Project closing report

PD-113022

Role of gaseous mediators in the development of adverse pregnancy outcomes

1. Role of oxygen and nitrogen derived free radicals in the prognosis of adverse pregnancy outcomes together with the measurement of uterine artery pulsatility index

Pregnancy, despite its physiological nature, may be associated with a number of pathological conditions. The prevalence of pregnancy related complications, like preeclampsia, gestational diabetes mellitus (GDM), intrauterine growth restriction (IUGR), etc. can be as high as 15-20% in the general population. In spite of their high prevalence, their pathomechanism is still not fully understood and we only have limited therapeutic approaches. The early identification of these complications, even before the appearance of their specific symptoms would allow the better understanding of their pathomechanism and help the development novel screening and therapeutic methods. The occurrence of these complications are shown to be related to placental function. Examination of the uterine artery's blood flow by ultrasound Doppler method at the end of the first trimester or in the second trimester with the determination of pulsatility index, resistance index or early diastolic notch may reflect the resistance of the placental circulation. Increased placental resistance may indicate the higher risk for preeclampsia and IUGR. The simultaneous measurement of other early markers, like maternal plasma pregnancy related plasma protein A (PAPP-A), soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng), placental growth factor (PlGF), etc. may increase the sensitivity and specificity of the prognosis, however they still not meet the expectations.

Our aim was to examine the possible connection between high uterine artery resistance and oxidative-nitrative stress on the 12th week of healthy pregnancy. And to examine the possible predictive value of these parameters on pregnancy outcomes. In the present study, we aimed to measure oxidative-nitrative stress markers from peripheral blood samples that can be collected in minimal invasive way and do not disturb pregnancy outcome. Systemic oxidative-nitrative stress measured in these samples presumably correlates with placental function.

In a prospective observational study (Scientific and Research Ethics Committee of the Hungarian Medical Research Council approval: 425/2014 and 392/2015), healthy pregnant women between the 12-13th gestational weeks were invited to participate in the study. Uterine artery pulsatility index was determined by transabdominal ultrasound. Age and BMI matched pregnant women were divided into two groups based on the mean of the right and left artery PI; low resistance group (LR), (PI < 2.4), (n=31) and high resistance group (HR), (n=30) (Figure 1A). Plasma total peroxide (PRX) by colorimetric method, serum nitrotyrosine (NT) by competitive ELISA, and peripheral mononuclear leukocyte NT by immunohistochemistry was measured from blood samples collected right after the ultrasound examination. We observed pregnancy complications in 9.1% of all cases, all of which was GDM. No preeclapsia or IUGR was diagnosed during the study. Uterine artery resistance did not influence the development of GDM as its prevalence was similar in both groups.

In the high resistance group, serum NT was significantly higher (Figure 1B), and plasma PRX (Figure 1C) was significantly lower compared to the low resistance group, intracellular NT was not different in the two groups (Figure 1D). Lactate dehydrogenase was significantly lower in the high resistance group (152.66±2.75 vs. 144.70±2.47 U/L; $p<0.05$). In the high resistance group the

newborns' weight and chest circumference were significantly lower (3517.4±77.02 vs. 3316.8±63.76 g; 34.4±0.29 vs. 33.6±0.26 cm; $p<0.05$). Beside its intergroup difference, serum NT showed correlation to plasma glucose values in the total study cohort. The level of intracellular NT showed a positive correlation with the incidence of Cesarean section and negative correlation with the one minute Apgar values of newborns (Table 1).

