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Serum estradiol concentration, estradiol-to-progesterone ratio and sputum IL-5 and IL-8 concentrations are increased in luteal phase of the menstrual cycle in perimenstrual asthma patients

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KEY WORDS

perimenstrual asthma; bronchial hyperreactivity; induced sputum; 17 β -estradiol; progesterone; testosterone; prolactin

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Introduction

Asthma is a chronic inflammatory lung disease leading to the pathologic triad of bronchial hyperresponsiveness, intermittent airway obstruction and chronic bronchial inflammation. Despite the fact that this definition has been approved and implemented by the authorities in all existing global asthma guidelines, there has been growing support for the concept that asthma consists of multiple phenotypes, which are easier to de-

Summary

Background. The results of the research on perimenstrual asthma (PMA) pathophysiology are inconsistent, and the role of sex hormones remains inconclusive. The aim of the study was to evaluate the influence of serum sex steroid (and other) hormones' concentrations on lower airway inflammation of PMA patients. **Methods.** Thirty-three women of childbearing age diagnosed as: PMA ($n = 13$), non-PMA asthmatics ($n = 10$), and healthy controls ($n = 10$) were prospectively followed for 12 weeks over 2 consecutive menstrual cycles. On the 10th and 26th day of each cycle serum 17 β -estradiol, progesterone, testosterone, androstendion, dehydroepiandrosteron, cortisol, thyroid-stimulating hormone and prolactin were measured, and sputum was induced. Sputum inflammatory cell count and IL-5, -6, -8, -10 concentrations were determined. **Results.** When compared to non-PMA asthmatics, the luteal phase of the cycle in PMA subjects was associated with increased serum estradiol concentration, estradiol-to-progesterone ratio ($p < 0.001$, $p = 0.001$, respectively) and sputum IL-5 and IL-8 concentrations ($p = 0.045$, $p = 0.039$, respectively). Decreased serum testosterone levels ($p < 0.05$) and a trend to increased serum prolactin levels in both phases of the menstrual cycle in PMA subjects were observed. Sputum analysis in PMA patients revealed increased total inflammatory cell count in both phases of the menstrual cycle ($p < 0.05$), when compared to non-PMA asthmatics. **Conclusions.** The luteal phase of the cycle in PMA patients is associated with increased serum estradiol levels with concurrent higher sputum concentration of IL-5 and IL-8. Serum testosterone levels are decreased, and total number of sputum inflammatory cells is increased in PMA patients in both phases of the menstrual cycle.

fine in patients with severe form of the disease (1-3). The Severe Asthma Research Program (SARP), a large network to study severe asthma pathomechanisms, has recently distinguished a female-predominant, late-onset asthma phenotype with cyclically recurrent perimenstrual worsening of the disease symptoms (4). The data from SARP Rao et al (3) aimed to identify specific clinical and immunologic characteristics that might contribute to perimenstrual asthma (PMA) phenotype, suggest that it usually represents a poorly controlled and exacerbation-prone

disease often associated with aspirin sensitivity and lower lung capacity (when compared to other asthma phenotypes). Women with PMA require more bursts of corticosteroids therapy and have higher risk for emergency room visits, hospitalization, admission to intensive care unit (3), intubations and near fatal and fatal events (5). Although PMA has been associated with severe and difficult-to-control asthma, it remains poorly characterized and understood. A host of data suggests that cyclical variations in sex steroid hormones in women influence asthma symptoms, with a worsening during the luteal phase of the menstrual cycle which occurs in one third of patients (3,6). Underlying these exacerbations are either high progesterone and estrogen concentrations (during the luteal phase of the cycle), or the pre-menstrual steep decrease in both hormones levels. It is suggested that sex steroid pathway might play a key role in breaking the threshold, that triggers a clinically apparent bronchial reaction (7). It has been documented that in adult women with stable, well-controlled asthma, PC20 decreases by more than half during the menstrual cycle, with the lowest PC20 occurring at peak estrogen and progesterone levels in the luteal phase (8). The cyclic changes in PC20 have been assigned to abnormal β_2 adrenoreceptor regulation in PMA (8,9). It has been suggested that β_2 adrenoreceptors are influenced by ovarian sex-steroid hormones, the hypothesis confirmed by down regulation of β_2 adrenoreceptors after progesterone administration to PMA women during the follicular phase of the menstrual cycle (9,10). However, female sex hormones have a wide variety of effects beyond the β_2 adrenoreceptor. The progesterone receptor expressed in airway epithelium and progesterone has been proved to inhibit the beat frequency of cilia, which may impact mucociliary clearance during the menstrual cycle (11). Progesterone has been demonstrated to reduce bronchial smooth muscle contractility (12) and to increase eosinophilia-related bronchial hyper-responsiveness (BHR) in sensitised mice (13). Both estradiol and progesterone have been proposed to activate mast cells, and the idea that they have an influence on the symptoms of mast cell-associated disorders has been discussed widely (14). In contrast to human research, animal work on the relationship between estrogens and asthma is conflicting. In mice, estrogen appears to protect against BHR (15), while in humans, high estrogen levels (during pregnancy or the luteal phase of the menstrual cycle) shift skew of the immune system towards a Th2-type response (16). In premenopausal women, these two sex steroids may have additive or synergistic effect on exacerbating asthma, while androgens appear to act anti-inflammatory by decreasing Th2 cell response (17). However, it still remains unclear as to whether fluctuation in sex steroids concentrations or a balance between them is responsible for premenstrual worsening of symptoms. Some of the research on PMA carried out so far suggest, that except sex hormones additional factors must

