

Assessment of airway and systemic inflammation in asthmatic pregnancy

PhD Thesis

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1. INTRODUCTION

Asthma is one of the most common chronic diseases complicating pregnancy, and a risk factor for several maternal and fetal complications. There is an interaction between the two conditions: asthma influences the outcome of pregnancy and pregnancy also may affect asthma severity; the disease deteriorates in one third of pregnant women. Underlying immunological mechanisms of this interference and possible predictive factors are currently being intensively studied, but up till now there are no systemic or exhaled biomarkers known that could predict the worsening of asthma. On the other hand, optimal asthma control reduces the risk of maternal and fetal complications during pregnancy, thus close monitoring of the disease and airway inflammation would be important in this patient population. Currently available methodologies for assessing airway inflammation (eg. bronchoscopy, induced sputum test), however, involve potential risks for the mother and the fetus. Non-invasive methods for airway inflammation assessment could aid the clinical decision-making in pregnant asthmatic women.

Analysis of exhaled breath condensate (EBC) is a novel, completely non-invasive method for assessing airway inflammation, and might have some value in the clinical management of asthma and asthmatic pregnancy. The best reproducible and most validated marker in EBC is pH, which is a surrogate marker of the acid–base status in the whole airway tract. Decreased pH is measured in various inflammatory airway disorders with elevated oxidative stress such as bronchial asthma. Assessment of EBC pH may be

helpful in identifying loss of asthma control in non-pregnant patients, but its potential value in asthmatic pregnant women has not been investigated.

Allergic asthma is an inflammatory disease characterized by Th2-type inflammation. Regulatory T cells (Tregs) have also been shown to influence inflammatory responses in asthma by inhibiting CD4⁺ CD25 effector T cells and suppressing Th2 responses to allergens. In non-pregnant atopic asthmatics, a reduction of peripheral Treg cell prevalence was observed. At the same time, pregnancy is characterized by an immunological tolerance that attenuates maternal immune responses against paternal antigens expressed by the fetus. A component of this tolerance is skewness toward Th2-type immunity but Treg cells are also key players in the maintenance of this peripheral tolerance. However, the recent discovery of a distinct T helper subset, referred to as Th17 cells based on their IL-17 production, led to the transformation of the Th1/Th2 paradigm of immunity into a novel viewpoint that incorporates Th1, Th2, Th17 and Treg cells as elements of a complex and mutually interacting network. IL-17 has been proposed to have an important role in the development of autoimmune disorders, and in induction and maintenance of chronic inflammation. Abnormal Th17 immunity may also be involved in the pathogenesis of allergic asthma and in adverse pregnancy outcomes such as preterm labor or systemic inflammation in preeclampsia. The effect of Th17 cells on the inflammatory balance may be opposed by CD4⁺ Tregs. These findings suggest that in addition to Th1 and Th2 cells, Th17 and Treg cells also may have crucial effects on the course of asthma and on the outcome of pregnancy as well.

Circulating T cell immune phenotype is known to correlate with airway inflammation in non-pregnant allergic asthmatics: the proportion of

circulating IL-4-producing CD4+ T cells correlate with exhaled nitric-oxide (FE_{NO}) levels and induced sputum eosinophilic counts, which are considered as markers of airway inflammation in asthma. Theoretically, these associations might be altered in asthmatic pregnancy due to physiological pregnancy-induced immune tolerance which defends the developing fetus. Despite of these results, the role of EBC pH just as the alterations in the balance of Th1, Th2, Th17 and Treg lymphocytes and the relation of circulating T cell profiles to airway inflammation and asthma control has never been studied in asthmatic pregnancy.

2. OBJECTIVES

As EBC pH alterations and Th1/Th2/Th17/Treg immune responses characterizing asthmatic pregnancy were not known, and the relationship of immune responses and exhaled inflammatory markers were also not described, our main goals were to answer the following questions:

1. Whether pregnancy itself influences EBC pH in healthy women and whether the possible pregnancy-induced alteration of EBC pH can be detected also in asthmatic pregnant women.
2. Whether EBC pH is related to clinical outcomes of asthma or fetal growth in asthmatic women during pregnancy.
3. Whether the known Treg proliferation characterizing healthy pregnancy can be detected also in asthmatic pregnancy.
4. Whether the balance of Th1, Th2, Th17 and Treg lymphocytes changes, in asthmatic compared with healthy pregnancy.

