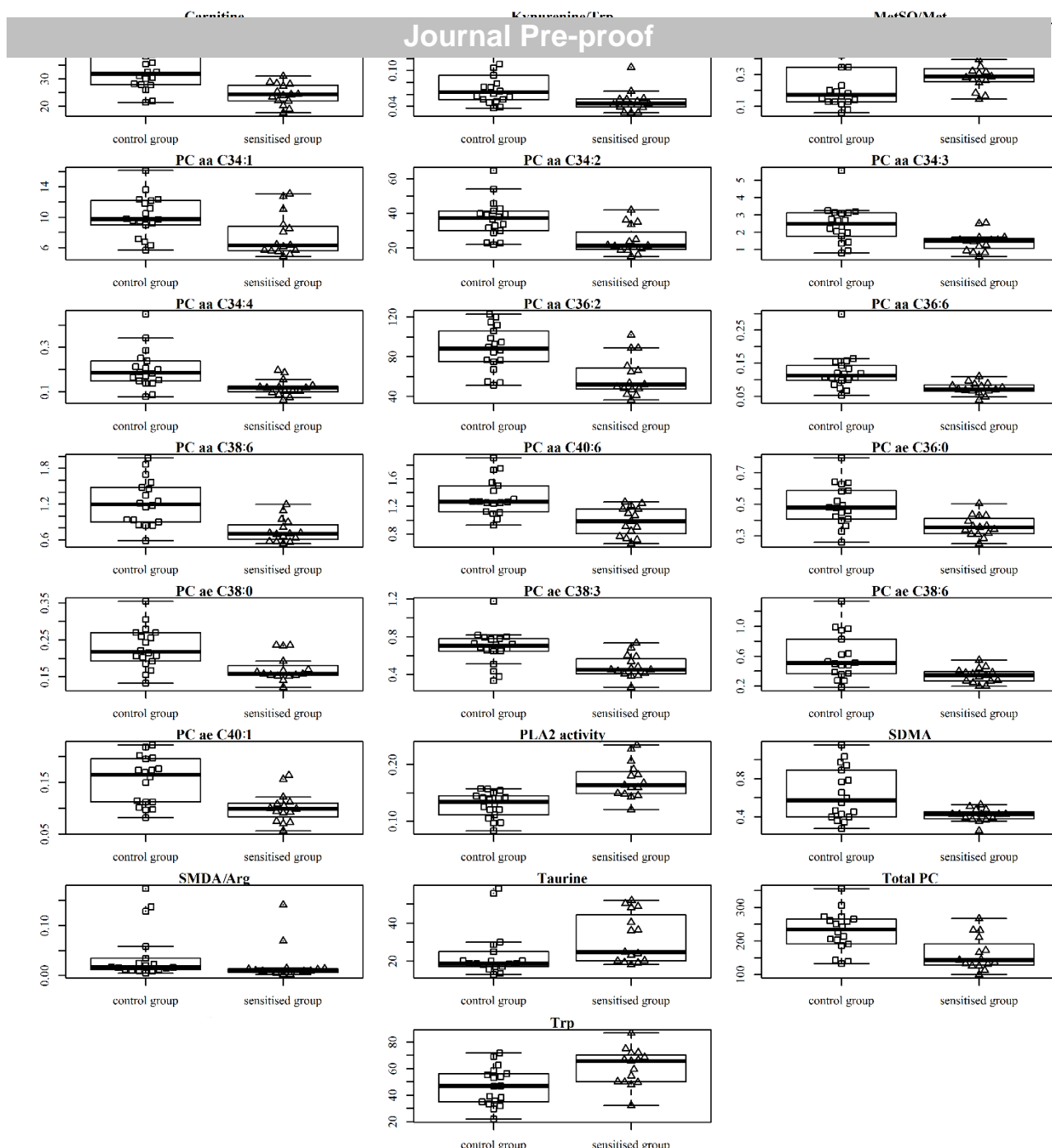


Figure 3 Boxplot of normalized concentrations (μmol ; normalization was performed using the QC2 sample) of the selected 22 metabolites for the control group (square) and the sensitized group (triangle). A boxplot exhibits the median of the data (thick horizontal line), the upper and lower hinge, a version of upper and lower quartile (upper and lower line segment in the box) and the whiskers are defined as a multiple of the Inter Quartile Range (for details see the R help to boxplot.stats function). Each boxplot is overlaid by a swarm plot of the data.

