Sex Differences in Plasma Metabolites in a Guinea Pig Model of Allergic Asthma

Romana BAROSOVA[1], Eva BARANOVICOVA[2], Jana ADAMCAKOVA[1], Kristian PRSO[3], Juliana HANUSRICHTEROVA[2], Daniela MOKRA[1]

[1]Department of Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovak Republic, [2]Biomedical Center Martin, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovak Republic, [3]Department of Pharmacology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovak Republic

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Summary

Sex seems to be a contributing factor in the pathogenesis of bronchial asthma. This study aimed to find sex-related differences in metabolome measured by hydrogen-1 nuclear magnetic resonance (^{1}H NMR) spectroscopy in healthy and ovalbumin (OVA)-sensitized guinea pigs. Adult male and female animals were divided into controls and OVA-sensitized groups. OVA-sensitization was performed by OVA systemic and inhalational administration within 14 days; on day 15, animals were killed by anesthetic overdose followed by exsanguination. Blood was taken and differential white blood cell count was measured. Left lung was saline-lavaged and differential cell count in the bronchoalveolar lavage fluid (BALF) was measured. After blood centrifugation, plasma was processed for ^{1}H NMR analysis. Metabolomic data was evaluated by principal component analysis (PCA). Eosinophil counts elevated in the BALF confirming eosinophil-mediated inflammation in OVA-sensitized animals of both sexes. Sex differences for lactate, glucose, and citrate were found in controls, where these parameters were lower in males than in females. In OVA-sensitized males higher glucose and lower pyruvate were found compared to controls. OVA-sensitized females showed lower lactate, glucose, alanine, 3-hydroxy-butyrate, creatine, pyruvate, and succinate concentrations compared to controls. In OVA-sensitized animals, lactate concentration was lower in males. Data from females (healthy and OVA-sensitized) were generally more heterogeneous. Significant sex differences in plasma concentrations of metabolites were found in both healthy and OVA-sensitized animals suggesting that sex may influence the metabolism and may thereby contribute to different clinical picture of asthma in males and females.

Key words

Allergic asthma • Sex • Metabolomics • Plasma • Guinea pig

Corresponding author

D. Mokra, Department of Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Mala Hora 4C, SK-03601 Martin, Slovak Republic. E-mail: daniela.mokra@uniba.sk

Introduction

Bronchial asthma is a heterogeneous disease involving chronic airway inflammation, hyperreactivity, and oxidative damage; however, pathomechanisms of the disease remain poorly understood [1]. Asthma can be divided into several endotypes distinguished according to type of inflammation, and into several phenotypes with different clinical characteristics [2]. In addition, differences among the endotypes/phenotypes contribute to a wide heterogeneity of appropriate treatment as various subtypes may exert variable response to given treatments [1,2].

Considerable differences in asthma patients may be found between the sexes [3] as well. Although the molecular mechanisms behind are not yet fully understood, sex hormones presumably play a key role [4]. Estrogen and progesterone mainly stimulate airway inflammation, while androgens have an opposite effect [5]. Sex hormones also influence the sensitivity of β_2-adrenoreceptors and mucociliary clearance, contributing to bronchoconstriction and airway obstruction [6].

The gaps in understanding asthma pathophysio-

logy and subtyping can be partially elucidated by novel methods of technology and bioinformatics that make the research design and data analysis more accessible. Progress in metabolomics uncovered that metabolites act not only as intermediates and nutrients, but they are very important mediators, signaling and regulatory molecules, neurotransmitters, modulators of immune responses etc. In the respiratory diseases, besides sputum, bronchoalveolar lavage fluid (BALF) and exhaled breath condensate (EBC), the blood plasma and urine seem to be adequate biological liquids with easy accessibility, convenient for metabolomics [7,8].

Several metabolomic studies were conducted in asthma patients and animal models, in order to elucidate possible asthma-associated pathways, to distinguish various endotypes/phenotypes of asthma, and to discover biomarkers valuable for asthma diagnostics and choice of appropriate treatment. Recent studies have identified metabolomic patterns related to changes in Krebs (tricarboxylic acid, TCA) cycle metabolism, phospholipid regulation, hypoxic and oxidative stress, and hypermetylation in asthma [8-10]. Similar results demonstrating alterations linked to energy gaining TCA cycle [11] or to energy and lipid metabolism [10] were shown in animal models of asthma. For instance, disturbances in glucose, 3-hydroxybutyrate, tyrosine, and creatine metabolism were observed in female guinea pigs with ovalbumin (OVA)-induced allergic asthma [12], while changes in amino acids were detected in OVA-treated mice [13]. Several studies pointed out an altered phospholipid metabolism [13-15] which plays a key role in surfactant function and immune response to allergen.