play role, either further sensitizing the airways or modifying their hormonal effects. Some of these suspected cofactors (such as aging, obesity, atopy) have already been evaluated, yielding conflicting results (1,3,4).

With this in mind, we aimed to investigate whether changes in serum concentrations of sex steroid hormones in the follicular and luteal phase of the cycle influence the severity of lower airway inflammation in patients with PMA, when compared to non-PMA. We also hypothesized that the specific inflammatory response in PMA might be generated by the interaction of other hormones, not directly related with the reproductive system, and therefore evaluated their serum concentration in both phases of the cycle with respect to markers of inflammation present in lower airways of PMA patients.

Materials and methods

Study population

A three-arm (women with PMA vs non-PMA asthmatics vs healthy controls) case-control clinical study was designed, in order to accurately determine the characteristics of airway inflammation in PMA and its relation to changes in selected serum hormone levels. The study was approved by ethics committee of the Medical Faculty, Silesian Medical University, Katowice, Poland. Each study participant provided written informed consent. At the time of enrolment all women completed a health questionnaire, to obtain information on demographic factors, asthma, pregnancy and smoking history, the use of antiasthmatic drugs and comorbidities. All women completed a menstruation card (reporting the first day of menstruation), asthma symptom questionnaire and asthma control test.

After rejection of women with: 1 - poor compliance during the study (n = 2), 2 - inadequate information on PMA / asthma status (n = 4), 3 - the use of exogenous hormones (n = 6), 4 - irregular menstrual cycles (n = 5), or 5 - other reasons (n = 11), 33 premenopausal women were enrolled in the study.

Of these, 13 women recruited from the outpatient pulmonology and gynecology units of the Central Clinical Hospital of the Silesian Medical University and from outpatient allergy clinic fulfilled all entry criteria, and were recruited to PMA group. PMA was diagnosed on the basis of typical clinical history of asthma (mild to severe according to current GINA recommendations (18), confirmed by the positive metacholine challenge test with cyclical clinical asthma worsening during luteal phase, and/or during the first days of menstruation, and/or $\geq 20\%$ reduction of PEF values up to 5 days before or during menstruation, for at least 5 consecutive years. During the study, all PMA patients were all treated according to current GINA guidelines (18). The exclusion criteria for entry were: 1 - age < 18 and > 45, 2 - irregular menstrual cycles (cycle-to-cycle variability >

3 [days]), 3 - use of exogenous hormones in a period shorter than 12 months before entering the study, 4 - pregnancy and/or breastfeeding 12 months prior to and during the study, 5 - reported polycystic ovary syndrome, 6 - cumulative smoking exposure of > 5 pack-years, 7 - history of infection for 4 weeks prior to, and in course of the study, 8 - any chronic diseases other than asthma, 9 - poor patient compliance.

Ten non-PMA asthmatics with typical clinical history of asthma (mild to severe according to current GINA recommendations (18) confirmed by the positive metacholine challenge test), with no perimenstrual symptoms worsening (defined as above), entered the study.

The study involved 10 healthy volunteers with no history of asthma or other chronic disease. Exclusion criteria for both non-PMA asthmatics and healthy control women were the same as for the PMA patients.

The study protocol

All women included in the study have prospectively been followed for 12 weeks, over 2 complete consecutive menstrual cycles. Asthma symptom questionnaire including the use of antiasthmatic drugs (daily) and the peak expiratory flow measurement (twice daily) have been recorded, during both menstrual cycles. Serum 17β -estradiol, progesterone, testosterone, androstendion, dehydroepiandrosteron, cortisol, thyroid-stimulating hormone and prolactin were measured, and sputum inductions were determined in the 10th and the 26th day of each of the two cycles. Sputum inflammatory cell count and concentration of IL-5, IL-6, IL-8 and IL-10 were measured by sandwich ELISA test. The values obtained from measurements during two successive menstrual cycles have been subjected to statistical analysis.

Measurements of hormone levels in serum

Venous blood samples were collected between 8.00 and 10.00 am, after an overnight fast.

