

Investigation of circulating biomarkers in asthmatic pregnancy

PhD Thesis

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1. INTRODUCTION

Asthma is one of the most common chronic diseases which require treatment during pregnancy. It represents a risk for several maternal and fetal complications. On the other hand, pregnancy has also an effect on asthma control and severity, leading to the deterioration of symptoms in one-third of pregnant women. The factors provoking loss of control during pregnancy are not clearly known; therefore, the identification of pregnant women at risk of not controlled asthma and complications is not completely solved yet. On the other hand, optimal asthma control during pregnancy is associated with lower risk of maternal and neonatal morbidities. However, monitoring of asthma during pregnancy may be difficult. Lung function parameters may be influenced by the pregnancy itself which may lead to non-recognition of early signs of asthma deterioration. Furthermore, the forced expiration maneuver may not be safe and applicable in every pregnant woman. Hence, non-invasively obtainable systemic markers related to symptoms, lung function, or loss of asthma control would be of great value in the management of asthmatic pregnancy.

Vascular endothelial growth factor (VEGF) is a main mediator of angiogenesis; its biological activity is regulated by its soluble receptor sFlt1. VEGF plays a critical role in the normal embryonic and placental angiogenesis during pregnancy. An impaired balance of angiogenic/angiostatic factors may lead to pregnancy complications. A significant increase in vascularization of airways is an early component of the airway remodeling caused by chronic

inflammation in asthma, in which process the stimulatory effect of VEGF is crucial.

Soluble urokinase plasminogen activator receptor (suPAR) is a sensitive indicator of both inflammation and the activation state of the immune system in general. It can be an ideal biomarker because its levels are not affected by diurnal variation and fasting state and it is resistant even to repeated freezing and thawing of plasma samples. Its serum level is elevated in many inflammatory diseases predicting a worse prognosis. Its concentration may increase during pregnancy indicating trophoblast cell activity and the physiologically activated state of the immune system. In preeclampsia even higher concentrations were documented.

Hyaluronic acid (HA) is a major component of the extracellular matrix. It degrades to low molecular weight HA fragments during pathological conditions such as inflammation and tissue injury and repair that have several proinflammatory and regulatory roles. HA was shown to be implicated in asthma pathophysiology as well. In addition, it is indispensable to the embryo implantation, decidualization and maintenance of healthy gestation; however its serum level further increases in pathological pregnancies.

Despite the increasingly recognized important roles of the above mentioned three potential biomarkers in inflammatory conditions such as in healthy or pathological pregnancies, there are no data about their peripheral blood concentrations in asthmatic patients or the possible relationships with clinical parameters of asthma, and these molecules were neither investigated in asthmatic pregnancy yet.

2. OBJECTIVES

The key aim of our studies was to detect a non-invasively obtainable biomarker showing correlation with the deterioration of asthma symptoms, which could be a candidate for further investigations in asthma and asthmatic pregnancy. We sought to answer the following questions:

- A) Do the peripheral levels of VEGF [1] and suPAR [2] differ in treated asthmatic non-pregnant patients compared to healthy non-pregnant people?
- B) Is there a correlation between the peripheral levels of VEGF [1], suPAR [2] and HA [3] and the clinical parameters of asthma in asthmatic non-pregnant patients?
- C) Are any of the investigated molecules able to detect suboptimal asthma control?
- D) Do the peripheral levels of VEGF [1] and suPAR [2] differ in asthmatic pregnancy compared to healthy pregnant women?
- E) Do the peripheral levels of VEGF [1], suPAR [2] and HA [3] differ in asthmatic pregnancy compared to asthmatic non-pregnant patients?
- F) Is there a relationship between the peripheral levels of VEGF [1], suPAR [2] and HA [3] and the clinical parameters of asthma in asthmatic pregnancy?
- G) Are any of the investigated molecules able to detect suboptimal asthma control during pregnancy?