Some variations in metabolomes between males and females exist in healthy humans [16,17]. Nevertheless, little is known about metabolomic differences between females and males suffering from asthma. To our knowledge, there is only one metabolomic study up to now evaluating the sex differences in OVA-challenged guinea pigs where changes in lipid metabolism between male and female animals in allergic asthma-model groups were found [18].

Therefore, the aim of this pilot study was to find sex-related differences in metabolome measured by hydrogen-1 nuclear magnetic resonance (^{1}H NMR) spectroscopy in the healthy guinea pigs and subsequently in OVA-sensitized animals to compare the response of animals of both sexes to allergic sensitization. As there is insufficient data in this field, we presume that our findings may contribute to understanding the sex-based differences in asthma.

Materials and Methods

Animals

The experiments were held in accordance with the European Guidelines on Laboratory Animal Care. The study protocol was approved by the local Ethics Committee and by the National Veterinary Board of Slovakia. For experiments, adult Dunkin-Hartley male and female guinea pigs with a mean body weight of 300-400 g were used. Animals were supplied by a certified animal breeding station of our faculty where they were kept in standard conditions (temperature 22-23 °C, humidity around 50 %), with food and water available *ad libitum*.

Design of experiments

Animals were semi-randomly divided into 4 groups: 1. female-ctrl (female healthy controls, n=7), 2. female-ova (OVA-sensitized female animals, n=7), 3. male-ctrl (male healthy controls, n=6), and 4. male-ova (OVA-sensitized male animals, n=5). The sensitization of animals was performed by OVA, an allergen from a chicken egg white (Ovalbumin, Sigma-Aldrich, USA). The process of sensitization was performed during 14 days so that 1 % OVA (dissolved in *aqua pro inj.*) was administered on the 1st day intraperitoneally (0.5 ml) and subcutaneously (0.5 ml), and on the 3rd day intraperitoneally (1.0 ml), while controls were given *aqua pro inj.* in the corresponding times. On the 14th day, 1 % OVA (dissolved in sterile saline) was inhaled for 30 s using an inhalation chamber (challenge), while controls were given saline aerosol only [19]. On the following day, animals were killed by a lethal dose of anesthetics (xylazine, Xylariem, Ecuphar N.V., Belgium, and tiletamin and zolazepam, Zoletil, Virbac, France) with subsequent exsanguination by a direct puncture of the heart.

Differential white blood cell (WBC) count in the blood and differential cell count in the BALF

Differential WBC count was measured by veterinary hematologic analyzer (Sysmex XT-2000i, Japan) and expressed as $\times 10^3/\mu$l. The left lung was lavaged by saline (2×10 ml/kg) and differential cell count was evaluated by the analyzer Sysmex and expressed as $\times 10^3/\mu$l.

Blood plasma collection and handling

Blood was collected in K3-EDTA-coated tubes, centrifuged at 4 °C, 2500 rpm, for 15 min. Plasma was deproteinized [20] as follows: the mixture obtained after adding 600 µl of methanol to 300 µl of plasma was shortly vortexed and stored at -20 °C for 20 min. After centrifugation at 14000 rpm (14800× g), 650 µl of supernatant were dried out. Before measurement, the dried matter was carefully mixed with 500 µl of deuterated water and 100 µl of stock solution (100 mM phosphate buffer, pH-meter reading 7.40, and 0.25 mM of 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4) as a chemical shift reference in deuterated water). For measurement, 550 µl of the final mixture was transferred into a 5 mm NMR tube.