Serum 17β -estradiol, progesterone, testosterone, androstendion, dehydroepiandrosteron, cortisol, thyroid-stimulating hormone and prolactin concentrations were measured by using electrochemiluminescence immunoassay (Cobas e601, Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's protocol.

Lung function and metacholine challenge test

Forced expiratory volume in 1s (FEV_1) and forced vital capacity (FVC) were measured with dry spirometer (MasterLab, Jaeger, Germany) according to the recommendations of the European Respiratory Society (19). Bronchial hyperreactivity (BHR) in metacholine challenge testing was assessed according to the ap-

proved consensus recommendations (20) by inhaling increasing concentrations of metacholine from ultrasonic nebulizer device (Thomex MB, Medbryt, UK). The provocative concentration required to achieve the FEV_1 fall by $\geq 20\%$ from its original value was determined by linear interpolation.

Sputum induction and analysis

Sputum was induced by hypertonic saline inhalation (3%, 4% and 5% for 7 min) by an ultrasonic nebulizer device (Thomex MB, Medbryt, UK) and processed according to a method previously described (21). Aliquots of the supernatant fluids were stored at -80°C until further analysis.

Measuring cytokine levels

Sputum IL-5, IL-6, IL-8 and IL-10 concentrations were measured by ELISA kits (R&D Systems, McKinley Place N.E. Minneapolis, USA) according to the manufacturer's protocol.

Statistics

Statistical evaluation was performed with software package (Statistica 6.0). Descriptive statistics are presented as medians and ranges. Kolmogorov-Smirnov test was used to test variables for normal distribution. As our data are generated from small patient populations and these data that are not normally distributed, we used the Mann-Whitney U-test for comparison between non-parametric results. Inter-group comparisons were performed using Kruskal-Wallis ANOVA and Mann-Whitney U test. Spearman's rank correlation coefficient test was used to examine the association between functional data and hormone levels. P values of < 0.05 were accepted as statistically significant.

Results

Baseline characteristics

Baseline characteristics of the study subjects are summarized in **table 1**. Groups were comparable with respect to age and number of pregnancies. Patients with PMA were more likely to be classified as severe asthma compared to non-PMA asthmatics. Four women (30.7%) in PMA group were classified as severe asthma (compared to 10% in non-PMA asthmatics). Ten (76.9%) of the PMA subjects had persistent asthma and the remaining three (23.1%) had intermittent asthma. "Average asthma control measured by asthma control test was significantly ($p = 0.011$) worse in PMA group, than in non-PMA asthmatics (**table 1**) and the average daily use of inhaled steroids in PMA patients was significantly higher than among non-PMA asthmatics (800 μg vs 400 μg BUD/day, $p < 0.001$). In PMA patients, asthma

Table 1 - Baseline characteristics of the study sample.

	PMA group No: 13	non-PMA asthmatics No: 10	healthy control No: 10
Age [years]	29.1 (20-39)	30.1 (21-43)	32.1 (22-45)
FEV ₁ [% predicted] ^{1,2}	87.1 (36.3-121.3)	95.3 (81.6-115.7)	105.4 (108.3-119)
FEV ₁ %VC [%] ^{1,2}	71.9 (41.2-98.1)	80.1 (59.6-98.4)	93.1 (81.8-97.5)
ACT [pts] ²	17 (12-25)	21 (19-25)	not applicable
PC _{20 metacholine} [mg/ml] ^{1,2}	0.1 (0.1-4.3)	1.9 (0.1-6.9)	> 25
No of pregnancies	2.1 ± 0.89	1.9 ± 0.8	2.3 ± 0.9

Data are presented as medians and ranges (maximal and minimal values are in brackets).

¹p < 0.05 for inter-group comparison.

²p < 0.05 between PMA and non-PMA asthmatics.

FEV₁ - forced expiratory volume in 1s (percentage of predicted value).

VC - vital capacity.

ACT - asthma control test.

PC₂₀ - the first provocative concentration of metacholine that caused a 20% fall in FEV₁.

control test was significantly ($p = 0.032$) worse during perimenstrual period (ACT = 14 pts) than in the remaining days of the cycle (ACT = 22 pts) and this was accompanied by higher daily use of inhaled steroids (1600 µg [perimenstrual period] vs 800 µmcg [other days of the cycle] BUD/day, $p < 0.001$). Both worsening of asthma symptoms and the increased daily use of inhaled corticosteroids have not been present in non-PMA asthmatic group throughout the menstrual cycle". As anticipated, the pulmonary function of PMA patients was lower than in non-PMA asthmatics and healthy controls. The FEV₁% predicted and FEV₁%VC was lower in PMA group when compared to non-PMA asthmatics ($p < 0.05$ for both variables) and the mean values for PC₂₀ were also significantly lower in PMA group than in non-PMA-asthmatics ($p = 0.003$). In both PMA group and non-PMA asthma group, there was one aspirin-sensitized patient. In the control group, no one was allergic to aspirin.