3. METHODS

Study populations

We involved four groups in our cross-sectional studies: asthmatic pregnant (AP), asthmatic non-pregnant (ANP), healthy pregnant (HP) and healthy non-pregnant (HNP) groups. In the VEGF study 31 AP, 29 ANP, 28 HP and 22 HNP subjects participated. In the same study, we carried out sFlt1 measurements in 85 plasma samples (21 AP, 23 ANP, 21 HP and 20 HNP) to assess the balance of VEGF/sFlt1 observed in peripheral blood. The latter molecule was measured only in second trimester samples in both pregnant groups because of the known alterations in its level characteristic for pregnancy. In the suPAR measurements 15 AP, 38 ANP, 58 HP and 29 HNP subjects were involved. As suPAR was not investigated in asthma earlier, we recruited in this study 11 and 10 men in the ANP and HNP groups, respectively, to draw conclusions about asthma in general. In this study interleukin-6 (IL-6) and CRP levels were also measured to obtain a more complex view about the inflammatory state of the patients. In the HA study 16 AP and 36 ANP women were enrolled.

Asthmatic patients were enrolled at the Department of Pulmonology, Semmelweis University. Healthy pregnant women were recruited when attending their scheduled visit at the 1st Department of Obstetrics and Gynaecology, Semmelweis University. Healthy non-pregnant control subjects were employees of the Department of Pulmonology and students of the University.

Asthma had been diagnosed according to the Global Initiative for Asthma (GINA) guidelines at least 6 months prior to the study. The

patients had persistent, in most cases controlled or well controlled disease and they were treated according to the GINA guideline. Exclusion criteria were any other chronic or acute disease (except for allergic rhinitis), smoking, known pregnancy complications and multi-fetal gestation.

Sample collection and laboratory procedures

Plasma was isolated from EDTA anticoagulated peripheral venous blood samples and stored at -80°C until measurement. The investigated molecules were measured using commercially available ELISA assays as reported in the kit guidelines. VEGF and sFlt1 were measured with DVE00 and DVR100B assays, respectively (R&D Systems, Abingdon, UK). The detection limits for plasma VEGF and sFlt1 were 9 and 2.5 pg/mL, respectively. To study the interference between sFlt1 and the VEGF ELISA kit, we plotted VEGF standard curve (ranging from 0 to 500 pg/mL) in a presence of different amounts of sFlt1 (4000, 2000, 1000, 500 and 250 pg/mL). Plasma suPAR concentrations were measured with the suPARnostic Flex ELISA assay (ViroGates A/S, Birkerød, Denmark). Serum hyaluronan was determined by ELISA assay, too (Coragenix, Inc., Broomfield, Co, USA). CRP and IL-6 levels were measured using commercially available tests (Roche Diagnostics GmbH, Mannheim, Germany). The measurements were carried out in the Department of Pulmonology and Department of Laboratory Medicine, Semmelweis University.

Lung function and exhaled nitric oxide measurements, and asthma control evaluation

Lung function was measured by means of electronic spirometer (PDD-301/s, Piston, Budapest, Hungary) according to the current guideline. Asthma control was assessed with the Asthma Control Test (ACT). Fractional exhaled nitric oxide (FE_{NO}) was measured by a NIOX MINO electrochemical analyzer (Aerocrine; Solna, Sweden) at an expiratory flow of 50 mL/s as per guideline recommendations.

Recording obstetrical data

We recorded the main parameters of obstetrical outcome in both pregnant groups, such as neonatal birth weight, gestational age at delivery, gender of the newborn, Apgar scores, maternal or fetal complications.

Statistics

The normality distribution of the data was assessed by Kolmogorov-Smirnov and D'Agostino-Pearson tests. Normally distributed variables were expressed as mean \pm SD, while not normally distributed variables were expressed as median [interquartile range]. Among parametric tests unpaired Student t-test, while among non-parametric tests Kruskal-Wallis, Dunn's *post hoc*, Mann-Whitney *U*, Wilcoxon and Spearman tests were used. The necessary corrections were made with Quade's rank analysis of covariance and partial rank correlation.