NMR data acquisition

NMR data were acquired using 600 MHz NMR spectrometer (Avance III, Bruker, Germany) equipped with triple resonance (TCI) cryoprobe. Initial settings were performed on an independent sample and adopted for measurements. Samples were stored in Sample Jet at approximately 6 °C before measurement for maximal time of 2 h. Standard Bruker profiling protocols were modified as follows: profiling 1D NOESY with presaturation (noesygppr1d): FID size 64k, dummy scans 4, number of scans 128, spectral width 20.4750 ppm; COSY with presaturation was acquired for randomly chosen 10 samples (cosygpprqf): FID size 4k, dummy scans 8, number of scans 1, spectral width 16.0125 ppm; homonuclear *J*-resolved (jresgpprqf): FID size 8k, dummy scans 16, number of scans 4; profiling CPMG with presaturation (cpmgpr1d, L4=126, d20=3 ms): FID size 64k, dummy scans 4, number of scans 128, spectral width 20.0156 ppm. All experiments were conducted with a relaxation delay of 4 s; all data were once zero-filled. An exponential noise filter was used to introduce 0.3 Hz line broadening before the Fourier transformation. Samples were measured at 310 K and randomly ordered for acquisition.

Data analysis

A chemical shift of 0.000 ppm was assigned to TSP-d_4 signal. All spectra were binned to bins of the size of 0.001 ppm, starting from 0.500 ppm to 9.500 ppm. No normalization method was applied on NMR data, as exactly the same amount of blood plasma was taken from all samples. Spectra were solved using internal metabolite database, online human metabolome database (www.hmdb.ca) [21], chenomx software free trial version and literature [20]. For all compounds the multiplicity of peaks was confirmed in J-resolved spectra and homonuclear cross peaks were confirmed in cosy spectra. After the metabolites were identified we chose the spectra sub regions with only single metabolite assigned. In 0.001 ppm binned spectra we summed integrals of selected signals. These values were handled as relative concentrations of metabolites in the blood plasma. Metabolites not having appropriate signals for the evaluations or with unambiguous peak assignment were excluded from a further evaluation.

The Mann-Whitney U test was used to test the null hypothesis of stochastical equality of distributions of metabolite concentrations in the groups under comparison, and medians were used to calculate fold changes. Principal component analysis (PCA) was performed using online tool Metaboanalyst [22]. Comparisons of eosinophil counts in the blood and BALF were performed with GraphPad Prism software 9.0 (GraphPad Software Inc., La Jolla, CA). Statistical tests to compute P-values were calculated for median ± SEM values. Results were considered as significant at $P \leq 0.05$. To compare the means of two groups, Student's *t*-test was used for parametric data, and the MannWhitney test was used for nonparametric data.

Results

OVA sensitization resulted into increased mean of the eosinophil count in the blood; however, the differences did not reach statistical significance (Fig. 1).

In total, 22 metabolites were quantitatively evaluated from NMR spectra (glucose, lactate, leucine, isoleucine, valine, ketoleucine, ketoisoleucine, ketovaline, alanine, acetate, glutamine, 3-hydroxy-butyrate, citrate, creatine, creatinine, phenylalanine, tyrosine, tryptophan, histidine, pyruvate, succinate, uridine). In the first step, we used the principal component analysis (PCA), a method of transformation the multidimensional metabolomic data onto a set of orthogonal axes to find the directions of maximum variance. As input variables, relative concentrations of plasma metabolites in the blood plasma determined by NMR spectroscopy were used. This method helps to estimate data variability among the groups. Principal component 1 (PC1) on x-axis represents the most variation in the data and PC2 on the y-axis the second most variation in the data. The PC values are imaginary

variables resulting from a multilinear combination of the variables in the data sets. In fact, if the data within a group are of low variance, the points are well clustered together with small 95 % confidence ellipse (ellipse that defines the region that contains 95 % of all samples). If the data within the group vary, the confidence ellipse grows. The distance between ellipses can be seen as difference between the data of particular groups. As displayed in Figure 2 and Figure 3, metabolome from female animals in comparison to that of males appears to be more variable, as the metabolic features were visually more spread over the space. This sex dissimilarity was "true" for both naive (ctrl) animals as well as for OVA-affected animals.

The statistical tests for multiple comparison among the groups evaluated differences in the blood plasma glucose, lactate, alanine, 3-hydroxybutyrate as significant using threshold value of 0.05 and differences in blood plasma citrate and pyruvate as boundary significant (P<0.1). These and the results for pairwise comparisons for female/male and OVA/ctrl groups are provided in Table 1 and Table S1. Pairwise comparison showed changes between control female/male and OVA-affected female/male guinea pigs as well as sex-dependent differences in the plasma metabolites in the OVA-sensitized animals and controls (Table S1, Fig. 4). Changes in all 22 metabolites are provided in Figure S1. Not listed metabolites did not show significant differences between the tested groups. However, due to a small sample size, the probability of type II error is extremely high. The chances for false negativity are up to 90 % due to low power of hypothesis tests in values around 0.1.