5. Whether there is a relationship among peripheral T cell profile, airway inflammation, and the control of asthma symptoms.

3. METHODS

Study subjects

Asthmatic pregnant women (AP), healthy pregnant (HP), asthmatic non-pregnant (ANP) and healthy non-pregnant (HNP) controls participated in all the three conducted studies. We examined the EBC pH in 23 HNP, 17 HP, 22 ANP and 21 AP individuals, peripheral Th1, Th2, Th17 and Treg lymphocyte profile in 24 HNP, 23 HP, 15 ANP and 15 AP patients, and investigated the relationship between the T cell profile and asthmatic inflammation in 22 AP women. There was no difference regarding age or gestational age among the groups. All asthmatic women had mild or moderate, well-controlled or partly controlled persistent disease, and inhaled corticosteroids (ICS) were prescribed to all of them. Asthma had been diagnosed according to the current guidelines at least 6 months before the study. Exclusion criteria were hypertensive disorders, diabetes mellitus, autoimmune disease, angiopathy, renal disorder, maternal or fetal infection, fetal congenital anomaly, multifetal gestation, current smoking or more than 5 pack-years (number of daily smoked cigarettes' packs by the years of smoking) of smoking history, any other chronic disease (except for allergic rhinitis), and acute infection within 4 weeks of measurement. Women with a body mass index >30 kg/m² were not included.

All studies had a cross-sectional design. Asthmatic women were assessed at their regular visit at the outpatient clinic of the Department of Pulmonology,

Semmelweis University. Healthy non-pregnant volunteers were recruited from students and workers of the institution. Healthy pregnant individuals were recruited when attending their scheduled visit at the 1st Department of Obstetrics and Gynecology, Semmelweis University. All pregnant women were in the 2nd or in the 3rd trimester. Patients were asked not to use their medication for 12h before visits. All enrolled subjects signed an informed consent and our study was reviewed and approved by an independent ethical committee of the institution.

Measurement of EBC pH

Condensate samples were collected for 10 minutes using a handheld device (R Tube, Charlottesville, VA, USA) without wearing a nose-clip. The chiller tube was held at -80°C before condensate collection. Samples were stored at -80°C in microcentrifuge tubes for no longer than 4 weeks. EBC pH was measured at room temperature of $22\text{--}25^{\circ}\text{C}$ after argon deaeration for 10 minutes using the glass electrode SevenEasy S20 (Mettler Toledo, Schwerzenbach, Switzerland).

Evaluation of lung function, asthma control and blood gas parameters

Forced expiratory volume in 1 s (FEV1), peak expiratory flow (PEF) and airway resistance (Raw) were measured by means of electronic spirometer and plethysmography (PDD-301/s; Piston, Budapest, Hungary) according to the American Thoracic Society guidelines. Three technically acceptable maneuvers were performed, and the highest one was recorded. Asthma control was assessed using the Asthma Control Test (ACT) suggested by the current Global Initiative for Asthma guideline. Blood gases and blood acid–

base status were analyzed in arterialized capillary blood samples (GEM Premier 3000; Instrumentation Laboratory, Bedford, MA, USA).

Measurement of FE_{NO}

Exhaled nitric oxide was measured using NIOX MINO Airway Inflammation Monitor (NIOX MINO; Aerocrine AB, Solana, Sweden) according to the latest guidelines.

