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Original Article

Sphingolipid profiles and their relationship with inflammatory factors in asthmatic patients of different sexes

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Abstract

Background: Asthma is a heterogeneous disease with distinct prevalence and manifestation between sexes. This study was to identify sex-specific features of asthma via metabolomic analysis of sphingolipids.

Methods: Forty-two asthma patients (27 women and 15 men) admitted to the Peking University Third Hospital from January 2015 to December 2015 were enrolled. Peripheral venous blood was collected for metabolomic analysis by targeted liquid chromatography-mass spectrometry. Sex hormones(estradiol, progesterone, testosterone, and androstenedione) and multiple inflammatory factors (periostin, leptin, IgE, IL-4, IL-5, IL-10, IL-13, IL-17A, and IFN-γ) were also assessed. The eosinophil percentage in induced sputum was also detected. All these data were applied to comparative analysis between sexes.

Results: Testosterone was negatively related to periostin ($\rho = -0.420$, $P = 0.009$) and IL-5 ($\rho = -0.540$, $P = 0.012$), while estradiol was positively related to the blood eosinophil percentage ($\rho = 0.384$, $P = 0.025$). Among the eighteen species of sphingolipids detected in the 42 patients, five ceramide (Cer) species (Cer16:0, Cer:20:0, Cer22:0, Cer24:0, and Cer26:0) and one sphingomyelin (SM) species (SM38:0) were significantly higher in male than in female patients. Further investigation found that the correlation between Cer20:0 and IL-5 was positive in males ($\rho = 0.943$, $P = 0.005$) but negative in females ($\rho = -0.561$, $P = 0.030$).

Conclusions: Testosterone was negatively correlated with eosinophil inflammatory factors, but estradiol was positively correlated. Male asthma patients had higher ceramide and sphingomyelin levels than female patients. Different sexes had opposite correlations with ceramide and IL-5, respectively, suggesting that therapeutic strategies targeting ceramide should be different between sexes.

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Introduction

Asthma is a heterogeneous disease characterized by chronic airway inflammation and reversible airflow obstruction.[1] Asthma phenotypes are variable, and their underlying pathophysiological mechanisms and treatment are distinct. Considerable efforts have been devoted to identifying phenotype-specific biomarkers and customizing treatment.

Sex disparity has been well established in asthma and changes throughout life.[2] As adults, women have an increased asthma prevalence compared to men. Epidemiological data show that women are more likely to have a later onset of asthma and a more severe phenotype than men.[3] Animal studies show that estrogen increases and testosterone decreases type 2 airway inflammations, but the mechanisms remain unclear.[4,5] Therefore, understanding gender disparity in adult asthma is important for providing effective education and personalized management plans for patients with asthma throughout their lives.

Sphingolipids are a family of lipids with sphingosine as the backbone and includes ceramides (Cer) and sphingomyelins (SM). Sphingolipids represent an important structural component of cell membranes and are instrumental to the regulation of membrane fluidity, signal transduction, and other pathophysiological processes.[6] Genome-wide association studies have identified The ORM1 (*Saccharomyces cerevisiae*)-like protein 3(ORMDL3), a protein of the endoplasmic reticulum, as a significant asthma risk factor. ORMDL3 can negatively regulate serine palmitoyl-coenzyme A (CoA) transferase (SPT), the rate-limiting enzyme for sphingolipid synthesis.[7] Owing to this finding, extensive attention has been given to sphingolipid metabolism in asthma.

Studies have found a close correlation between sex and sphingolipid metabolism. Rauschert et al.[8] showed significant sex-specific differences in plasma sphingomyelin concentrations in young adults. Michelle et al.[9] reported that women had significantly higher serum levels of ceramide and dihydroceramide species than men, and the levels increased dramatically with age. However, to our knowledge, no studies have examined sex differences in sphingolipid metabolism in asthma patients. We hypothesized that the levels of sphingolipid metabolites may serve as biomarkers or determinants of sex disparity in adults with asthma.

In this pilot study, we analyzed the metabolomic profiles of adult asthma patients and sought to identify differences in sphingolipid metabolism between sexes and to evaluate whether sex hormones affect sphingolipid metabolism and asthma pathogenesis.