Serum hormone concentrations

Serum hormone concentrations in PMA patients, non-PMA asthmatics and healthy controls are presented in **table 2**. When compared to non-PMA asthmatics, the luteal phase of the cycle in PMA subjects was associated with increased serum estradiol concentration ($p < 0.001$; **figure 1**) and increased serum estradiol-to-progesterone ratio ($p = 0.001$). Patients with PMA

had decreased serum testosterone levels in both, follicular ($p = 0.011$; **figure 2**), and luteal ($p = 0.019$; **figure 3**) phase of the cycle. The estradiol / testosterone ratio in luteal phase of the cycle in PMA patients was significantly higher, when compared to

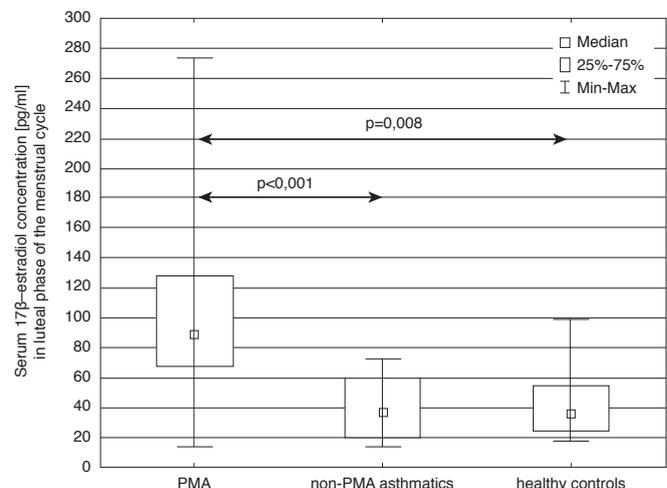
Figure 1 - Serum 17β-estradiol concentration in luteal phase of the menstrual cycle in groups studied.

Table 2 - Serum hormones concentrations in follicular (FF) and luteal (LF) phase of the menstrual cycle in groups studied.

Hormone	PMA		non-PMA asthmatics		healthy controls	
	FF	LF	FF	LF	FF	LF
17β-estradiol (pg/ml)	42.7 (18.1-104.2)	89.6 ^{1,2} (14.2-273.8)	22.8 (10.4-60.0)	37.1 (12.6-72.1)	40.1 (10.1-61.2)	36.1 (17.4-98.4)
Progesterone (ng/ml)	0.9 (0.1-4.2)	0.9 (0.1-6.4)	0.7 (0.2-3.7)	1,5 (0.1-4.2)	0.2 (0.1-5.2)	0.4 (0.1-6.4)
Testosterone (ng/ml)	27.8 ^{1,2} (0.8-77.5)	24.7 ^{1,2} (1.6-62.6)	46.2 (12.4-75.4)	47.0 (21.0-87.4)	38.9 (15.3-64.7)	43.7 (11.6-72.8)
Androstendion [ng/ml]	3.7 (0.9-4.3)	2.5 (0.4-5.3)	0.8 (0.1-2.1)	2.0 (0.8-4.2)	2.7 (0.2-5.1)	1.1 (0.2-1.6)
Dehydroepiandrosteron [ng/ml]	11.1 (3.4-16.4)	9.5 (0.1-20.1)	13.6 (4.7-25.2)	15.9 (3.9-20.1)	10.1 (2.2-16.8)	17.8 (5.3-28)
Cortisol [ng/dl]	18.8 (3.1-40.8)	12.6 (6.9-25.8)	14.4 (7.0-29.7)	21.6 (2.0-39.6)	24.3 (5.2-30.1)	20.5 (8.1-50.8)
Thyroid-stimulating hormone (TSH) [mIU/ml]	2.2 (0.8-3.1)	2.2 (0.4-3.7)	1.8 (1.1-2.9)	2.1 (1.6-3.4)	1.6 (0.5-3.1)	1.9 (0.9-3.3)
Prolactin [ng/ml]	23.8 (0.3-31.7)	21.6 (7.5-27.9)	17.6 (8.4-19.1)	10.2 (4.3-20.1)	16.9 (5.8-20.9)	15.1 (4.8-19.8)

Data are presented as medians and ranges (maximal and minimal values are in brackets)

¹p < 0.05 for inter-group comparison in corresponding phase of the menstrual cycle.

²p < 0.05 between PMA and non-PMA asthmatics in corresponding phase of the menstrual cycle.

non-PMA asthmatics, (p < 0.001). A trend to increased serum prolactin levels in both follicular (p = 0.076) and luteal phase of the menstrual cycle (p = 0.09) in PMA subjects (compared to

non-PMA asthmatics and healthy controls) was observed (**table 2**). No association between serum hormones levels and respiratory variables was found.