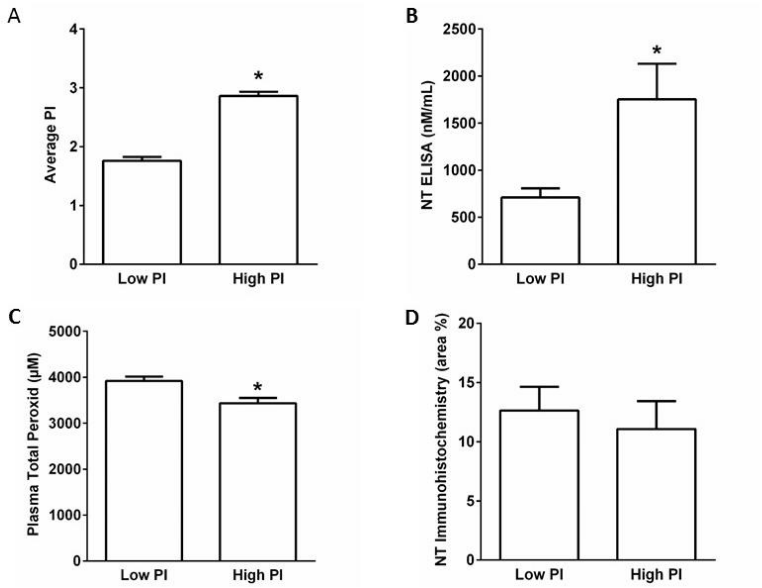

Figure 1. Pulsatility index and oxidative-nitrative stress markers. Panel A. Mean pulsatility index *of the uterine artery.* ***Panel B. Plasma total peroxide*** *levels were significantly lower in the high PI group.* ***Panel C. Serum 3-nitrotyrosine*** *levels were significantly higher in the same group.* ***Panel D. NT immunohistochemical staining intensity*** *of peripheral mononuclear cells were similar in the two study groups. Data are presented as mean±SEM, *:p<0.05.*

Table 1. Clinical parameters correlating to systemic nitrative stress markers measured on the 12-13th weeks of gestation. *Intracellular tyrosine nitration of circulating mononuclear cells positively correlated to γGT levels and the occurrence of Cesarean session and negatively with the 1 minute Apgar points. While serum free NT positively correlated to plasma glucose values.*

Independent variable: Leukocyte NT		
Dependent variables	*R*	*p*
γGT	0.407	0.026
Cesarean session	0.391	0.048
Apgar 1	-0.408	0.039
Independent variable: Serum NT		
Dependent variable	*R*	*p*
Glucose	0.295	0.042

In order to determine the odds ratio and relative risk of the two birth conditions (Cesarean section occurrence, worse one-minute Apgar value) we created two groups from the total study cohort based on their intracellular NT level. The cut-off value was 14 %. We found that the relative risk of

Cesarean section was 5.1 in for the high nitrative stress group. In the case of the one-minute Apgar value, the relative risk of newborns getting lower Apgar1 points (Apgar1 < 10) was 4.1 (Table 2).

Table 2. Predictive value of leukocyte tyrosine nitration above 14% of positive cellular area on the occurrence of later Cesarean session and lower (9 or lower) 1minute Apgar points.

	Cesarean session	*Apgar 1*
p	0.006	0.019
Relative risk	5.1	4.1
Odds ratio	12.3	8.4
Sensitivity	0.78	0.78
Specificity	0.78	0.71
Positive predictive value	0.64	0.58
Negative predictive value	0.88	0.86

We may hypothesize that pregnant women with high pulsatility index have lower concentrations of serum LDH because their placental vascular transformation is altered. This hypothesis is also supported by our other observation of decreased oxidative stress in these women that may be the result of reduced oxygenation of the placenta. On the other hand increased nitrative stress may reflect a compensatory increase in nitric oxide production, however it may also play role in placental alterations.

In conclusion, in parallel with higher PI, lower oxidative stress and higher nitrative stress were observed during the first trimester together with lower birth weight and chest circumference of the newborns. Elevated nitrative stress was associated with higher rate of Cesarean sections. Parallel uterine artery flowmetry and oxidative – nitrative stress parameters might predict newborns' and maternal outcomes.

The results of this study were presented at Hungarian and international conferences and a manuscript has been submitted to Fertility and Sterility (Manuscript #: FandS26054).

2. Role of oxygen and nitrogen derived free radicals in the prognosis of pregnancy outcome in gestational diabetes

Our other group of interest was women having GDM according to their oral glucose tolerance test between the 24^{th} and 28^{th} week of gestation and their healthy counterparts. Our aim was to examine the possible predictive value of 24-28^{th} week systemic oxidative-nitrative stress measured in blood components on pregnancy outcomes. Methods used were similar than in the previous study.

Plasma total peroxide, intracellular NT and protein poly(ADP-ribosylation) (PAR) values were significantly higher (3103.4± 118.1 vs. 2512.1± 130.6 μM; 32.8± 9.1 vs. 7.3±1.9%; 42.2±8.3 vs. 14.0±7.5%; $p<0.05$ respectively). The measured oxidative-nitrative parameters showed correlation with the concomitant BMI, metabolic laboratory parameters and gravidity Leukocyte tyrosine nitration also correlated to the development of perinatal hypoglycemia of the newborns (Table 3).

Table 3. Clinical parameters correlating to systemic nitrative stress markers measured on the 24-28th weeks of gestation.