Flow cytometry measurements

Peripheral blood mononuclear cells (PBMCs) were separated by standard density gradient centrifugation (Ficoll Paque; Amersham Biosciences AB, Uppsala, Sweden; 27 min, 400 3 g, 22 C) from freshly drawn blood collected in lithium heparin-treated tubes (BD Vacutainer; BD Biosciences, San Jose, CA, USA). The cells were analyzed on a BD FACSAria flow cytometer (BD Biosciences); 200 000 cells were recorded. The population of lymphocytes was gated from PBMCs according to Forward Scatter Characteristics and Side Scatter Characteristics. This was followed by the gating of the CD4+, CD8+ or CD56+ subsets. Then, within these subsets, CXCR3+, CCR4+, CD25+FoxP3+, IFN γ +, IL-4 + and IL-17+ cells were gated, respectively. Isotype-matched PE-, APC- and FITC-conjugated mouse IgG1 antibodies were used as controls (eBioscience and BioLegend, respectively).

Statistics

Statistical analysis was performed using GraphPad Prism (Version 4, Los Angeles, CA, USA) and the R software (R Development Core Team; R Foundation for Statistical Computing, Vienna, Austria). Data distribution

was analysed by Kolmogorov–Smirnov test in all studies. P value < 0.05 was considered significant.

Normally distributed data are presented as mean \pm SD, while in the case of not normally distributed data, median (interquartile range) is given.

Comparisons between sample populations were made by parametric and non-parametric analyzes depending on the type of data. As parametric test we used unpaired t-test, Pearson test and analysis of variance followed by Newman–Keuls post-hoc test. As non-parametric test Mann–Whitney test and Spearman test were performed.

4. RESULTS

4.1. Changes in EBC pH in healthy and asthmatic pregnant women

There was a significant difference in EBC pH among the four groups (analysis of variance; $p = 0.007$, Figure 1). EBC pH was the highest in HP women (8.02 ± 0.43), significantly higher than that of the HNP control group (7.75 ± 0.27 ; $p = 0.017$). HNP and ANP groups had similar EBC pH (7.75 ± 0.27 vs. 7.54 ± 0.57 ; $p = 0.118$) probably indicating the sufficient control of airway inflammation in ANP women. In the AP group EBC pH (7.65 ± 0.38) was also similar to that of the ANP and HNP women (7.54 ± 0.57 and 7.75 ± 0.27 , $p = 0.470$ and $p = 0.300$, respectively).

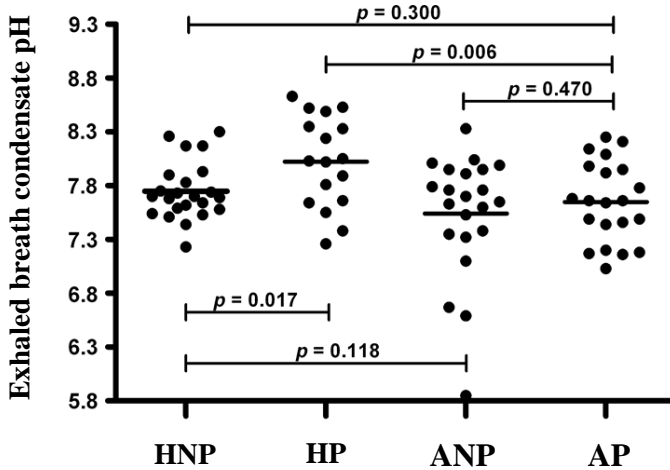


Figure 1. Comparison of exhaled breath condensate pH among the four groups

No relationship was detected between maternal EBC pH and birthweight of newborns in HP women ($r = 0.25$, $p = 0.427$, $n = 12$). Regarding asthma control determinants, a positive correlation was observed between EBC pH and forced expiratory vital capacity values in AP women ($r = 0.45$, $p = 0.039$, $n = 21$; Figure 2), and there was also a correlation between maternal EBC pH and birthweight ($r = 0.49$, $p = 0.047$, $n = 17$; Figure 3) in this group together, with a weak trend towards a negative correlation between FE_{NO} and birthweight ($p = 0.092$, $r = 0.45$). However, there was no relationship between ACT total scores and birthweight ($r = 0.09$; $p = 0.704$). EBC pH and ACT values ($r = 0.10$, $p = 0.652$) or FE_{NO} levels ($r = 0.37$, $p = 0.126$) were also not related in asthmatic pregnancy.