Figure 2 - Serum testosterone concentration in follicular phase of the menstrual cycle in groups studied.

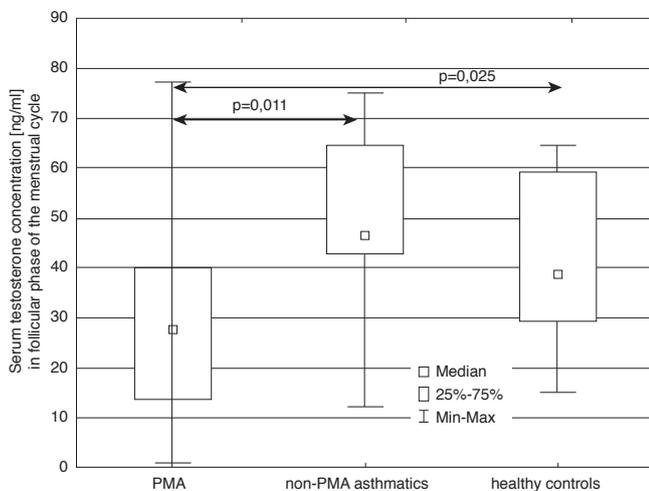
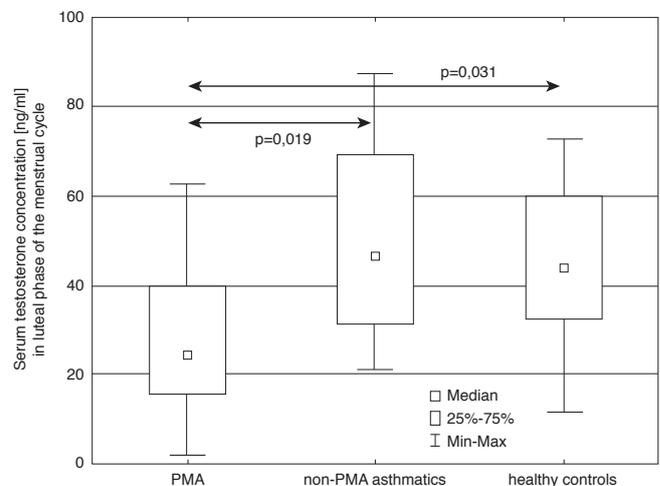


Figure 3 - Serum testosterone concentration in luteal phase of the menstrual cycle in groups studied.



Induced sputum cell count and cytokine levels

Sputum analysis in PMA patients revealed significantly increased total inflammatory cell count in both phases of the menstrual cycle when compared to non-PMA asthmatics and healthy controls ($p < 0.05$; **table 3**). No significant differences in differential sputum cell count between PMA and non-PMA asthmatics were noticed ($p > 0.05$; **table 3**). Measurable concentrations of IL-5, IL-8 and IL-10 were found in all sputum samples assayed. Sputum IL-6 levels were undetectable in one person in non-PMA asthmatic. Sputum cytokine levels in groups studied are presented in **table 3**. The luteal phase of the cycle in PMA subjects was associated with significantly increased sputum IL-5 ($p = 0.045$; **figure 4**) and IL-8 ($p = 0.039$; **figure 5**) concentrations, when compared to non-PMA asthmatics and healthy controls ($p = 0.008$; $p < 0.001$, respectively). No differences ($p > 0.05$) were noticed in sputum levels of IL-6 and IL-10 between studied groups (**table 3**).

Discussion

Our study adds the key evidence of the immuno-hormonal cross-talk in the pathogenesis of PMA, which is now considered one

of the burdensome asthma phenotypes. Although the pathogenesis of asthma remains incompletely understood, it is now clear that it comprises several different endotypes, and hence there is an apparent shift in asthma perception that focuses on matching the therapy with the presence of specific, corresponding biomarkers. There is now an array of evidence demonstrating a female predominance in asthma prevalence by adulthood (22,23). In a large population-based cohort study, women over the age of 35 years were shown to have a 20% higher risk of developing asthma, and demonstrated higher predominance of non-allergic asthma than men (23). In addition, women are especially burdened with hard-to-treat, corticosteroid-resistant asthma (3-5) and studies conducted so far revealed that asthma-related deaths were up to 30% higher for women (22,24). Female predominance in asthma has generated an interest in studying the sexual dimorphism in the immune system, and the role of sex hormones on airway pathophysiology. A number of clinical and epidemiological studies demonstrated that 30-40% of asthmatic women experience worsening of asthma symptoms during the perimenstrual phase of the cycle (being the time point of large sex hormone fluctuations) (3,6,8,9). However, the role of estrogen and progesterone in PMA exacerbations remains conflicting and inconclusive, with studies reporting both the protective

Table 3 - Sputum variables in follicular (FF) and luteal (LF) phase of the menstrual cycle in groups studied.