Independent variable: Plasma total peroxide		
Dependent variables	*R*	*p*
BMI	0.713	<0.001
120min glucose of OGTT	0.705	<0.01
Triglyceride	0.419	<0.05
Cholesterol	0.413	<0.05
LDL	0.449	<0.05
Gravidity	0.404	<0.05
Independent variable: Leukocyte NT		
Dependent variable	*R*	*p*
BMI	0.754	<0.05
Triglyceride	0.920	<0.001
Perinatal hypoglycemia of the newborn	0.639	<0.05
Independent variable: Leukocyte PAR		
Dependent variable	*R*	*p*
120min glucose of OGTT	0.916	<0.05

The results of this study were presented at Hungarian and international conferences.

3. Oxidative-nitrative stress and poly(ADP-ribose) polymerase activation 3 years after pregnancy

Oxidative-nitrative stress and poly(ADP-ribose) polymerase-1 (PARP) activation have been previously observed in healthy and gestational diabetic pregnancies, and they were also linked to the development of metabolic diseases. Our aim was to examine these parameters and their correlation to known metabolic and cardiovascular risk factors following healthy and gestational diabetic pregnancies.

Fasting and 2h post-load plasma total peroxide level, protein tyrosine nitration and PARP activation were measured in circulating leukocytes three years after delivery in women following healthy, 'mild' (diet treated) or 'severe' (insulin treated) gestational diabetic pregnancy during a standard 75g OGTT. Nulliparous women and men served as control groups.

Fasting plasma total peroxide level was significantly elevated in women with previous pregnancy ($B=0.52\pm0.13$; $p<0.001$), with further increase in women with insulin-treated gestational diabetes ($B=0.36\pm0.17$; $p<0.05$) ($R^2=0.419$). Its level was independently related to previous pregnancy ($B=0.47\pm0.14$; $p<0.01$), and current CRP levels ($B=0.06\pm0.02$; $p<0.05$) ($R^2=0.306$).

Elevated oxidative stress but not nitrative stress or PARP activation can be measured three years after pregnancy. The increased oxidative stress may reflect the cost of reproduction and possibly

play a role in the increased metabolic risk observed in women with a history of severe gestational diabetes mellitus.

After reconsidering the manuscript according to the suggestions of the reviewers at Diabetes and its Complications, it has been submitted to Oxidative Medicine and Cellular Longevity on the 18th of January in 2018, and it is still under review (Manuscript #:1743253).

Another manuscript describing that the severity of prior GDM may have an impact on cardiovascular risk, which is in correlation with oxidative-nitrative stress and PARP activation was published in Orvosi Hetilap in 2015. (http://real.mtak.hu/27113/)

A short review about the role of oxidative stress is GDM was published as a book chapter in "Oxidative stress and diseases" edited by the Hungarian Society for Free-Radical Research. (http://real.mtak.hu/28141/)

4. The role of vitamin D deficiency and hyperandrogenism in the development of polycystic ovary syndrome and its metabolic and cardiovascular consequences in a rat model of polycystic ovary syndrome (PCO).

We continued the collaborative study with Szabolcs Várbíró (Semmelweis University, 2nd Department of Gynecology and Obstetrics), which was approved by the review board of the present grant after the first period closing report.

As hyperandrogenic state in females, which is one of the characteristic phenomenon in PCO, is accompanied with metabolic syndrome, insulin resistance and vascular pathologies. High number of hyperandrogenic women (67-85%) also suffer from vitamin D deficiency. Our aim was to examine the potential interplay between hyperandrogenism, vitamin D deficiency and possible oxidative-nitrative stress in the pathogenesis of insulin resistance and vascular pathologies using animal models.

Adolescent female rats were divided into four groups, 11–12 animals in each. Transdermal testosterone-treated and vehicle-treated animals were kept either on vitamin D-deficient or on vitamin D-supplemented diet for 8 weeks. At the end or the chronic treatment period, oral glucose tolerance test was performed and plasma insulin, leptin and vitamin D levels were measured. Testosterone treatment resulted in impaired glucose tolerance reflected by the increased 2hr value of OGTT. It also increased leptin levels, however only in the vitamin D supplemented group. Vitamin D deficiency elevated postprandial insulin levels and homeostatic model assessment insulin resistance (HOMA-IR).

In order to test peripheral insulin resistance in coronary resistant arteries insulin-induced relaxation was measured in vitro on isolated arterial segments. Insulin receptor and vitamin D receptor expressions were tested by immunohistochemistry. Testosterone treatment diminished insulin relaxation but did not affect the expression of insulin and vitamin D receptors in vascular tissue. On the other hand, vitamin D deficiency besides reducing insulin-induced coronary arteriole relaxation, it raised the expression of vitamin D and insulin receptors in the endothelial and medial layers of the vessels.