4. RESULTS

4.1. VEGF and sFlt1

There was no difference in either the demographic (age, gestational age) or clinical data (FEV_1 and FVC, FE_{NO} levels, ICS dose, birth weight, gestational age at delivery, Apgar scores) among the 4 groups. Free VEGF was detectable in all 29 samples (100%) in the ANP and in 20 (91%) samples in the HNP group; however only in 18 samples (58%) in the AP and in 10 samples (36%) in the HP group. Significant differences were observed between AP and ANP ($p < 0.001$) and HP and HNP groups ($p < 0.001$) with decreased levels of free VEGF in the two pregnant groups (Figure 1).

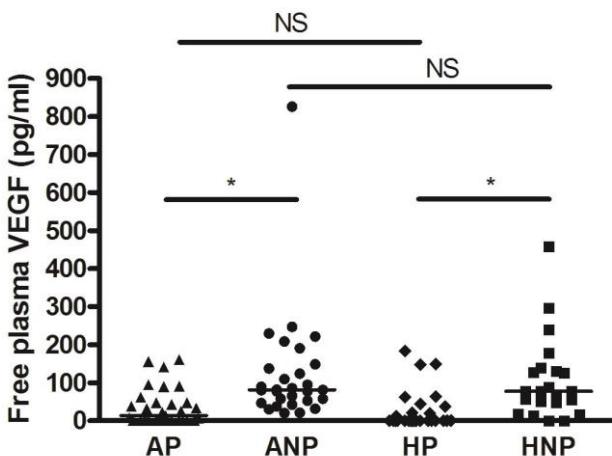


Figure 1. Free plasma VEGF levels in the four study groups.
AP, asthmatic pregnant; ANP, asthmatic non-pregnant; HP, healthy pregnant; HNP, healthy non-pregnant; NS, not significant; * $p < 0.05$.

Free VEGF levels were similar in the second- and third-trimester AP subjects ($p = 0.54$, Figure 2A). There was no change from the

second to third trimester in VEGF levels examining individual samples of AP women ($p = 0.67$, Figure 2B).

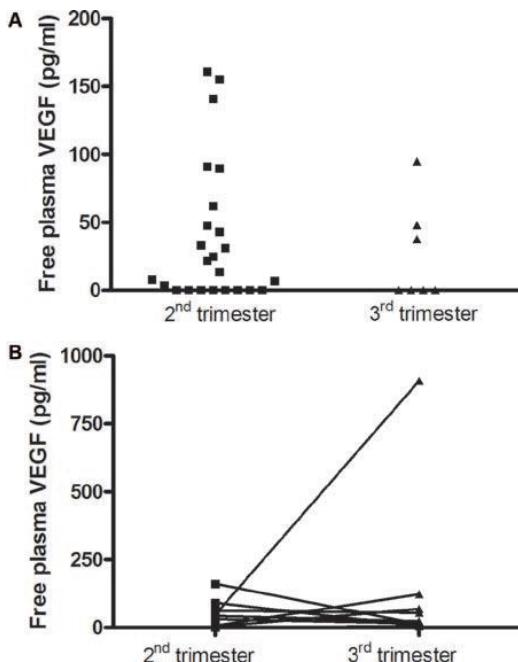


Figure 2. Free plasma VEGF levels in second- and third-trimester AP subjects. AP, asthmatic pregnant.

In the AP group, there was a significant inverse relationship between free plasma VEGF and FE_{NO} levels ($p = 0.01$, $r = -0.51$); however, this relationship was absent after adjustment on ICS dose ($p = 0.22$), suggesting steroid-dependence of FE_{NO} .

sFlt1 levels were elevated in AP and HP compared to ANP and HNP groups ($p < 0.001$) without any difference between AP vs. HP or ANP vs. HNP groups ($p > 0.05$; Figure 3).