In ANP patients no correlation was found between EBC pH and ACT values ($r = 0.03$, $p = 0.875$) or FE_{NO} levels ($r = 0.18$, $p = 0.443$). Similarly, there was no significant relation between EBC pH and forced vital capacity or any other lung function parameter ($p > 0.05$, Figure 4).

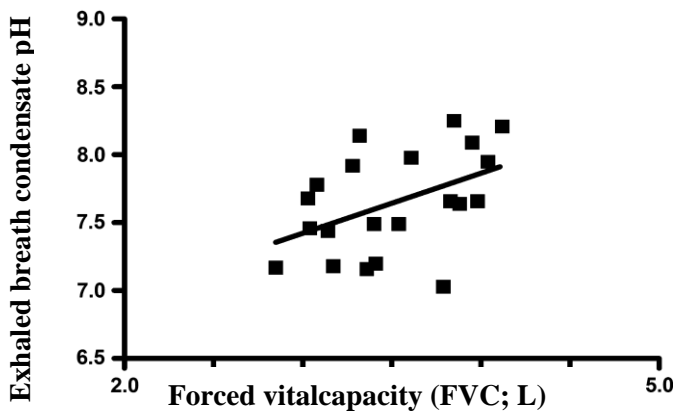


Figure 2. Relation between exhaled breath condensate pH and forced expiratory vital capacity in asthmatic pregnant ($r = 0.45$, $p = 0.039$, $n = 21$).

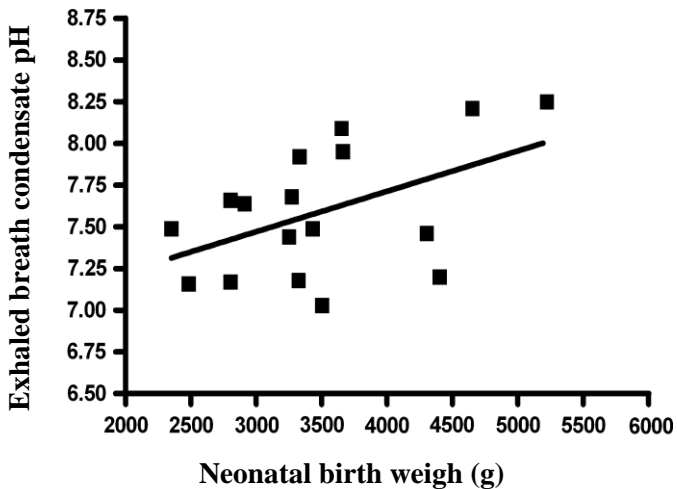


Figure 3. Relation between exhaled breath condensate pH and neonatal birthweight in asthmatic pregnant women ($r = 0.49$, $p = 0.047$, $n = 17$).

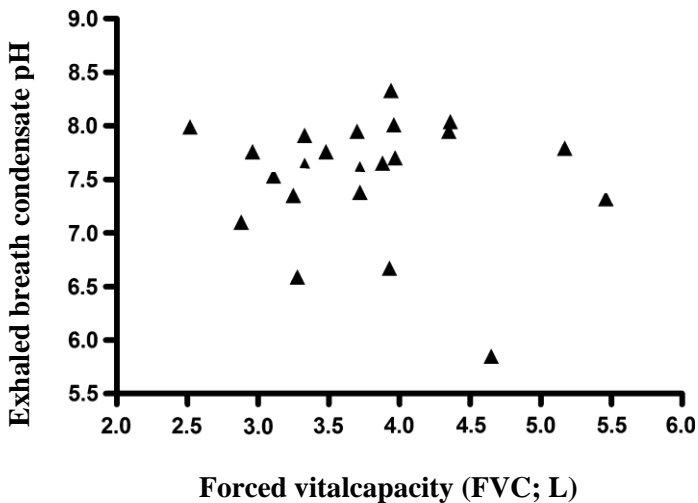


Figure 4. Lack of correlation between exhaled breath condensate pH and forced expiratory vital capacity values in asthmatic non-pregnant women ($r = 0.11$, $p = 0.637$, $n = 22$).