In summary testosterone treatment elevated postprandial sugar without hyperinsulinemia and HOMA-IR elevation, however serum leptin was augmented. Vascular response to insulin

diminished without alterations in insulin receptor expression. On the other hand, vitamin D deficiency raised only postprandial insulin concentrations, elevated HOMA-IR values and diminished vascular response to insulin despite substantial elevation of vascular insulin receptor expression in both layers of the vascular wall. According to our results, two different ways for vascular insulin insensitivity seem to be existing, both aggravating the pathological situation in our experimental model of PCO.

These results have been published in Diabetes and Vascular Disease Research in 2018. (http://journals.sagepub.com/doi/pdf/10.1177/1479164118758580)

In the same animal model, the function of the aortae was also analyzed. Estrogen induced endothelium dependent relaxation of isolated aorta segments was examined ex vivo by wire myography. Vitamin D deficiency but not testosterone treatment reduced the estrogen induced relaxation of the vessel segments. The nitric oxide synthase (NOS) inhibitor Nω-Nitro-L-arginine methyl ester (L-NAME) was able to diminish this relaxation in all cases, however the cyclooxygenase-2 (COX-2) inhibitor NS-398 only had effect in vitamin D deficiency (Figure 2). The involvement of COX-2 mediated mechanisms in estrogen induced relaxation can be involved in compensatory processes for reduced estrogen sensitivity in vitamin D deficiency. This hypotheses is reinforced by the reduced estrogen receptor expression of the vessel walls examined by immunohistochemistry. Reduced estrogen receptor expression was accompanied by reduced vitamin D receptor, endothelial nitric oxide synthase and COX-2 density.