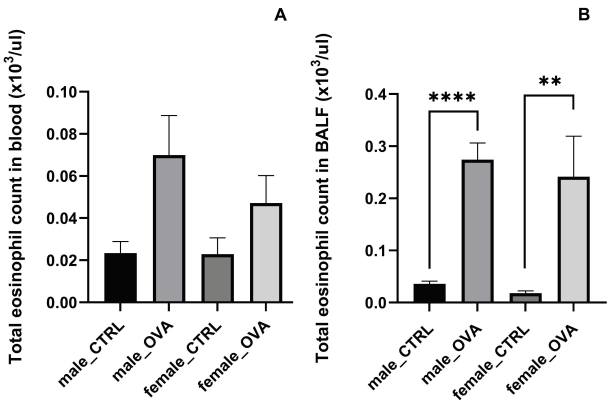

Fig. 1. Eosinophil count in the blood (**A**) and BALF (**B**). Statistical differences for eosinophil count in the blood (A): male_OVA vs. male_CTRL group (P=0.1104); female_OVA vs. female CTRL group (P=0.1358). Statistical differences for eosinophil count in the BALF (B): ** P<0.001; **** P<0.0001.

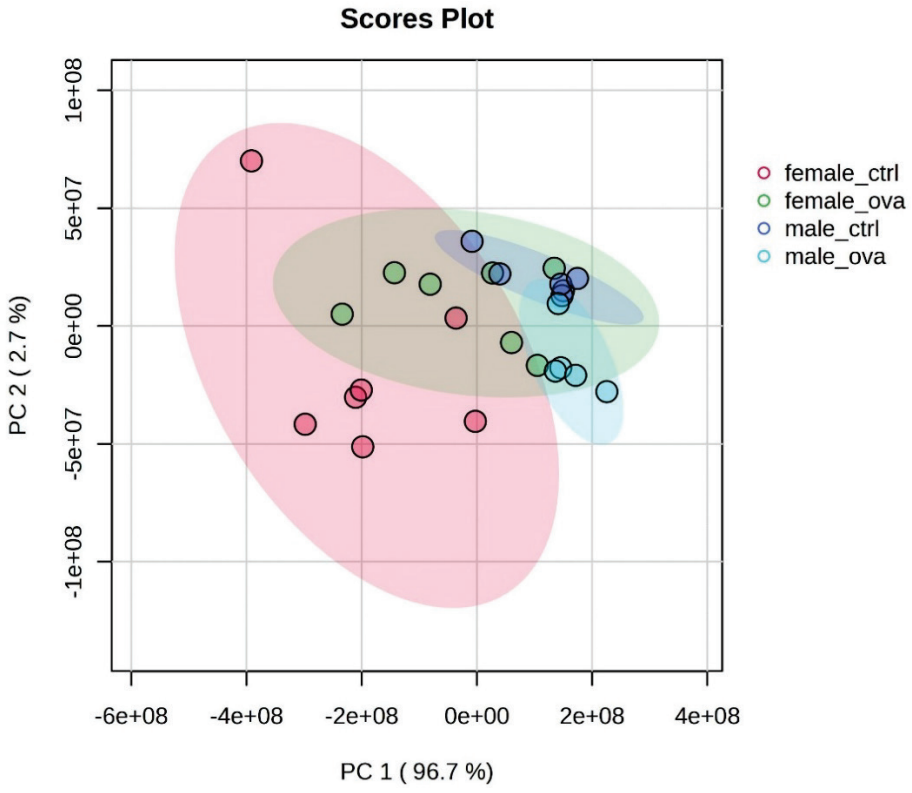

Fig. 2. PCA analysis of blood plasma metabolome in all analysed groups: female and male guinea pigs, both genders affected by OVA and unaffected as controls with 95 % confidence ellipse (ellipse that defines the region that contains 95 % of all samples).