Methods

Ethical approval

This single-center cross-sectional study was approved by the Research Ethics Committee of Peking University Third Hospital (No. 2014071). All procedures were in accordance with the *Declaration of Helsinki*. All participants were informed about the study protocols and signed informed written consent forms.

Study population

Forty-two adult patients (27 women and 15 men) admitted to the Department of Respiratory in Peking University Third Hospital from January 2015 to December 2015 were enrolled in the study. Patients who met the diagnostic criteria for asthma, as specified on the 2014 Global Initiative for Asthma guidelines were included. Subjects were excluded if they had acute exacerbation of asthma, bronchiectasis, pneumonia, chronic obstructive pulmonary disease, obstructive sleep apnea-hypopnea syndrome, severe cardiovascular disease, acute or chronic respiratory failure, or malignant tumors.

Collection of demographic and clinical data

Demographic data, including sex, age, body mass index (BMI), smoking history, and asthma control test (ACT) scores, were recorded. All subjects underwent standard pulmonary function tests using spirometry (Elite series, MGC Diagnostics, St Paul, MN, USA). The predicted % forced expiratory volume (FEV) in 1 second (FEV1%pred) and FEV1/forced vital capacity (FVC) values were recorded. The eosinophil count was measured as part of routine peripheral blood testing, and 2 mL of peripheral blood was collected to analyze

the serum phospholipid profile. Induced sputum was also obtained for total and differential cell counts.

Serum sample collection

Two mL blood samples were obtained after an 8- to 12-hour overnight fast. Blood samples were allowed to clot at room temperature for 30 min and then centrifuged at 1500×*g* for 5 min, and serum was stored at −80 °C until analysis.

Preparation of sputum for cytology analysis

Sputum induction and preparation for cytology analysis were performed according to the methods described in our previous publication.[10] Specifically, the patients were instructed to gargle with water and blow their nose before nebulization. Then they inhale nebulized 3% sodium chloride and expectorate into a sterile sputum container after 20–30 min. An equivalent volume of 0.4% (w/v) dithiothreitol (DTT) was added, and the sample was incubated at 37 °C for 30 min. The sample was centrifuged for 5 min at 1500×*g*. The cells were sorted after Wright-Giemsa staining.

Measurement of inflammatory factors

Total serum IgE levels were determined by ELISA (BioLegend). Cytokines including IL-4, IL-5, IL-10, IL-13, IL-17A, and IFN-γ were tested by ELISA (Multi-Analyte Flow Assay Kit, BioLegend). Serum leptin levels were tested by radioimmunoassay, and periostin levels were detected by ELISA. All experiments were performed according to the manufacturers' instructions. Half of the OD450 value of the lowest detectable standard was used as the lower limit of detection.

Serum sex steroid hormones

All serum sex hormones, including estradiol, progesterone, testosterone and androstenedione, were analyzed by chemiluminescence immunoassay at Peking University Third Hospital Reproductive Centre. The lower limits of detection for the assays were as follows: estradiol 73.4 pg/mL, progesterone 0.64 ng/mL, testosterone 0.69 ng/dL, and androstenedione 1.05 μg/dL. For the purpose of mathematical analyses, individuals with measurements below the limit of detection were considered to have the value of the lower limit of detection.

Liquid chromatography-mass spectrometry

A total of 18 sphingolipids were analyzed, including Cer (8 species), SM (10 species).

Lipid was extracted from patients' serum as described previously.[10] Briefly, 100 μL of serum was mixed with 400 μL of 75% ice-cold methanol (with 10 μL of the lipidomic standard mixture mother liquor added) and the mixtures were ultrasonicated for 2 min followed by addition of 1 mL Methyl tertiary butyl ether (MTBE). The samples were vortexed for 1 hour at room temperature before 250 μL of H_2O was added. After stratification for 10 min, the samples were centrifuged at 12,000×*g* at 4 °C for 10 min to allow fat-soluble substances to distribute into the upper layer. The upper layer was removed to and dried in a new tube. Followed by adding 100% methanol, the sample was filtered into a vial. The lipid omic internal standard mixture consisted of Cer (d18:1/17:0), sphingosine (So, d17:1), SM (d18:1/17:0), and sphingosine-1-phosphate (S1P, d17:1), 20 μg/mL each.