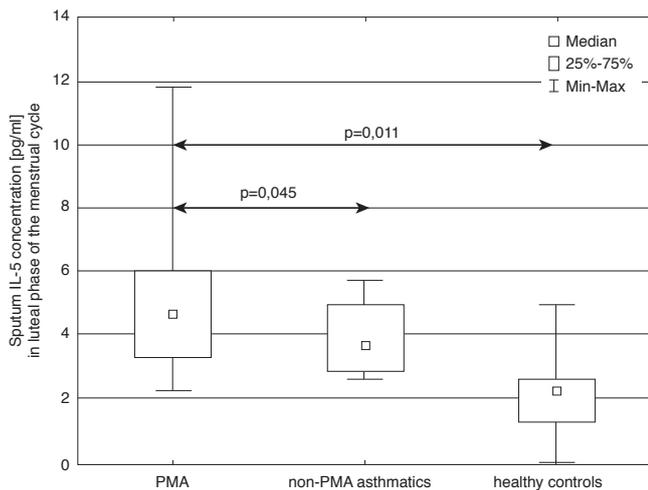
Variable	PMA		non-PMA asthmatics		healthy controls	
	FF	LF	FF	LF	FF	LF
Total cell count [nx 10 ⁶ /g]	1.9 ^{1,2} (0.6-6.2)	1.7 ^{1,2} (0.5-3.8)	1.2 (0.4-4.1)	1.4 (0.6-5.2)	0.9 (0.1-1.4)	1.1 (0.1-1.3)
Neutrophils [nx 10 ⁶ /g]	0.7 (0.14-3.1)	0.6 (0.2-3.9)	0.9 (0.4-5.5)	0.7 (0.1-2.9)	0.7 (0.6-1.9)	0.8 (0.7-3.5)
Eosinophils [nx 10 ⁶ /g]	0.1 (0.0-0.75)	0.04 (0.0-0.7)	0.05 (0.0-1.0)	0.06 (0.1-0.8)	0.06 (0.0-0.7)	0.03 (0.0-0.8)
Macrophages [nx 10 ⁶ /g]	1.0 (0.18-2.5)	1.0 (0.31-3.2)	1.1 (0.3-3.9)	0.8 (0.2-4.1)	0.8 (0.6-1.9)	0.7 (0.5-2.2)
IL-5 [pg/ml]	3.1 (1.23-7.8)	4.7 ^{1,2} (2.24-11.8)	3.1 (2.29-7.1)	3.5 (2.5-5.6)	2.7 (0.5-6.6)	2.4 (0.4-4.9)
IL-6 [pg/ml]	121.5 (56.8-170.2)	87.5 (67.1-111.8)	101.9 (23.5-131.0)	76.9 (0.0-186.2)	66.5 (10.8-95.3)	71.0 (6.5-153.9)
IL-8 [pg/ml]	335.4 (197.1-1004.5)	404.7 ^{1,2} (164.2-901.4)	302.3 (118.2-479.7)	301.6 (94.2-545.4)	227.6 (102.4-450.4)	216.7 (99.4-469.8)
IL-10 [pg/ml]	41.9 (2.5-110.1)	62.8 (15.6-90.8)	39.8 (21.4-70.5)	29.9 (1.8-59.3)	51.7 (18.6-89.0)	45.5 (4.2-101.6)

Data are presented as medians and ranges (minimal and maximal values are in brackets).

¹ $p < 0.05$ for inter-group comparison in corresponding phase of the menstrual cycle.

² $p < 0.05$ between PMA and non-PMA asthmatics in corresponding phase of the menstrual cycle.

Figure 4 - Sputum IL-5 concentrations in luteal phase of the menstrual cycle in groups studied.



(12,15) and deteriorating (9-11,13,14) effect on lung function and airway inflammation in these patients.

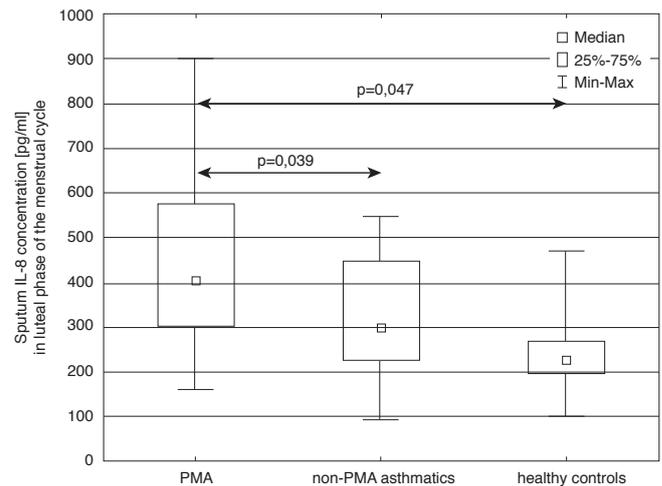
Different methodologies and lack of uniformity between studies approaches used to investigate the hormonal hypothesis in PMA significantly limit drawing reliable conclusions. Finally, although some of the clinical data support the role of sex hormones, the results are confounded by many other factors. In a recent large-scale analysis of women with severe asthma, PMA has additionally been associated with aspirin sensitivity, higher BMI, gastroesophageal reflux and lower IgE levels (3).