4.2. Peripheral Th1/Th2/Th17/regulatory T-cell balance in asthmatic pregnancy

Peripheral Th1/Th2/Th17/regulatory T cell balance was examined in all four groups. The prevalence of the CD4+ CXCR3+ subset (Th1 cells, cell surface marker detection) was lower in the HP, ANP and AP groups than in the HNP group, whereas that of CD4+ CCR4+ cells (Th2 subset, cell surface marker detection) did not differ between the four groups.

The prevalence of CD4+ IFN-gamma+ lymphocytes (Th1 cells, intracellular cytokine detection) was lower in HP, ANP and AP samples compared with HNP data (Fig. 5a), while the CD4+ IL-4+ prevalence (Th2 cells, intracellular cytokine detection) was elevated among HP, ANP and AP women compared with HNP controls (Fig. 5b). Healthy pregnancy and asthma alone both decreased the IFN-gamma+/IL-4+ CD4 (Th1/Th2) cell ratio (with intracellular cytokine detection), but no further decrease was observed in asthmatic pregnancy (Fig. 5c).

Asthma itself increased CD4+ IL-17+ (Th17) cell prevalence, as it was significantly higher in ANP samples compared to HNP ones (Fig. 5d). HP did not change Th17 prevalence compared to HNP control data. Supporting compromised pregnancy-specific immune tolerance as a result of asthma, Th17 prevalence was significantly higher in the AP group than in the HP group. Moreover, healthy pregnancy was accompanied by a significant elevation of CD4+ CD25+ FoxP3+ regulatory T-cell prevalence compared with HNP subjects, and this elevation of Tregs was absent in AP patients (Fig. 5e). As a result, the ratio of Th17/Treg cells decreased in HP but not in AP subjects in comparison with HNP controls. In the AP group, the

Th17/Treg ratio was significantly higher than in HP women (Fig. 5f), which also suggests an attenuated immune tolerance caused by asthma that complicates pregnancy

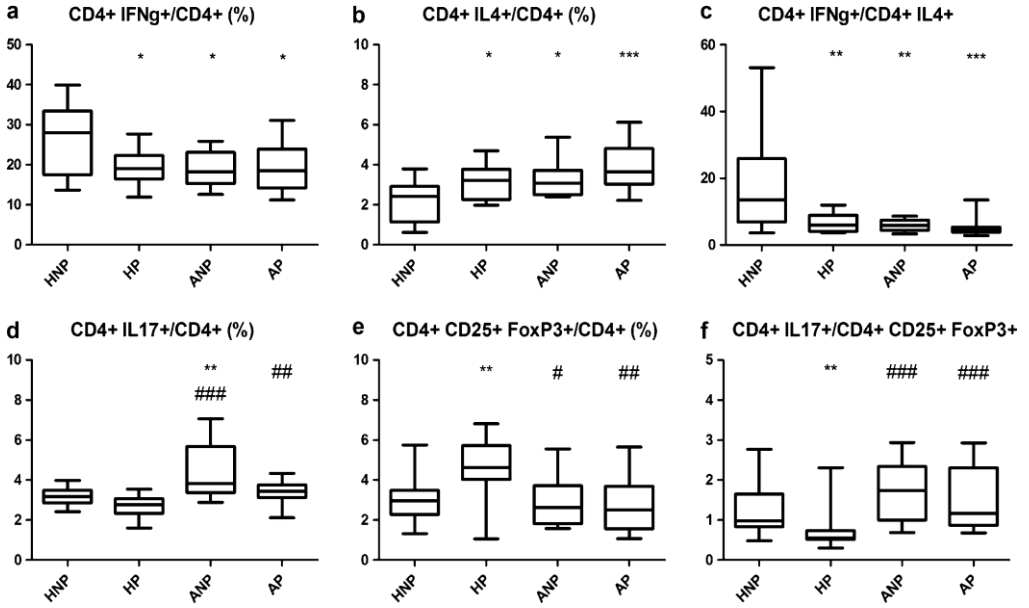


Fig. 5. Box plots representing the prevalence of IFN- γ + (a), IL-4+ (b), IL-17+ (d) and CD25+ FoxP3+ (e) cells among CD4+ lymphocytes as well as the Th1/Th2 (c) and Th17/Treg (f) ratios in HNP, HP, ANP and AP women. Horizontal line, median; box, interquartile range; whisker, range. P values <0.05 were regarded significant. *P < 0.05 versus HNP; **P < 0.01 versus HNP; *** P < 0.001 versus HNP; #P < 0.05 versus HP; ##P < 0.01 versus HP; ###P < 0.001 versus HP.