Serum metabolite components were analyzed by liquid chromatography-mass spectrometry (LC-MS) on the Waters ACQUITY UPLC system (Milford, MA). UPLC BEH C18 columns (1.7 μm, 100 × 2.1 mm id) were used with the setting of temperature at 25 °C, flow rate at 0.25 mL/min. The A liquid was 60% acetonitrile (5 mmol/L ammonium acetate) and the B liquid wasf isopropanol/acetonitrile (9/1).The sequential setup for B liquid gradient within a 20 min period is 15% for 0–3 min, 15%–99% for 3–15 min, 99% B for 15–17 min, 99%–15% for 17–19 min min, and 15% for the last minute. The AB Sciex 5500 QTRAP mass spectrometer with a Turbo Ion Spray electrospray ionization (ESI) ion source was used with a multiple reaction monitoring (MRM) scan mode and and ion source parameters as followings CUR = 40 psi, GS1 = 30 psi, GS2 = 30 psi, IS = −4500 V, CAD = MEDIUM, TEMP = 350 °C. The detailed scanning strategy used for multiple reaction monitoring is shown in supplementary Table S1.

Statistical analysis

Normally distributed data are presented as the median ± standard deviation (SD), whereas non-normally distributed data are presented as the median (interquartile range, IQR). A *t*-test was used to compare the data with a normal distribution, and the

Mann–Whitney U-test was used to compare the data with a nonnormal distribution. Spearman correlation analysis was used to detect correlations of nonnormally distributed data. All statistical analyses were performed using the SPSS 22.0 software package (IBM Corp., USA). Univariate P-values < 0.05 were considered to indicate statistical significance.

Results

Subject demographics

Forty-two patients (27 women and 15 men) were included in current study. The two sexes had similar ages, BMIs, family histories of asthma, complications (allergic rhinitis and eczema), ACT scores, and pulmonary function values (FEV1/FVC and FEV1%pred). The total number of smokers was significantly higher in the male group than in the female group. The basic clinical information of the study population is shown in Table 1.

Comparison of sex hormones between sexes

Estradiol levels were significantly higher in females than in males, while testosterone levels were higher in males. Progesterone levels were higher in females than in males, but the difference between sexes was not significant. Likewise, androstenedione levels were higher in males than in females, but the difference was also not significant (Supplementary Table S2).

Comparison of inflammatory factors between sexes

As shown in Supplementary Table S3, serum leptin levels were significantly lower in males than in females. However, there were no significant differences between sexes in the serum level of IgE, serum periostin level, blood eosinophil percent, or sputum eosinophil percentages. Due to insufficient serum samples, we only tested cytokines in 11 male patients and 19 female patients, and there was no difference in cytokines between the sexes (Supplementary Table S4).

Correlation between inflammatory factors and sex hormones

We further assessed the relationship between sex hormones and inflammatory factors and cytokines in different genders, and the results are shown in Table 2. We found that testosterone was negatively related to periostin and IL-5, while estradiol was positively related to the blood eosinophil percentage. In males, this correlation was not significant (Supplementary Table S5), but in females, testosterone was significantly negatively related to periostin and IL-5, while estradiol was positively related to blood and sputum eosinophil percentage (Supplementary Table S6).

Comparison of sphingolipids between sexes

Five species of ceramides were significantly higher in males than females: Cer16:0, Cer20:0, Cer22:0, Cer24:0, and Cer26:0 (Supplementary Table S7). For

Table 1
Basic demographic information in both sexes.

Characteristics	Male ($n = 15$)	Female ($n = 27$)	Statistical values	P
Age (years)	38.8 ± 15.3	40.4 ± 11.8	−0.380[a]	0.706
BMI (Kg/m^2)	24.6 ± 3.0	23.4 ± 3.2	1.273[a]	0.213
Smoking history	7 (46.7)	1 (3.7)	11.543[b]	0.003
Family history of asthma	3 (20.0)	7 (25.9)	0.187[b]	0.995
Age at onset of asthma (years)	27.9 ± 17.9	30.2 ± 14.2	−0.450[a]	0.655
Complications (allergic rhinitis)	10 (66.7)	18 (66.7)	0.000[b]	1.000
Complications (eczema)	0	2 (7.4)	1.167[b]	0.530
Asthma control test	18.0 ± 6.1	17.4 ± 5.8	0.308[a]	0.760
FEV_1/FVC	75.6 ± 10.1	74.0 ± 11.5	0.406[a]	0.687
$FEV_1\%_{pred}$	93.0 ± 15.6	94.4 ± 18.7	−1.072[a]	0.290

Data were shown as mean ± standard deviation or n (%). BMI: body mass index; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity.
[a] t value.
[b] Chi-square value.