As the hormonal impact in PMA patients seems complex and not completely understood, in our study we aimed to investigate the relation between serum sex hormones levels and lower airway inflammatory markers in the pre-selected and well-defined group of patients with previously confirmed, recurrent PMA symptoms.

According to our knowledge, this is the first study that demonstrated that PMA is associated with significantly increased serum estradiol levels and significantly increased estradiol-to-progesterone ratio in luteal phase of the cycle when compared to non-PMA asthmatics. This is also the first study in which lower serum testosterone levels and the trend to increased serum prolactin levels in both phases of the menstrual cycle in PMA (when compared to non-PMA asthmatics) have been observed. Our analysis also supports evidence of the increased sputum total inflammatory cell count, along with the increased IL-5 and IL-8 concentrations in sputum of PMA patients in luteal phase of the menstrual cycle.

The result of our study, which revealed decreased serum levels of testosterone in both phases of the cycle in PMA patients, is

Figure 5 - Sputum IL-8 concentrations in luteal phase of the menstrual cycle in groups studied.



consistent with some earlier (25,26), but not with all (27,28) studies. The association between androgens and PMA has weakly been examined yet. Testosterone is secreted by ovaries and by the adrenal gland, and it appears to act as an immunosuppressive and anti-inflammatory, by decreasing the Th2-response via androgen receptor (by activating androgen responsive elements of target genes) (29). Studies show that the severity of asthma in men remains stable from puberty, until serum testosterone levels start to decrease with aging (when higher asthma prevalence in men is observed) (30) and that castration in man exacerbates asthma (31). Consistent with our findings, Wulfsohn et al demonstrated (28) that female asthmatic patients treated with testosterone demonstrated a 90% improvement of asthma symptoms, while Mileva et al (25) found low levels of serum testosterone in patients with severe (when compared with mild) asthma. The impact of testosterone on the function of lower airways might be explained by the results of the study by Monatano et al (26) who found that testosterone induces bronchial relaxation via blockage of calcium channels, thus relaxing airway smooth muscle and increasing the airflow. It is therefore possible that reduced serum testosterone level in PMA patients found in our study is one of the factors increasing BHR in these patients. Our study adds decreased serum testosterone (but not androstendion or dehydroepiandrosteron) levels to the suspected hormonal fluctuations in PMA, suggesting that in addition to changing concentrations of female sex steroids, the loss of balance between male and female sex hormones may be crucial to trigger the exacerbation of asthma symptoms. It is possible that the effect of individual sex hormones on lower airways in PMA depends not only on their absolute concen-

tration, but also on the timing, the duration and especially the context of exposure. The increased estradiol / testosterone ratio in luteal phase in PMA patients may also be important, given the so far obtained evidence for the impact of these hormones on the immune system. In light of the recent findings about the significant androgen-dependent increase of Foxp3 expression in human T-cells from women, it is possible that momentary disturbance in this hormonal balance may lead to lower activity of regulatory T-cells (thus, reducing the immune tolerance in luteal phase of the menstrual cycle). The concomitant increased estradiol concentration may in such circumstances more easily lead to shifting the immune response towards Th2-dependent, thus triggering asthma exacerbation.

Data on the impact of prolactin in women with asthma are sparse. In this context, it is difficult to critically assess the trend to increased serum prolactin levels found in our study. Prolactin is a peptide hormone secreted from pituitary gland which acts as a cytokine on regulatory T-cells (that express the PRL receptor constitutively) (32). The recent study by Ochoa-Amaya et al (33) proved that hyperprolactinemia induced before antigen challenge in rats, decreased allergic lung inflammation, suggesting that prolactin may act as immunomodulator in asthma. This is consistent with findings of other authors who reported that prolactin promotes Th1-type of cellular response (32,34). The observed trend to increased serum prolactin levels in PMA patients found in our study (as well as increased sputum IL-8 concentrations), could therefore contribute to the development of the distinct immune response in lower airways of this specific asthma phenotype. We assume that a larger asthmatic sample would possibly provide significant statistical findings.