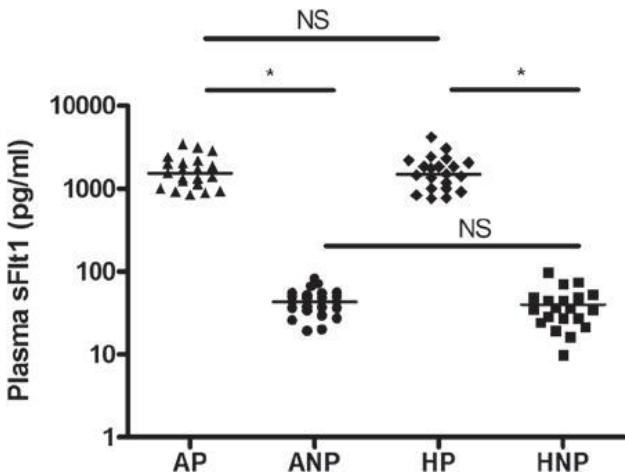


Figure 3. Plasma sFlt1 levels in the four study groups. AP, asthmatic pregnant; ANP, asthmatic non-pregnant; HP, healthy pregnant; HNP, healthy non-pregnant; NS, not significant; * p < 0.05.

Besides FE_{NO} no other clinical or obstetrical variable was related to free VEGF or sFlt1 levels in any group.

4.2. suPAR

In this study, the median age of participants was higher in the ANP group compared to the HP and AP groups ($p < 0.001$) and gestational age at blood collection was lower in the AP than in the HP group ($p < 0.001$). Gestational age at delivery and fetal birth weight were comparable in the pregnant groups. No difference was detected in parameters describing the severity or control of asthma between the ANP and AP groups (FEV₁, PEF, R_{aw}, ACT total score, ICS dose). Circulating suPAR values were lower in the HP and AP than in the HNP and ANP subjects, respectively ($p < 0.001$; Figure 4). ANP and HNP groups had similar circulating suPAR levels and

no difference was found between AP and HP groups ($p > 0.05$; Figure 4).

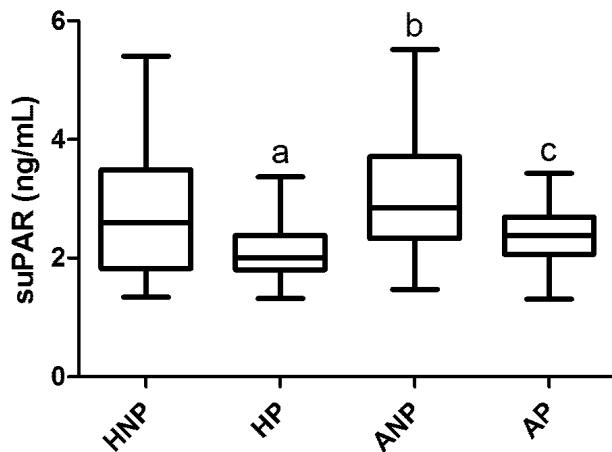


Figure 4. Circulating suPAR levels in the four study groups.
HNP, healthy non-pregnant; HP, healthy pregnant; ANP, asthmatic non-pregnant; AP, asthmatic pregnant; ^a $p < 0.05$ vs. HNP, ^b $p < 0.05$ vs. HP, ^c $p < 0.05$ vs. ANP.

IL-6 levels were comparable in all study groups ($p > 0.05$). CRP values were comparable in the HNP, ANP and HP groups and were higher in the AP compared to the HNP group ($p = 0.005$).

Importantly, considering the relationship between suPAR levels and asthma control determinants, there was a significant positive correlation between R_{aw} and suPAR levels ($p = 0.004$, $r = 0.47$) as well as R_{aw} and IL-6 levels ($p = 0.047$, $r = 0.35$) in the ANP group (Figure 5).