4.3. Relation of circulating T cell profiles to airway inflammation and asthma control in asthmatic pregnancy

Circulating Th1, Th2, Treg and NK cell proportions were not significantly correlated to airway inflammation assessed by FE_{NO} (all T cell subsets $p > 0.05$; study power $> 75\%$) (Fig. 6 and Fig. 7). However, there was a significant negative correlation between proportions of Th2 cells and ACT total scores ($r = -0.48$, $p = 0.03$; Fig. 8) which was attenuated to a trend after adjustment to the dose of inhaled corticosteroid therapy ($p = 0.061$).

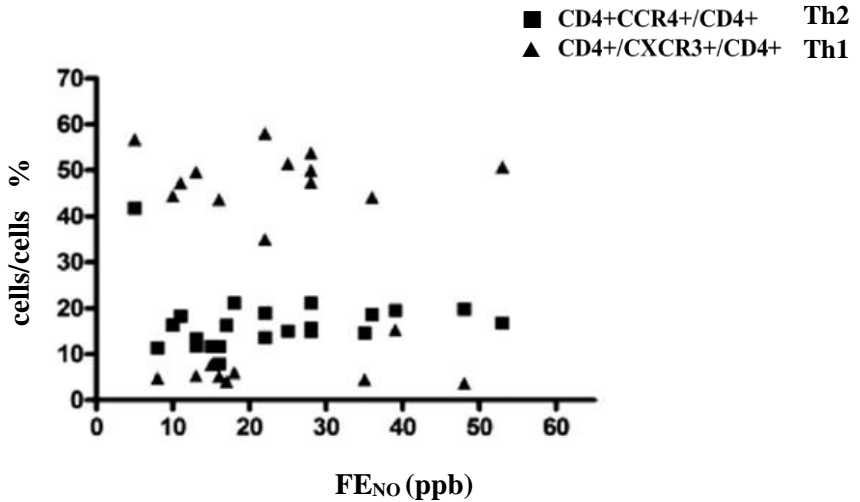


Fig. 6. Lack of significant correlation between FE_{NO} and the prevalence of Th1 and Th2 cell subsets ($n = 22$; $p > 0.05$; study power $> 75\%$ for both cell types) in asthmatic pregnancy. (CD – cluster of differentiation, FE_{NO} – fractional exhaled nitric oxide, CD4+CCR3+/CD4+ – T helper cell 1 (Th1), CD4+CCR4+/CD4+ – T helper cell 2 (Th2), ppb – parts per billion)

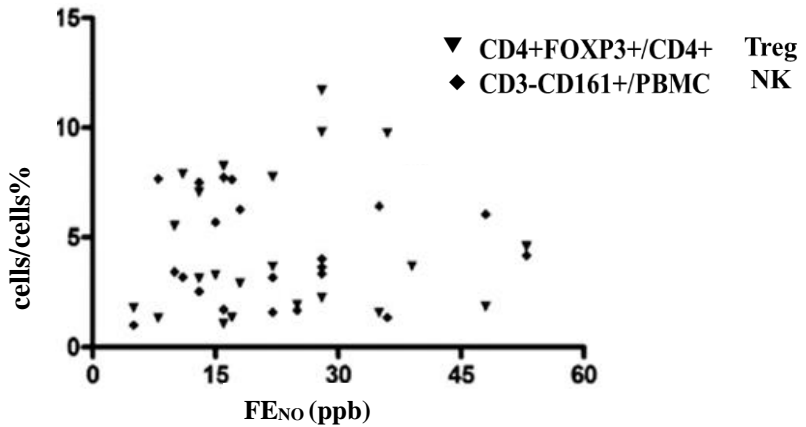


Fig. 7. Lack of significant correlation between FE_{NO} and the prevalence of Treg and NK cells (n = 22; p > 0.05; study power > 75% for both cell types) in asthmatic pregnancy.