The result of ROC curve analysis showed that the panel of the 22 selected potential biomarkers had an area under a curve (AUC) value 0.87, and the sensitivity and specificity of the metabolites panel were 94.4% and 73.3%, respectively, at the best cut-off value (Fig. 4). Using PLS-DA method, as one of the most well-known classification procedures in chemometrics, we could

presence of differences between both groups of animals (Fig. 5).

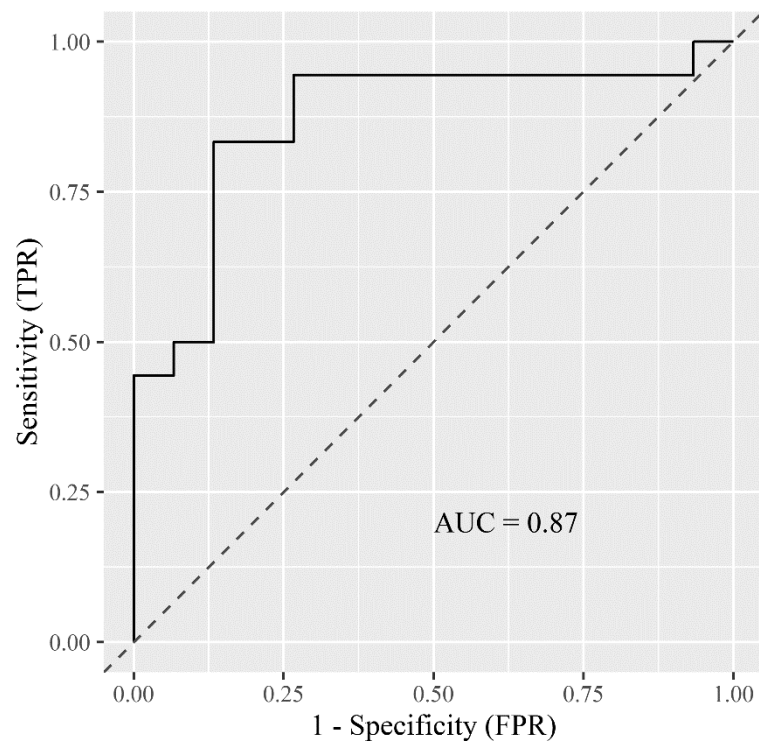


Figure 4 Performance of Random forest-based method diagnostic algorithm. Multiparametric receiver operating characteristic (ROC) classifier curves for the differentiation of sensitised guinea pigs and non-sensitised guinea pigs using 22 selected metabolites. The continuous line represents the discrimination power of the classifier. The corresponding area under the curve (AUC) value is shown. The x-axis and y-axis show the false-positive rate (FPR; 1-specificity) and true-positive rate (TPR; sensitivity), respectively. The diagonal dashed line indicates no discriminatory power.