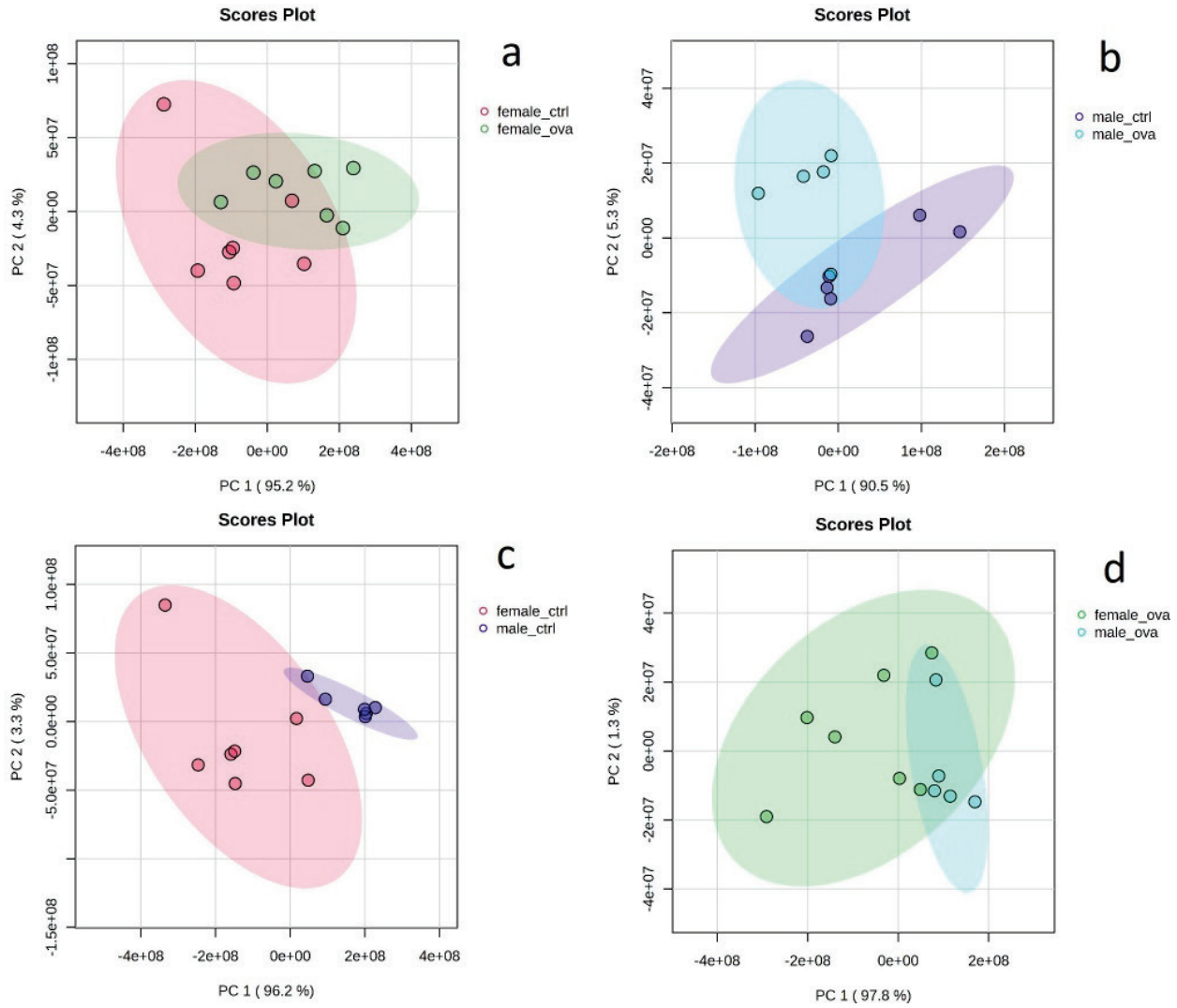

Fig. 3. PCA analysis of blood plasma metabolome for individual pairs of groups: (**a**) female ctrl vs. female ova, (**b**) male ctrl vs. male ova, (**c**) female ctrl vs. male ctrl, (**d**) female ova vs. male ova with 95 % confidence ellipse (ellipse that defines the region that contains 95 % of all samples).

Table 1. Statistical evaluation of relative plasma concentrations between the groups. Results from Mann-Whitney U test for a pairwise comparison, only significant changes with a P<0.05 are listed, percentage change derived from medians. Abbreviations: ova: OVA-sensitized animals, ctrl: control animals.