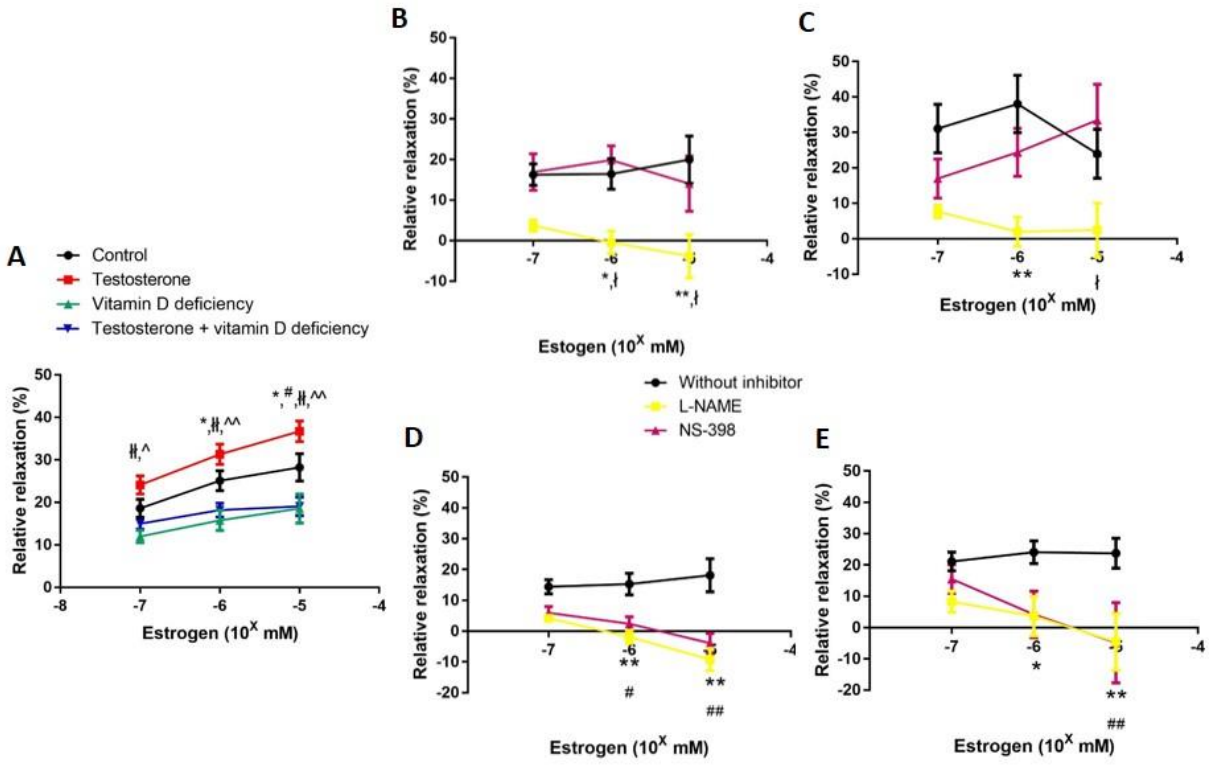

Figure 2. Estrogen induced aortic relaxation. Panel A. *Vitamin D deficiency significantly reduced the* ***estrogen induced aortic relaxation****. Mean±SEM, *: p<0.05 Control vs. Vitamin D deficiency, #: p<0.05 Control vs. Testosterone + vitamin D deficiency, łł: p<0.01 Testosterone vs. vitamin D deficiency, ^: p<0.05 Testosterone vs. Testosterone + vitamin D deficiency, ^^: p<0.01 Testosterone vs. Testosterone + vitamin D deficiency. P****anel B-E. Effect of NOS and COX-2 inhibition on***

estrogen induced relaxation. *Mean±SEM, *: p<0.05 L-NAME vs. Without inhibitor, **: p<0.01 L-NAME vs. Without inhibitor, ł: *: p<0.05 L-NAME vs. NS-398, #: p<0.05 NS-398 vs. Without inhibitor, ##: p<0.05 NS-398 vs. Without inhibitor* ***Panel B. In control animals*** *estrogen induced relaxation was only inhibited by NOS blockade,* ***Panel C.*** *similarly to the* ***testosterone treated group****. In both vitamin D deficient groups; in* ***Panel D. Vitamin D deficient*** *and* ***Panel E. Testosterone treated + vitamin D deficient*** *rats, both NOS and COX-2 inhibition diminished estrogen induced relaxation.*

According to our results the estrogen induced relaxation of big arteries that was shown to play an important role in the lower cardiovascular risk of fertile women is altered by vitamin D deficiency. The observed phenomenon may contribute to the cardiovascular pathologies in women with vitamin D deficiency frequently seen in PCO.

5. Oxidative Stress-Related Parthanatos of Circulating Mononuclear Leukocytes in Heart Failure

In collaboration with the Heart and Vascular Center of Semmelweis University, Budapest, Hungary we were able to test the possible predictive value of oxidative-nitrative stress and PARP activation of blood components in another disease; chronic heart failure (CHF).

In an observational cross-sectional cohort study patients with CHF (n = 20) and age- and body mass index-matched volunteers (n = 15) with a normal heart function were enrolled (Ethical approval: 7268-0/2011-EKU). C-reactive protein, N-terminal probrain-type natriuretic peptide (pro-BNP), plasma total peroxide level (PRX), plasma total antioxidant capacity (TAC), oxidative stress index (OSI), leukocyte lipid peroxidation (4-hydroxynonenal; HNE), protein tyrosine nitration (NT), poly(ADP-ribosyl)ation (PARylation), and apoptosis-inducing factor (AIF) translocation were measured in blood samples of fasting subjects.

Several markers of systemic oxidative-nitrative stress: plasma PRX, leukocyte HNE, NT, PARylation, and AIF translocation were significantly higher in the heart failure group. Pro-BNP levels that are strong predictors of mortality in chronic heart failure showed a significant positive correlation to PRX, OSI, leukocyte HNE, NT, PARylation, and AIF translocation in the total study cohort. Ejection fraction negatively correlated with the same parameters. Among HF patients, a positive correlation of pro-BNP with PRX, OSI, and PARylation was still present.

According to our results, markers of oxidative-nitrative stress, PARP activation, and AIF translocation in blood components shows correlation to reduced cardiac function and the clinical appearance of CHF. Moreover, oxidative stress and PARP activation may also indicate the progression of heart failure. The positive correlation between leukocyte PARP activation and pro-BNP may assign the minimal invasive measurement of this parameter to a possible diagnostic tool for heart failure progression monitoring. It may also serve as a potential early detection tool for incipient heart failure. These observations may also reinforce the consideration of PARP inhibition as a potential therapeutic target in CHF.