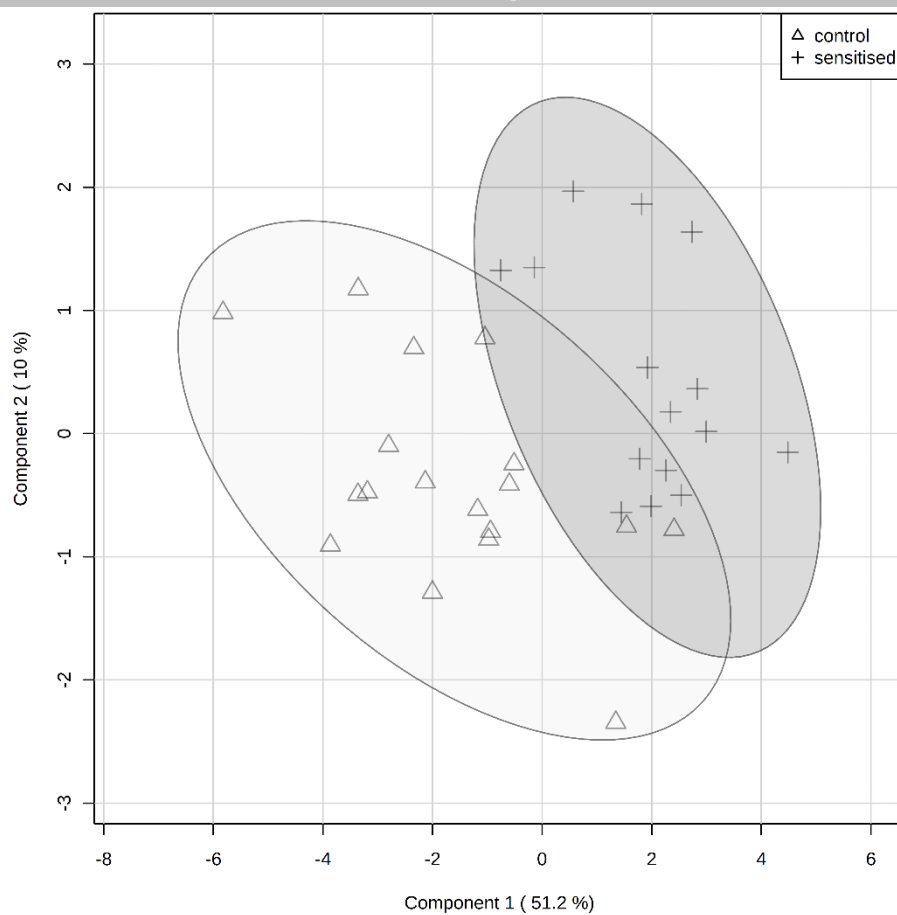


Figure 5 Partial least squares-discriminant analysis (PLS-DA) score plot from 22 selected metabolites. Data were log-transformed and Pareto scaling.

In asthma pathogenesis, many signalling molecules and cells including eosinophils are involved [30]. Nowadays, the term “eosinophilic asthma” is used for an asthma phenotype with a prevalence of eosinophils in airways and in peripheral circulation [31,32]. Due to ethical problems using human clinical studies, which might clear up pathophysiology of eosinophilic asthma, several animal models have been developed [33]. One of the most popular animal models is the sensitisation of guinea pigs by ovalbumin [34].

In this study, we present the first results using a targeted metabolomic platform for studying and better understanding of the detectable alterations in plasma metabolome of ovalbumin sensitised guinea pigs compared to naïve controls. The sensitisation of guinea pigs was confirmed by sRaw as an *in vivo* marker of airway reactivity, contraction of tracheal and lung tissue strips as an *in vitro* marker of airway reactivity, and by changes in white blood cells in the blood and BAL, as well as changes in concentrations of IL-4 and IL-5. The sensitisation by ovalbumin led to significantly increased airway reactivity; increased absolute counts of leukocytes elevated relative counts of eosinophils in the blood and BAL, and to increased concentrations of interleukins. These observations are in accordance with phenotype of allergic asthma [35,36], and with our previous studies with the ovalbumin sensitised guinea pigs [25,37].

The LC-MS/MS and FIA-MS/MS analyses were used for targeted metabolomic analysis using commercially available kit – AbsoluteIDQTM p180. For data processing, a combination of conventional and advanced data analysis methods were used. In our study, 22 metabolites were significantly changed in plasma of ovalbumin sensitised guinea pigs. We identified decreased plasma concentrations of 13 PCs with saturated or unsaturated long-chain fatty acids as well as decreased concentrations of total amount of PCs, carnitine, symmetric dimethylarginine (SDMA), ratio of SDMA to total unmodified arginine (SDMA/Arg), and ratio of kynurenine to tryptophan (Kynurenine/Trp). Contrary, the increased concentrations of tryptophan (Trp), taurine, a ratio of

total PCs as an indicator of phospholipase A2 (PLA2) activity has been observed.