Compared groups	**Metabolite**	**P**	**%change**
Male OVA/Male ctrl	Glucose	0.0043	46
	Pyruvate	0.014	-23
Female OVA/Female ctrl	Lactate	0.026	-38
	Glucose	0.026	-35
	Alanine	0.041	-32
	3-hydroxybutyrate	0.038	-21
	Creatine	0.0073	-35
Male ctrl/Female ctrl	Lactate	0.0023	-61
	Glucose	0.0034	-53
	Alanine	0.038	-28
	Citrate	0.0018	-21
Male OVA/Female OVA	Lactate	0.0025	-38

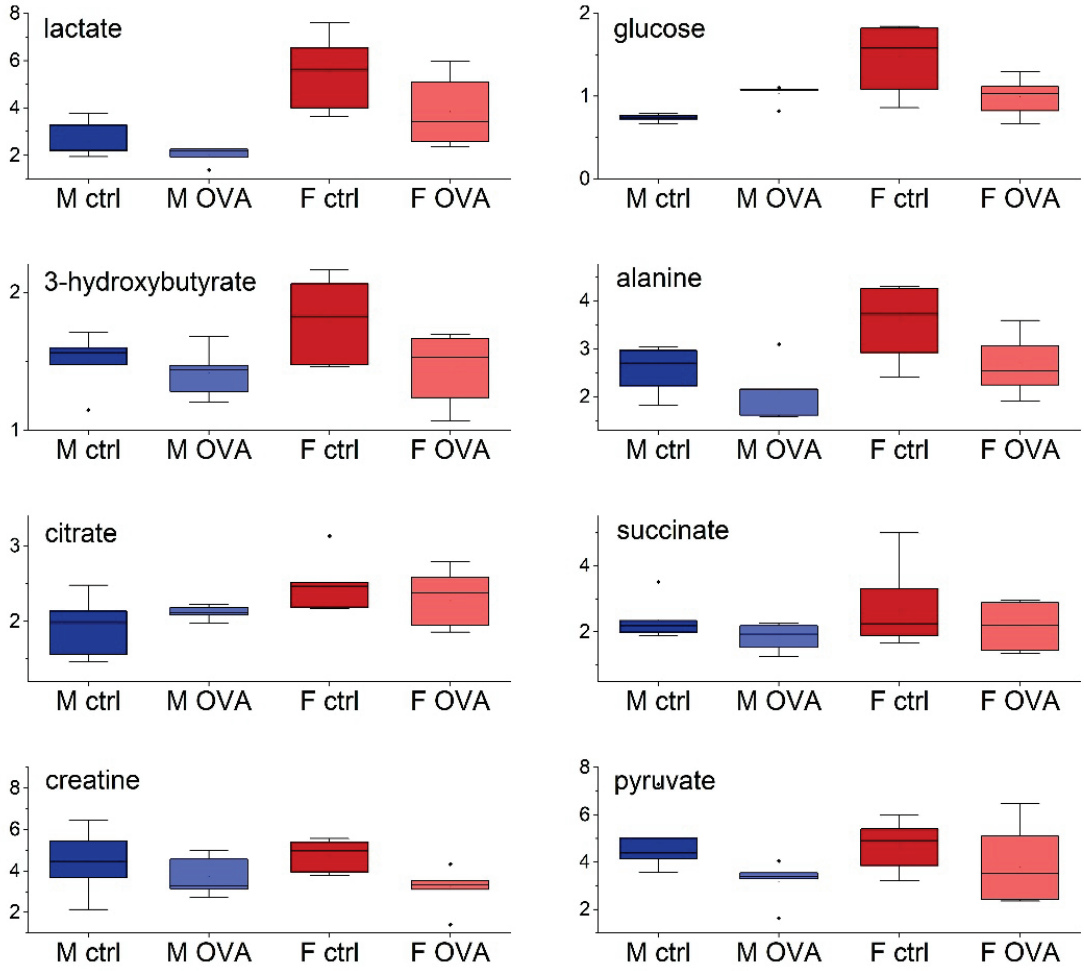

Fig. 4. Relative concentrations of plasma metabolites in all analysed groups, female/male, OVA-affected (ova) and unaffected as controls (ctrl). Abbreviations: ova: OVA-sensitized animals, ctrl: control animals, f: female animals, m: male animals.