Table 2
Correlation between inflammatory factors and sex hormones.

Inflammatory factors	Testosterone, ρ (P)	Estradiol, ρ (P)
Blood eosinophils		0.384 (0.025)
Periostin	−0.420 (0.009)	
IL-5	−0.540 (0.012)	

IL: interleukin. The sex hormone data did not conform to a normal distribution, and Spearman correlation analysis was used. The correlation coefficient is ρ.

sphingomyelins, SM38:0 was significantly higher in males than in females (Supplementary Table S8).

Since there is difference in the proportion of smokers between the two groups, we compared the sphingolipids in nonsmokers only.Four species of ceramides (Cer16:0, Cer22:0, Cer24:0, and Cer26:0) and SM38:0 were still significantly higher in non-smoking males than in females (Fig. 1 and Supplementary l Table S9).

Correlation between inflammatory factors and sphingolipids

Furthermore, we analyzed the relationships between inflammatory factors and sphingolipids. In male patients, the correlation of Cer16:0 and Cer20:0 with IL-5 was significantly positive (Table 3). In females, there was a negative correlation between Cer16:0 and IL-10, Cer20:0 and IL-5 and between Cer20:0 and periostin; in addition, there was a positive correlation between SM38:0 and leptin (Table 4).

Discussion

Although asthma is more common in boys than in girls, adult women have a higher prevalence and morbidity from asthma than adult men. There are

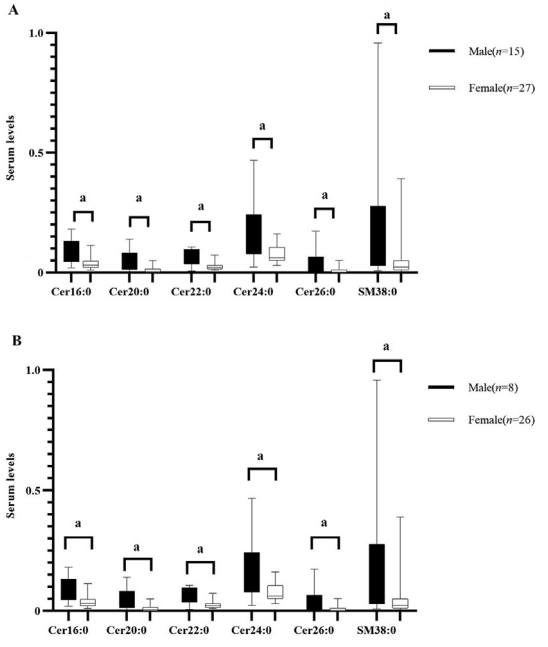

Fig. 1. Comparison of serum Ceramides(Cer) and sphingomyelin(SM) levels between male and female asthma patients. Comparison in total subjects between male and female patients (A); Comparison between nonsmoking patients only (B). [a]: $P < 0.05$.

Table 3
Correlation between inflammatory factors and sphingolipids in males.

Inflammatory factors	Cer16:0, ρ (P)	Cer20:0, ρ (P)
IL-5	0.829 (0.042)	0.943 (0.005)

The ceramide data did not conform to a normal distribution, and Spearman correlation analysis was used. The correlation coefficient is ρ.

reports of worsen asthma during pregnancy[11] or the perimenstrual period.[12] Changes in sex hormone levels during the life course may partly explain this disparity. Clustering analysis shows that women are likely to have more severe, less corticosteroid-responsive phenotypes of asthma than men.[13] Animal studies have shown that estrogen increases and testosterone decreases type 2-mediated airway inflammation, but how ovarian hormones and testosterone regulate airway inflammation pathways remains to be elucidated.[14]