The PCs are class of glycerophospholipids and one of the major components of biological membranes. Their molecules have diverse fatty acids combinations of differing length (16-, 18-, and 20-carbons fatty acids are the most common) and saturation, which are attached at the sn-1 and sn-2 positions and a phosphocholine moiety at the sn-3 position of glycerol [38]. PCs might be hydrolyzed to LPCs and fatty acids by PLA2 family of enzymes [39]. It is well known that PCs are essential elements of cell membranes and lipoproteins which play crucial roles in membrane structure and cellular signalling. Furthermore, PCs and lysoPCs serve as reservoirs and transporters of glycerophospholipid components: fatty acids (e.g. arachidonic acid-AA, docosahexaenoic acid), phosphate, glycerol, and choline. AA and related polyunsaturated fatty acids (PUFA) are precursors of eicosanoids; locally active signalling lipids that regulate a diverse set of homeostatic and inflammatory processes linked to numerous diseases [40,41], and consider as the key mediators of the asthma pathology [42,43]. Decreased concentration of PCs with long-chain fatty acids, as well as increase in indicator of PLA2 activity were observed in present study, which might indicate increased turnover of PCs for synthesis of pro- and anti-inflammatory eicosanoids as a result of induced inflammation. Our findings are consistent with previous metabolomic studies of ovalbumin sensitised mice [20–23], and asthma patients [44–46]. However, in the vast majority of those studies the untargeted metabolomic approach was used; therefore, the quantitative cross-comparison is not possible.

Carnitine, highly polar molecule, plays an essential role in the transfer of long-chain fatty acids from the cytoplasm into the mitochondria, where β -oxidation takes place [47]. Furthermore, carnitine is involved in the transport of peroxisomal β -oxidation products into mitochondria for completion of oxidation via tricarboxylic acid cycle (TCA cycle), storage of energy in the form of acetylcarnitines [48], decreasing leukotriene synthesis through inhibition of lipoxygenase enzyme [49], and scavenging of reactive oxygen species (ROS) to prevent oxidant injury [50]. Carnitine is

active [50]. In this study, plasma carnitine decreased in the sensitized guinea pigs, which may indicate utilization of carnitine to in mitochondrial fatty acid metabolism. Bronchial asthma is associated with a higher level of oxidative stress leading to the production of excessive amount of ROS [51,52], confirmed also in animal models of asthma [5,53,54]. As carnitine is also ROS scavenger, this might also contribute to its lower plasma concentrations. Our findings are in accordance with previous studies which found lower level of carnitine in asthma, and reduced risk of asthma attack, improved bronchoconstriction and bronchial inflammation after its supplementation [55–58]. In spite of the fact that many studies have been focused on association between carnitine and asthma, it is still unknown if the lower carnitine concentration is the cause or the consequence of asthma.

Methionine (Met), a sulfur amino acid, is one of the major targets of ROS, resulting in the formation of methionine sulfoxide (Met-So; [59]. Therefore, higher Met-So levels can be related to increased production of ROS and the ratio of Met-So/Met is considered as a marker of oxidative stress in *in vivo* conditions [60]. During our experiments, a higher ratio of Met-So/Met in sensitised guinea pigs was observed which might reflect higher ROS production.

Taurine is another metabolite that was significantly increased in the sensitised group of guinea pigs. As a ubiquitous sulfur-containing amino acid, taurine is widely distributed in animal tissues, and it is involved in maintaining of an intracellular osmotic pressure, has antioxidant activity, plays an important role in the development of the brain of certain animals, and acts as a neurotransmitter [61]. There are only a few studies focusing on taurine plasma level and asthma and the results are ambiguous. Cortijo et al. [62] and Santangelo et al. [63] found increased taurine concentration in BAL fluid and oral taurine supplementation showed beneficial effects in *in vivo* and *in vitro* model of experimental asthma. Contrary, Zinellu et al. [64] assessed selected possible markers of oxidative stress in asthma and chronic obstructive pulmonary disease including taurine, with no changes in plasma taurine concentrations. Finally, Comhair et al. [65] found out higher

correlation with plasma levels of arachidonic acid. Mechanisms of these changes can only be speculated.