Discussion

Because of differences in the clinical picture of asthma in male vs. female patients, use of metabolomics seems to be reasonable for searching and verification of clinically useful markers. This study investigated metabolomic profiles of healthy and OVA-sensitized guinea pigs using ^{1}H NMR spectroscopy, and for the first time compared these profiles between males and females.

Our measurements were performed on adult guinea pigs representing well-established species for preparing the model of allergic asthma because of similarity to humans in the airway autonomic control and response to allergen [23]. In our experiments, systemic and inhalational sensitization with OVA allergen was used to create asthma-like condition in animals as OVA induces Th_2-mediated immune response resulting in airway inflammation, hyperreactivity, and remodelling typical for human allergic asthma [24]. The model has been repeatedly used at our workplace and relevant tests including measurement of *in vivo* and *in vitro* airway reactivity and detection of markers of eosinophilic inflammation have demonstrated the induction of allergic airway inflammation [15,19]. In this article, presence of allergic inflammation was demonstrated by elevated eosinophil counts in the BALF.

Metabolomic analysis revealed female vs. male differences in the plasma concentrations of metabolites participating in the energy gaining biochemical pathways: lactate, glucose, alanine, and citrate. On the other hand, metabolites linked with immunoresponse (glutamine), liver and kidney function (phenylalanine and tyrosine), nitrogen homeostasis (glutamine besides altered alanine), protein synthesis and turnover (aminoacids), or with maintenance of body glutamate-glutamine level (BCAAs and BCKAs) did not show sex-based differences.

Some sex-dependent variations could be hormonally conditioned, as shown by e.g. pronounced differences in energy utilization and glucose kinetics across the menstrual cycle [25] or suppressed glycogen

utilization under the influence of 17β-estradiol [26], suggesting higher susceptibility of women for conserving energy and storing it as fat [27]. There are differences between males and females in lipid metabolism [28] and in mechanisms how energy regulating hormones respond to exercise where women preferentially burn a higher fat-to-glucose fuel mixture [29]. However, there is insufficient knowledge about the mechanisms that could satisfactory explain increased levels of blood lactate, glucose, alanine, and citrate in the females when compared to the males in our study. As lactate serves as a fuel for mitochondrial oxidation in muscles and other organs [30], increased lactate clearance in male guinea pigs may be linked to different muscle/fat ratio in the body or to other sex-dependent conditions. However, lactate influences metabolism, enhances production of reactive oxygen species in mitochondria, cell signaling or immunomodulation, suggesting a complex action of lactate [30]. The metabolites lactate, glucose, and alanine participate in Cahill and Cori cycles that partially overlap and link glucose production in the liver to energy production in other tissues. We may only speculate that increased energy-gaining metabolites in the plasma of mature female could be a sign of female tendency to store bigger energetic reserve or of lower consumption. Sex-based differences in lipid and carbohydrate metabolism were also observed in rats, suggesting a higher ability of the healthy male rat liver to respond to increased energy demands [31]. In humans, metabolomics found higher glucose and lactate in men and higher fatty acids levels in women, but fatty acids metabolism and amino acids were higher in men [17]. In other study, higher alanine level was observed in men, while citrate elevated in women [16].

From metabolomics point of view, bronchial asthma is associated with serious changes in metabolisms of carbohydrates, amino acids, and lipids, and in oxidation-reduction imbalance [8,14,32]. In situations associated with abnormal lung respiration, inflammation, and hypoxia, such as in asthma, lactate as a product of anaerobic glycolysis may be elevated in the tissues. However, lactate also has pleiotropic signaling and immunomodulatory roles [33], e.g., increases production of CXCL8 in airway smooth muscle cells associated with airway hyperresponsiveness [34]. Hyperlactatemia was also described in patients with asthma [35] where it was associated with delivery of β_2-agonists [36]. Although elevated blood lactate was expected in this study, OVA-sensitized animals of both sexes showed a decrease in blood lactate. This may be attributable rather to enhanced lactate utilization in stress condition as lactate can be an alternative energy source for the brain, heart, and other tissues [30] than to its underproduction. This effect was similar in both genders of animals. Our results disagree with clinical studies that observed higher lactate in serum or EBC from asthma patients [37]. However, hyperlactatemia in acute asthma may be more related to the effect of delivered treatments than to increased respiratory muscle workload leading to increased oxygen demands and lactic acidosis [36].