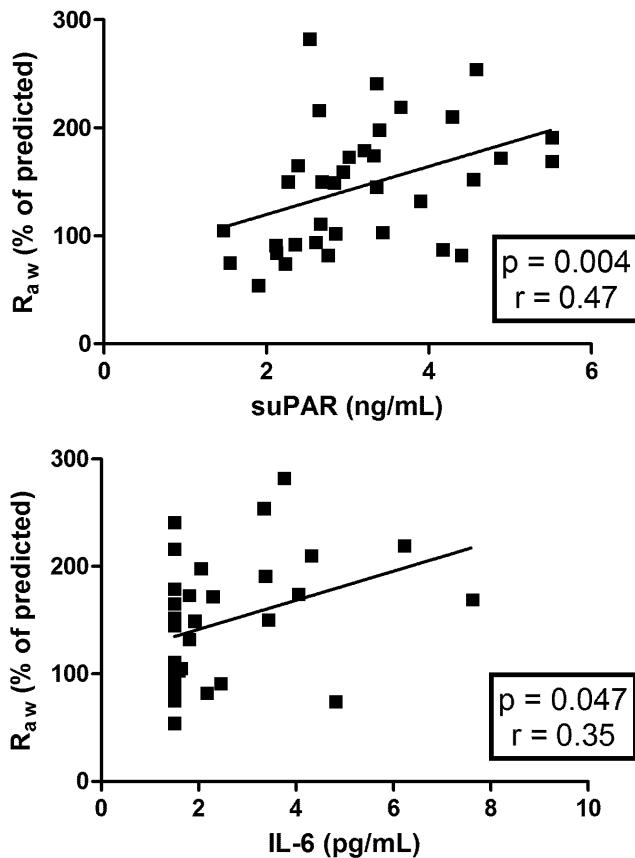


Figure 5. Positive correlation of airway resistance to circulating suPAR and to circulating IL-6 levels in ANP patients.
ANP, asthmatic non-pregnant; R_{aw}, airway resistance.

As current asthma guideline suggests PEF $\geq 80\%$ and ACT total score ≥ 20 as the main determinants of well-controlled asthma, ROC analyses of asthmatic patients' data were performed in subgroups of AP and ANP with PEF above and below 80%, and ACT total score above and below 20. Both analyses showed significant values in ANP group. ROC analysis of suPAR values in ANP patients with

PEF above and below 80% yielded an AUC of 0.75 with a cut-off value of 4,04 ng/mL ($p = 0.023$; Figure 6). ROC analysis of suPAR values in ANP patients with ACT score above and below 20 yielded an AUC of 0.80 with a cut-off value of 4,04 ng/mL ($p = 0.006$, Figure 6).

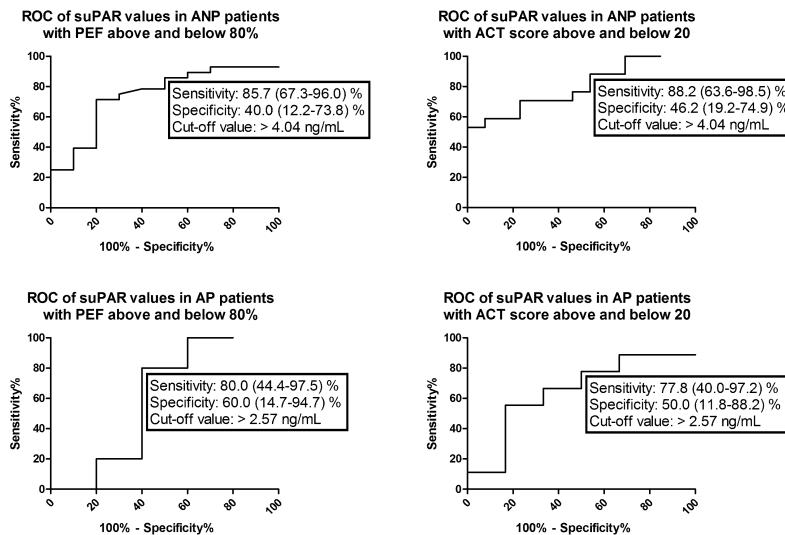


Figure 6. ROC analyses of circulating suPAR values in ANP and AP groups, based on PEF $\geq 80\%$ and ACT total score ≥ 20 .
ANP, asthmatic non-pregnant; AP, asthmatic pregnant; PEF, peak expiratory flow; ACT, Asthma Control Test.