The results obtained in our study demonstrate that it is not only the change in absolute concentrations of estradiol, but also significantly increased estradiol-to-progesterone ratio that contribute to perimenstrual worsening of the disease in luteal phase of the cycle in PMA patients. Estrogen peaks during ovulation and both hormone levels are elevated during the luteal phase (with progesterone being at its highest), then reaching the lowest values during menses (35). Our results indicate that high concentrations of serum progesterone overlap higher than normal estradiol levels in the luteal phase in women with PMA. Animal studies have provided evidence that both estrogen and progesterone have effects on humoral and cellular immunity and smooth muscle function (36). Estrogen receptor come in two nuclear subtypes ER- and ER- (acting as transcription factors on gene expression) and is expressed in airway epithelial cells (37,38). Eosinophils bind estradiol (39) and show increased degranulation and adhesion (39,40). Estradiol shows biphasic, dose-dependent response to Th1 and Th2 signaling, developing different effects depending on its concentration (41). Higher estrogen levels shift the female immune system towards a Th2 re-

sponse increasing IL-4, IL-13, eosinophil recruitment and mast cell degranulation (16). In a study by Oliveira et al, female rats that either underwent ovariectomy or were treated with selective estrogen receptor antagonist, developed less allergic airway inflammation compared with control animals (42). Estrogen replacement-therapy reestablished airway inflammation in these animals (42). The increased sputum IL-5 concentrations in the luteal phase in PMA patients found in our study could thus reflect a temporary shift towards local Th-2 response, in particular, in the context of simultaneously elevated serum estradiol level. A larger number of PMA patients could perhaps reveal significant correlation between the two indicators. The obtained results are consistent with the conclusions of some former studies (43,44) in which the enhanced local airway inflammation reaching its maximum shortly before menstruation has also been demonstrated.

Progesterone acts via two main receptors (PR-A and PR-B) present on airway epithelial cells, activation of which has been shown to reduce cilia beat frequency by 40-50% (45). This sex steroid also appears to inhibit Th1 cells in humans and mice and induce IL-4 and IL-10 secretion (45). In animal studies progesterone increased eosinophilia-related BHR in sensitized mice (13). It is possible, that in PMA patients the impact of high luteal serum progesterone levels (which shifts the balance of the immune system towards Th2-response) on local airway tissue is amplified by higher, than normal, estradiol levels, thus having additive or synergistic effect on exacerbating asthma. It has been demonstrated that some women with PMA observe relief of their perimenstrual exacerbations if they use oral contraceptives that suppress large fluctuations in circulating hormones (46). In the context of our results, it is possible that such treatment leads to restoring of estradiol-to-progesterone ratio to such a level that will not affect the induction of exacerbations.

However, it is obvious that the results of our study must be treated with caution, as hormone concentrations were only studied in peripheral blood and our analysis does not include local tissue metabolism.

Strengths and limitations of the study

A major limitation of most of the PMA studies conducted so far is their cross-sectional design not including serial measures of hormones or repeated assessments of lung function throughout menstrual cycle. That is why we designed a three-arm, case-control study to simultaneously analyze changes in sputum proinflammatory markers in PMA patients in relation to fluctuations in serum hormones concentrations at pre-selected time-points of the menstrual cycle. The clear advantage over most of the previous PMA studies is that metacholine challenge test was used at the time of enrollment to exclude women with inadequate asthma status, and that women with irregular menstrual cycles

and using exogenous have also been excluded at baseline. As in other population-based studies (47-49) we have used questionnaire-based definition of PMA, but other questionnaires used in our study allowed us to additionally keep abreast of asthma symptoms, daily antiasthmatic drug use and the day of menstruation. This symptom timing enabled a more reliable determination of the associations of hormones with respiratory variables which were lacking in previous studies. Blood sampling and respiratory function testing were performed concurrently in morning hours, to avoid potential confounding from diurnal variation of hormonal levels and lung function. The number of patients in the present study was relatively small, which has obviously led to a lower statistical power. However, to have more patients would pose a challenge, as the study protocol assumed sharp exclusion criteria and was logistically burdensome for patients. On one hand, this enabled recruiting of a relatively homogeneous group of PMA patients, but on the other it led to the resignation of many women from participation in the study. Another reason of poor recruiting into all groups was the exclusion of patients using exogenous hormones, as it is quite difficult nowadays to find a large group of young females naïve to oral contraceptives. However, it cannot be excluded that the lack of correlation between hormones levels and respiratory variables might be attributed to the small number of cases in our study population. Therefore, these results should be interpreted cautiously, and further studies with larger number of patients are appropriate. Due to financial constraints, we have not evaluated hormone levels in sputum, which makes it impossible to assess the impact of local tissue metabolism in the interpretation of the results.

Conclusions

The luteal phase of the cycle in PMA patients is associated with increased serum estradiol levels and estradiol-to-progesterone ratio, with concurrent higher sputum concentration of IL-5 and IL-8 (when compared to non-PMA asthmatics).

Serum testosterone levels are decreased, and total number of sputum inflammatory cells is increased in PMA patients in both phases of the menstrual cycle (when compared to non-PMA asthmatics).

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