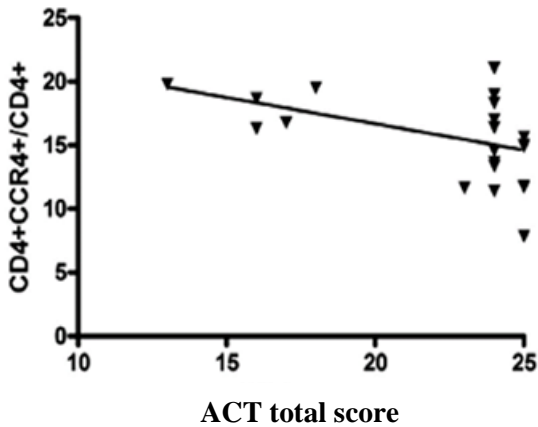


Fig. 8. Significant negative correlation between the prevalence of Th2 cells and ACT total scores in asthmatic pregnant patients (n = 22; Spearman r = -0.48; p = 0.03). (ACT – Asthma Control Test, Th2 – T helper cell 2)

Examining other T cell subpopulations, no significant relationship was found to ACT total scores. Furthermore, no correlation was detected between circulating T cell proportions and any of the lung function parameters. Considering lung function values, airway resistance was significantly correlated with exhaled nitric oxide levels ($r = 0.49$, $p = 0.02$; Fig. 9).

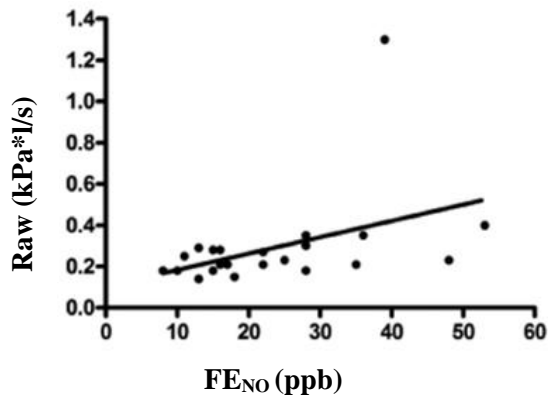


Fig. 9. Significant positive correlation between FE_{NO} and airway resistance in asthmatic pregnancy ($n = 22$; Spearman $r = 0.49$; $p = 0.02$). (FE_{NO} – fractional exhaled nitric oxide, ppb – parts per billion, Raw – airway resistance)

6. CONCLUSIONS

1. Healthy, but not asthmatic pregnancy is characterized by higher EBC pH compared with HNP womens data, indicating that oxidative inflammatory processes induced by asthma may compromise the

regulatory mechanisms causing alkaline pH in the airways during pregnancy.

2. EBC pH is related to lung function and birthweight in asthmatic pregnancy; more acidic pH is associated with worse lung function parameters and lower birth weight.
3. Increased Treg cell prevalence characterizing healthy pregnancy is missing in asthmatic pregnancy.
4. The sensitive balance of Th1, Th2, Th17 and Treg cells that characterizes healthy pregnancy is altered by concomitant well or partially controlled asthma:
 - a. Healthy pregnancy and asthma are both characterized by lower Th1/Th2 cell ratios, but we observed no further decrease in asthmatic pregnancy.
 - b. The prevalence of circulating Th17 cells is higher and that of Treg cells is lower in asthmatic than in HP women.
 - c. The altered prevalence of Th17 and Treg cells results in decreased Th17/Treg ratio in healthy, but not in asthmatic pregnancy, compared with the HNP state.
5. There is no relation between peripheral T cell subsets, lung function and airway inflammation assessed by FE_{NO} in asthmatic pregnancy, although we observed a negative correlation between asthma control and blood Th2 cell prevalence.

In summary, both healthy and asthmatic pregnancy markedly alter EBC pH and circulating Th/Th2/Th17/Treg profile, which alterations are partly associated with changes in the clinical parameters of gestation and asthma.

LIST OF PUBLICATIONS

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