Production of nitric oxide (NO) from L-arginine by the NO synthase family of isoenzymes plays an important role in the maintenance of airway tone [66]. Asymmetric and symmetric dimethylarginine (ADMA and SDMA, respectively) are arginine post-translational modifications products. The biological role of ADMA as an endogenous inhibitor of NO synthase is well known, while less attention has been paid to SDMA [67]. In this present study, we observed lower plasma levels of SDMA and lower ratio SDMA/Arg in the sensitised group and no changes in ADMA. This is in contrast with previous works, where Tajti et al. [68] and Bediwy et al. [69] described an increased level of ADMA in asthma patients. Scott et al. [70] found increased lung concentrations of ADMA and SDMA in a murine model of asthma and asthma patients, respectively. On the other hand, SDMA concentration in sputum was decreased, suggesting differences in SDMA metabolism and transport between compartments. Therefore, further investigations are necessary to understand the possible role of SDMA in asthma pathophysiology.

Tryptophan is the least abundant of the essential amino acids, with the main role in the human body as a constituent of protein synthesis. Tryptophan is also the precursor of two important metabolic pathways, kynurenine synthesis (accounts for approximately 90% of tryptophan catabolism) and serotonin synthesis [71]. There is growing evidence that the tryptophan metabolic process plays an important role in the development of allergic diseases [72]. In a present study, increased plasma levels of tryptophan and a reduced ratio of kynurenine/tryptophan in the sensitised group were observed. A ratio of kynurenine to tryptophan is widely used as a marker of indoleamine 2,3-dioxygenase (IDO) activity, an enzyme that metabolizes tryptophan to kynurenine [73]. Some studies have demonstrated an association between IDO activity and the pathogenesis of pollen allergy, allergic asthma and allergic rhinitis [74–76]. Hu et al. [77] found lower expression of IDO in the airways of children with asthma. van der Sluijs et al. [78] showed

downstream metabolites of IDO-mediated tryptophan degradation in patients with allergic asthma. Our results bring additional evidence of decreased activity of IDO in asthma, but underlying causes of these observations still remains to be elucidated.

There are some limitations concerning our study which should be recognized. The metabolite panel used in our study contains only selected metabolites from seven classes with interesting biological activity. However, they do not cover the whole metabolome. In addition, some metabolites were not specific enough (mainly the detailed structure of PCs) which should be explored in more detail. We admit that the number of animals recruited was relatively small, and our findings cannot be fully extrapolated to human asthma patients as only a model of allergic asthma was chosen here. Moreover, the plasma level of selected metabolites has been compared with the plasma profile of healthy humans (male, aged 19-22 years). Unfortunately, direct comparison of our results and their interpolation to human asthma patients is not possible due to discrepancy between reference metabolome of naïve control guinea pigs and human metabolome (differences vary from 5% for SDMA to more than 2000% for PC aa 34:1). Therefore, further investigations are necessary to determine the same changes as were observed in guinea pig model of allergic asthma in human asthma patients.

Conclusion

In summary, we identified alterations in the PCs profiles, carnitine levels and metabolites involved in inflammation and oxidative stress in ovalbumin sensitised guinea pigs. The majority of these metabolites have been associated with asthma or allergy. Moreover, we identified the change in the plasma levels of SMDA that has not yet been noticed in asthma and allergy-related diseases. In spite of the limitations of targeted metabolomic studies, we believe that this technology has huge potential to investigate possible candidate markers and underlying pathophysiology of asthma.

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Journal Pre-proof

The authors declare that they have no conflict of interests.

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