Results of this study was published in Oxidative Medicine and Cellular Longevity in 2017. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5700485/)

6. Role of PARP activation in inflammatory diseases

The role of increased production of nitric oxide and related nitrogen derived free radicals and the concomitant activation of PARP are shown to play important role in chronic inflammatory diseases. In collaboration with Dr. Gábor Veres at the 1st Department of Pediatrics of Semmelweis University we were able to examine the role of these parameters in the pathogenesis of pediatric Crohn's disease. However, in contrast to previous in vitro and animal model studies of the field, surprisingly we found decreased PARP protein expression and activation in colonic biopsies of children with Crohn's disease. Although our results contradict to the in vitro and animal model findings, there are prior studies that support our observations. Makowitz et al. in 1988 showed that hydrogen peroxide-induced PARP-1 activation was reduced in isolated mononuclear cells of IBD patients and even the involvement of their first-degree relatives was raised. The defect of DNA repair was hypothesized to play a role in the pathogenesis of the disease [Markowitz MM et al. Gut. 1988; 29:1680-1686]. Few years later, the production of autoantibodies against PARP-1 (zinc-finger motifs F1 and F2) was also demonstrated in Crohn's disease patients, where the level of the specific autoantibody seemed to correlate with the Crohn's disease activity index (CDAI) [Reumaux D et al. Clinical immunology and immunopathology. 1995; 77:349-357]. While examining the possible cause of decreased PARP protein expression and activity we were able to identify the increased expression of microRNA-223 that was shown to have PARP-1 as direct target in vivo in Barrett's esophagus. Our results have been presented on Hungarian and international conferences and the manuscript is under reconstruction according to the major comments of reviewers at the Journal of Pediatric Gastroenterology and Nutrition.

Other publication

The revised version of the methodological book chapter describing the determination of PARP activity in human samples that was published five years ago has been issued in the 2nd edition of Poly(ADP-ribose) Polymerase Methods and Protocols published as a member of the Methods in Molecular Biology series in 2017.

Achievements of PhD and undergraduate students

Dr. Tamás Bárány PhD student working under my supervision fulfilled the publication and credit point requirements of the Doctoral School of Basic and Translational Medicine of Semmelweis University and prepares for his final exam and writes his doctoral thesis. Undergraduate students working in our research team held 10 oral presentations on the Research Student Conference of Semmelweis University and received three 1st and two 2nd prizes. Áron Penyige presented his talk on the National Scientific Students' Associations Conference last year, and Dóra Gerszi will present it next year.

Difficulties and setbacks

Difficulties in the measurement of H_2S in plasma samples set back the progression of this aspect of the study. Methylene blue method used for the determination for H_2S level in snap frozen plasma showed high inter- and intra-assay variability, and the data of repeated measurements showed low

correlation. We also tried to establish the method for H_2S measurement for H_2S electrode. Sulfide-sensitive electrodes measure sulfide concentration from aqueous solutions, characterized by the electromotive force (EMF). Amongst other biological samples, it is also used for blood plasma measurements [Guo R et al. Mech Ageing Dev 2017; 162:46-52.]. However, we were not able to adjust the method for reliable human plasma H_2S measurements. The accurate measurement of H_2S in biological systems seems to address difficulties even in the clinical trials of H_2S donors; the termination of a trial was reported due to the lack of fast and reliable method (NCT00879645). Measuring H_2S with the more sensitive novel fluorescent probe 7-AZ [Gersztenkorn D. et al. Invest Ophthalmol Vis Sci. 2016] was unfortunately too expensive to our budget. We also tried to breach for new cooperators, but we were not able to establish a cooperation in the last grant period.

In the first two years of the grand period we faced difficulties by the low rate of patient recruitment that fell short of expectations due to a low rate of 10-13 week pregnancies turning up at our cooperator. Another difficulty of recruitment, especially in case of GMD pregnancies, was the need of an extra blood collection, as they usually have to make their clinical laboratory measurements at an outpatient clinic. This routine also increases the variability of data we are going to use from these measurements. In order to overcome this difficulty were agreed to perform these clinical laboratory measurements at the Central Laboratory of Semmelweis University with the generous financial help of the Department of Physiology. In the last one and a half year of the grant period we were able to increase recruitment rate by breaching for new cooperators at the 2nd Department of Obstetrics and Gynecology of Semmelweis University.

Between November 2014 and August 2015 our lab faced a temporary loss of a colleague, Rita Benkő (assistant professor), who was on sick leave. Her absence meant an unfortunate setback for the execution of measurements related to the study. In 2015, our research team joined the Department of Physiology due to the reorganization of physiology teaching at Semmelweis University. The official rearrangement and moving took place after September 2015.

Budapest, 31th of March 31, 2018

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Dr. Eszter M. Horváth