4.3. Hyaluronic acid

In this study the mean age of participants was higher in the ANP group compared to the AP one ($p < 0.001$). No difference was detected in asthma severity or control (FEV₁, PEF, R_{aw}, ACT total score, ICS dose). HA level was related to age in the whole asthmatic cohort ($n = 52$; $p = 0.006$; $r = 0.37$), and within the ANP group as

well ($p = 0.019$; $r = 0.39$). There was no difference between HA levels of women in the second and the third trimester ($p = 0.27$).

HA values were lower in AP than in ANP subjects ($p = 0.006$; Figure 7). After adjusting for age, significance softened to a trend ($p = 0.056$)

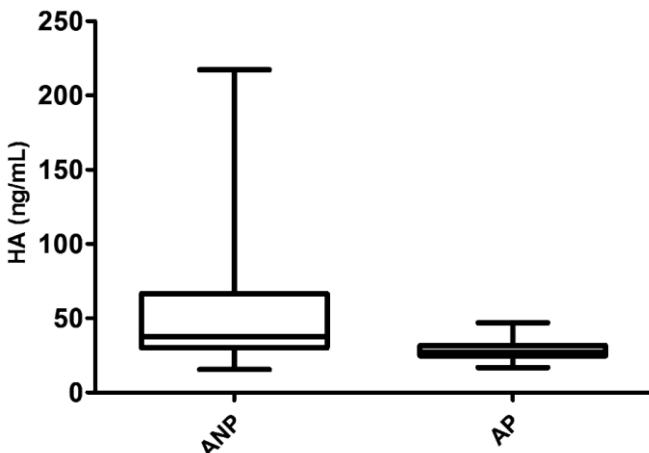


Figure 7. Circulating HA levels in the two study groups*. HA, hyaluronic acid; ANP, asthmatic non-pregnant; AP, asthmatic pregnant; $p = 0.006$; *data not adjusted for age.

Considering the relationship between HA levels and asthma control determinants, there was a significant correlation between HA levels and airway resistance in the whole asthmatic cohort ($p = 0.0055$; $r = 0.38$) and in the ANP group ($p = 0.004$, $r = 0.467$; Figure 8) as well.

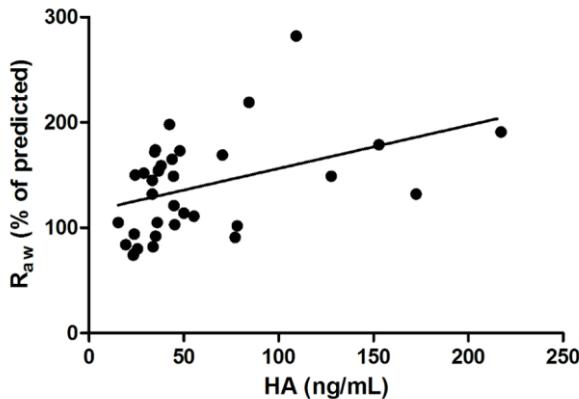


Figure 8. Correlation between HA levels and airway resistance in the ANP group. HA, hyaluronic acid; ANP, asthmatic non-pregnant; R_{aw}, airway resistance.

Moreover, in the ANP group an inverse correlation was revealed between serum HA levels and ACT total score ($p = 0.01$, $r = -0.437$; Figure 9). All these associations remained significant ($p < 0.05$) after adjustment for age.