Asthma may be also associated with hypoglycemia caused by excessive energy consumption within the diseased airways [37] or hyperglycemia triggered by delivered medication and the patient´s metabolic predisposition, smoking etc. [38]. Our results showed slightly decreased glucose level in female OVA-sensitized guinea pigs. Similarly to our results, glucose levels declined in urine of female OVA-challenged guinea pigs [12]. We can exclude hypoglycemia from insufficient intake of saccharides leading to enhanced ketone bodies production, as plasma 3-hydroxybutyrate also decreased in the OVA-sensitized animals, thus, another mechanism should be responsible for lower blood glucose. Interestingly, glucose concentration increased in male OVA-challenged group, representing the most pronounced difference observed between both sexes, when compared to controls. In humans, metabolic dysregulation including insulin resistance and altered glucose metabolism have been associated with asthma, particularly with obesity-associated asthma [39].

Glucose breaks down to pyruvate which after decarboxylation to acetyl-coenzyme A enters the TCA cycle, a main metabolic pathway which is crucial for energy homeostasis regulation and cell metabolism, represented by a series of reactions in a closed loop. In asthma, plasma levels of TCA cycle intermediates such as pyruvate, citrate, and succinate may change. In pediatric asthma patients, decreased plasma pyruvate and citrate were found [40]. In our study, pyruvate levels lowered in both OVA-challenged females and males, while citrate declined in OVA-challenged females when compared to control females. In female guinea pigs with asthma model, urine succinate level was depleted [12], while higher concentration of succinate was found in sera from adult asthmatics [37]. Our experiments showed lower succinate in OVA-challenged females compared to controls. Transamination of pyruvate gives rise to alanine [11]. Concentrations of alanine may also change in

asthma; however, the changes may be inconsistent as demonstrated by higher alanine levels in severe asthmatics compared with non-severe asthmatics [41] but lower alanine in sera from asthma patients in other study [37]. Our experiments showed elevated alanine in the control males compared to control females, but no significant changes were observed between OVA-challenged males and females and their controls.

Other metabolites influenced by asthma are ketone bodies represented e.g. by 3-hydroxybutyrate. 3-hydroxybutyrate is an alternative product of fatty acid oxidation which can be used as an energy source in the absence of sufficient blood glucose [42]. 3-hydroxybutyrate plays a role in maintaining redox balance during environmental and metabolic challenges. In this study, significantly decreased 3-hydroxybutyrate level was observed in OVA-challenged females compared with controls.

As demonstrated in the results, lactate, alanine, 3-hydroxybutyrate, pyruvate and succinate are following very similar pattern, a decrease in the plasma of OVA-affected animals when compared with controls with particular sex-dependent differences. All these metabolites are in direct connection to energy gaining processes, glycolysis, and TCA cycle. Besides that, lactate, alanine and glucose are the key players in Cahill and Cori cycles. Their decrease in the plasma may suggest a slowdown of reactions in which amino groups and carbons from muscles are transported to the liver, or ridding the muscle tissue of the toxic ammonium ion, as well as indirectly providing glucose to energy-deprived muscle tissue.

Another metabolite, creatine, is closely linked to energy metabolism in the muscles including airway smooth muscle, heart, and brain, but not directly interconnected with glycolysis [14]. In our study, decreased creatine was observed in the OVA-affected animals compared to controls. Similarly to our results, lower urine creatine was detected in female guinea pigs with asthma model [12].

Of course, we are aware of some limitations of this study. Relatively small number of animals in this pilot study may be responsible for absence of significant differences in some of the metabolites. Thus, further analysis is warranted with higher number of animals to confirm our results. In addition, the data should be correlated with plasma levels of female hormones to reveal any possible relationships between the phases of sexual cycle and metabolomic changes.

Conclusions

An understanding of the pathophysiologic background plays a key role in precise asthma management. Metabolomic profiling may offer a novel platform for understanding pathogenesis of asthma, explore new therapeutic targets or search for biomarkers of treatment effectiveness. In our model of allergic asthma, ^{1}H NMR spectroscopy revealed significant changes in the plasma metabolites the alterations of which can be bridged into energy metabolism, activated immune response, protein catabolism etc. These metabolomic profiles were for the first time compared between males and females. We presume that detection of sex-related differences in the metabolome may be helpful for translational applications “from the bench to the bedside” to set targeted diagnostic and treatment approaches in asthma in the context of personalized medicine.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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