In our study, the correlation between type 2 inflammatory factors (IL-5 and periostin) and testosterone was negative, especially in female patients with asthma, consistent with previous animal studies. The US National Health and Nutrition Examination Survey (NHANES) reported that higher levels of serum free testosterone were associated with a lower risk of asthma in women but not in men.[15] In a murine model, testosterone was shown to inhibit the secretion of IL-5 and IL-13 by reducing the expression of lung type 2 innate lymphoid cells (ILC2s).[5] Testosterone was shown to decrease dust mite-induced eosinophilic inflammation in the lungs, partially through androgen receptor (AR) signaling.[16]

We also found that serum leptin levels in female patients were significantly higher than those in male patients. Leptin can activate the extracellular regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) signal transduction pathways and promote eosinophil-mediated inflammation.[17] Thomas et al.[18] found that serum leptin levels were positively correlated with bioavailable estrogen in postmenopausal women and negatively correlated with bioavailable testosterone levels in men.

Table 4
Correlation between inflammatory factors and sphingolipids in females.

Inflammatory factors	Cer16:0, ρ (P)	Cer20:0, ρ (P)	SM38:0, ρ (P)
IL-5		−0.561 (0.030)	
IL-10	−0.539 (0.017)		
Periostin		−0.481 (0.041)	
Leptin			0.405 (0.040)

The ceramide and sphingomyelin data did not conform to a normal distribution, and Spearman correlation analysis was used. The correlation coefficient is ρ.

Previous studies have shown that sphingolipids play a role in the pathogenesis of asthma.[19] In our study, we found that five kinds of ceramides and one kind of sphingomyelin were significantly higher in males than in females. Previous studies have found that sex hormones impact sphingolipid metabolism. For example, Sukocheva O et al.[20] reported that sphingosine kinase (SphK) is a lipid kinase that catalyzes the phosphorylation of sphingosine to S1P, and estrogen is a strong activator of SphK in human breast cancer cells. These relationships indicate that estrogen may affect sphingolipid metabolism by regulating the SphK. Frode Norheim et al.[21] reported that testosterone suppresses the relative levels of specific kinds of ceramides in male mice. Thus, we assumed that sphingolipid sex-based differences may contribute to sexual disparities in asthma.

Basic studies have shown that a series of inflammatory reactions in asthma, including activation of FceR1 receptors to activate mast cells, airway smooth muscle contraction, airway hyperresponsiveness and airway remodeling, can be regulated by sphingolipids.[22,23] Sphingomyelin forms ceramide and sphingosine through catabolism, and sphingosine is phosphorylated to form S1P. The ceramide-sphigosine-S1P pathway promotes lymphocyte recruitment, neutrophil infiltration, mononuclear macrophage activation, and mast cell activation. These immune responses are closely related to the occurrence of asthma.[24] Sphingomyelin decomposition and ceramide formation also play an important role in the regulation of T cell functions, as they can mediate the CD3/CD28 costimulatory pathway, promote T cell activation, participate in CD95-induced T cell apoptosis, and promote T cell differentiation and cytokine production.[25] When we separately analyzed the correlation between sphingolipids and inflammatory factors in different sexes, we found a positive correlation between Cer20:0 and IL-5 in male patients but a negative correlation in female patients. It is not clear why the correlation between ceramide and inflammatory factors was opposite in patients of different sex. A previous study proved that testosterone affected the enzymes in the sphingolipid metabolism pathway to regulate the level of sphingolipids,[26] which may be an explanation for this phenomenon. Therapies targeting ceramide in different groups of different sex may be a future direction for the individualized treatment of asthma.

There are some limitations of this study. First, this study was a cross-sectional study, and the number of cases included was small. Second, women's sex hormone levels fluctuate. Different factors, such as pregnancy, breastfeeding, menopause, and oral contraceptives, can affect the levels of estrogen and progesterone. This study did not collect such information from female patients. In the future, a larger sample with targeted metabolic data is needed, and more severe asthma patients should be included to reveal the role of sphingolipids in asthma airway inflammation. In addition, varieties of enzymes participate in the metabolic pathways of sphingolipids and play an important regulatory role. Future research should include these enzymes.

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Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cdtm.2021.04.002.

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