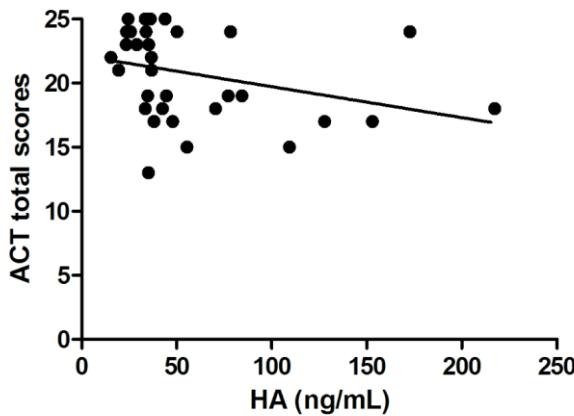


Figure 9. Inverse correlation between HA levels and ACT total scores in the ANP group. HA, hyaluronic acid; ACT – Asthma Control Test; ANP, asthmatic non-pregnant.

HA was found to be associated with asthma control level as ROC analyses of HA data in ANP patients with ACT total score above and below 20 yielded an AUC of 0.826 with a cut-off value of 37.4 ng/mL ($p = 0.006$; Figure 10).

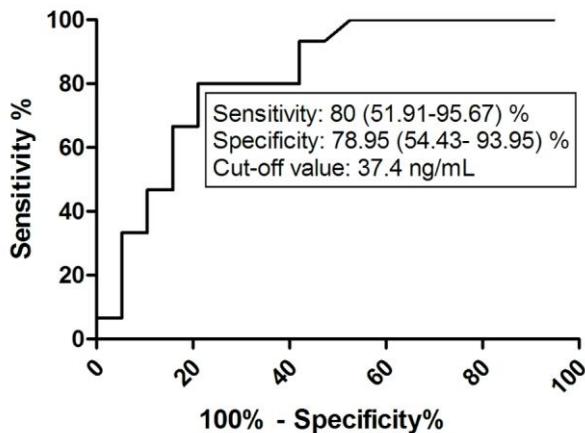


Figure 10. ROC analysis of HA values in the ANP group according to ACT total score ≥ 20 . HA, hyaluronic acid; ANP, asthmatic non-pregnant; ACT, Asthma Control Test.

5. CONCLUSIONS

- A) There is no difference in the peripheral level of VEGF [1] and suPAR [2] in treated asthmatic non-pregnant patients and healthy non-pregnant control subjects.
- B) In non-pregnant asthmatic patients the peripheral level of VEGF [1] is not related to the clinical variables of asthma. However, peripheral level of suPAR [2] shows a significant correlation with airway resistance. Peripheral level of HA [3] correlates with airway

resistance and inversely correlates with ACT total score, hence both suPAR [2] and HA [3] are related to clinical parameters of asthma.

C) Peripheral level of suPAR [2] is associated with loss of asthma control. There is a cut-off value, which can discriminate between patients with controlled or uncontrolled disease based on both the PEF and ACT total scores, according the guidelines. Also, there is a cut-off value of HA [3], which can discriminate between patients with controlled or uncontrolled disease based on the ACT total scores. Therefore, measuring both the circulating suPAR [2] and HA [3] levels is suitable to detect uncontrolled asthma in non-pregnant patients.

D) There is no difference in the peripheral level of VEGF [1] and suPAR [2] in antiinflammatory-treated asthmatic pregnant and healthy pregnant women.

E) Peripheral level of VEGF [1], suPAR [2] and HA [3] are all lower in asthmatic pregnancy compared to asthmatic non-pregnant patients, therefore the concentrations of these inflammatory markers are presumably influenced by the effect of pregnancy-induced immunosuppression and hemodilution.

F) In asthmatic pregnancy, VEGF [1] is inversely correlated to FE_{NO} values. Peripheral levels of suPAR [2] and HA [3] are not significantly associated with clinical variables of asthma in asthmatic pregnancy.

G) None of the investigated molecules is able to detect uncontrolled asthma in asthmatic pregnancy.

Besides the above listed answers obtained to the questions we asked, we documented the following new results:

H) There is no difference in the peripheral level of sFlt1 [1], IL-6 [2] or CRP [2] between asthmatic pregnant and healthy pregnant women. Furthermore, there is no difference in the peripheral level of IL-6 [2] or CRP [2] between asthmatic pregnant and asthmatic non-pregnant patients.

I) However, peripheral level of sFlt1 [1] is markedly increased in asthmatic pregnancy compared to asthmatic non-pregnant patients.

J) In asthmatic non-pregnant patients the peripheral level of IL-6 [2] correlates with the airway resistance, thus there is an association between lung function and inflammatory status.

In summary, measurements of the circulating concentrations of both suPAR and hyaluronic acid are suitable to detect uncontrolled asthma with good sensitivity in non-pregnant asthmatic patients, and are related to clinical parameters of the disease.

6. LIST OF PUBLICATIONS

Publications relevant to the dissertation

Cumulative impact factor: 8,947, as first author: 3,534

Publications in English peer-reviewed journals:

1. **Ivancsó I***, Toldi G*, Bohács A, Eszes N, Müller V, Rigó J Jr, Vásárhelyi B, Losonczy G, Tamási L. Relationship of circulating soluble urokinase plasminogen activator receptor (suPAR) levels to disease control in asthma and asthmatic pregnancy. *PLoS One.* 2013; 8(4): e60697. IF: 3,534
2. Eszes N, Toldi G, Bohács A, **Ivancsó I**, Müller V, Rigó J Jr, Losonczy G, Vásárhelyi B, Tamási L. Relationship of circulating hyaluronic acid levels to disease control in asthma and asthmatic pregnancy. *PLoS One.* 2014; 9(4): e94678. IF: 3,534
3. Bikov A, Bohacs A, Eszes N, Weiszhar Z, **Ivancso I**, Muller V, Rigo J Jr, Losonczy G, Tamasi L, Horvath I. Circulating and exhaled vascular endothelial growth factor in asthmatic pregnancy. *Biomarkers.* 2012; 17(7): 648-654. IF: 1,879
4. **Ivancsó I**, Bohács A, Eszes N, Losonczy G, Tamási L. Asthma in pregnancy. *EMJ Respir.* 2013; 1: 92-100.

Publications in Hungarian peer-reviewed journals:

1. **Ivancsó I**, Eszes N, Toldi G, Bohács A, Müller V, Rigó J Jr, Losonczy G, Vásárhelyi B, Tamási L. A perifériás hialuronsav és a betegsgékontroll kapcsolata asztmában és asztmás terhességeben. *Medicina Thoracalis.* 2015; 1: 40-45.

Publications out of the theme of the dissertation

Cumulative impact factor: 0,882

Publications in English peer-reviewed journals:

1. **Ivancsó I**, Böcskei R, Müller V, Tamási L. Extrafine inhaled corticosteroid therapy in the control of asthma. *J Asthma Allergy*. 2013; 6: 69-80.
2. Eszes N, Bohács A, Cseh A, Toldi G, Bikov A, **Ivancsó I**, Müller V, Horváth I, Rigó J Jr, Vásárhelyi B, Losonczy G, Tamási L. Relation of circulating T cell profiles to airway inflammation and asthma control in asthmatic pregnancy. *Acta Physiol Hung*. 2012; 99(3): 302-310. IF: 0,882

Publications in Hungarian peer-reviewed journals:

1. **Ivancsó I**, Böcskei R, Müller V, Tamási L. Extrafinom részecskeméretű inhalációs corticosteroid-terápia az asztma kezelésében. *Medicina Thoracalis*. 2013; 4: 235-238.
2. **Ivancsó I**, Vincze K, Juhász M, Tamási L. Sarcoidosis atípusos mycobacteriosissal. *Medicina Thoracalis*. 2011; 6: 397-401.
3. Tamási L, **Ivancsó I**. Foster NEXThaler®, az extrafi nom részecskét kibocsátó új szárazpor-inhalátor. *Medicina Thoracalis*. 2014; 6: 431-438.
4. Tamási L, Böcskei R, **Ivancsó I**. Epidermális növekedési faktor receptor gátló gyógyszerek a nem kissejtes tüdőrák kezelésében. *Onkológia*. 2013; 5: 201-205.