Spontaneous preterm birth as a multifactorial disease in pregnancy

Ph.D. theses

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INTRODUCTION

Preterm delivery is a multifactorial condition requiring a complex interaction between genetic and environmental factors. Preterm delivery develops when the physiological balance between factors necessary to maintain pregnancy vs. factors triggering uterine contractions is disrupted. The resulting imbalance may be of maternal, fetal or placental origin. Some of the disorders giving rise to premature birth may respond to medical treatment, while others progress unaffected.

Preterm delivery is defined as birth of an infant before the 37th gestational week. In certain cases when gestational age cannot be determined with precision, preterm delivery is also diagnosed on the basis of neonatal birth weight. Correspondingly, neonatal birth weight of less than 2500 grams is regarded as criterion for preterm delivery. Since nearly 75 percent of perinatal mortality and about half of overall postnatal morbidity are associated with preterm delivery, this condition is a major challenge not only for medical professionals but for the society as a whole.

Multiple genetic and environmental factors can be identified in the background of preterm delivery. Due to the wide spectrum of potential factors, it is often difficult to identify the one most influential in a specific case; the complexity of mechanisms operating in the etiology of premature birth renders both family planning and preventive medical care during pregnancy a challenging enterprise. A large set of physiological mechanisms may be affected by genetic factors in the background of preterm delivery. In addition, genes found to be responsible for several other obstetric conditions or conditions related to other organ systems may also play a role in the etiology of premature birth.

Environmental factors affecting preterm delivery are also widely heterogeneous. Some of these factors may respond favorably to appropriate medical care during pregnancy combined with a healthy lifestyle by the mother, while others remain largely unaffected by these measures. Due to a close interaction between environmental and genetic factors, the significance of environmental factors cannot be fully understood without considering the specific genetic background in an individual case. Similar to the situation in other hereditary problems, a complex interplay between genetic and

environmental factors affecting the risk for preterm delivery are necessary for this clinical condition to develop. Thus, any attempt to grasp the etiology of preterm delivery in a specific individual should involve a thorough understanding of the environmental-genetic background regarded as a complex system.

STUDY AIMS

The main objective of the studies described below was to investigate alterations of (1) gene expression patterns of IGF, (2) activity of apoptotic genes and (3) the activity of the 11β-hydroxysteroid-dehydrogenase 2 gene (the latter being crucial in the regulation of the feto-maternal glucocorticoid metabolism) in human placental samples from preterm delivery cases compared to placental samples from normal pregnancy. This provided an opportunity to clarify the role played by the IGF system, apoptosis and feto-maternal glucocorticoid metabolism in the etiology of preterm delivery.

The interpretation of genetic results was performed considering the clinical context with several relevant clinical factors analyzed including maternal age, gestational age at delivery, fetal gender distribution, gestational weight gain and increase of body mass index (BMI) during pregnancy, mode of induction of preterm delivery, history of preterm delivery including previous preterm delivery or preterm birth of the mother herself, vaginal colonization by group B streptococcus (GBS) and smoking during pregnancy.

Specific study questions included:

1. Is there a significant difference in median maternal age between mothers delivering preterm infants vs. those delivering term infants? What is the difference in maternal age distribution between the preterm and term delivery groups? What is median gestational age in the preterm delivery group?

2. What is the difference between the preterm and term delivery groups in gestational weight gain and increase of BMI during pregnancy?

3. What is the distribution of different modes of induction of preterm delivery? What is the fetal gender distribution within the preterm delivery group?

4. What role does a positive history for preterm delivery or preterm birth of the mother play in the etiology of preterm delivery?

5. How does the presence of vaginal colonization by GBS within the last 4 weeks before delivery affect the risk for preterm delivery? What is the role of smoking in increasing the risk for preterm delivery?

6. What is the difference between placental gene expression patterns of IGF-I, IGF-II and IGFBP-3 genes between the term and preterm delivery groups? What is the influence of altered gene expression patterns on the condition of the fetus in the preterm delivery group?

7. Is there a correlation between gene activity of IGF-I, IGF-II and IGFBP-3 genes and either gestational age at time of delivery or fetal gender?

8. Is there a difference between gene expression patterns for either the proapoptotic Bax or the antiapoptotic Bcl-2 genes in the preterm vs. term delivery groups? What is the role of apoptosis in the induction of preterm delivery?

9. Is there a correlation between the placental activity of Bax and Bcl-2 genes and gestational age at the time of delivery or fetal gender?

10. Is there a difference between placental gene activity of the 11β-hydroxysteroid-dehydrogenase 2 gene (11β-HSD2) between the preterm and term delivery groups? What is the role of abnormal feto-maternal glucocorticoid metabolism in the induction of preterm delivery?

11. Is there a correlation between placental activity of the 11β-HSD2 gene and gestational age at delivery or fetal gender?

MATERIALS AND METHODS

Between January 1, 2010 and January 1, 2011 we examined placentas from 104 pregnancies that ended in preterm delivery at the Second Department of Gynecology and Obstetrics. We compared placental gene expression patterns and several clinical characteristics (as described below) in the preterm delivery group to full term pregnancy controls gained from 140 normal pregnancies in the same time period. Preterm delivery was diagnosed on the basis of gestational age less than 37 weeks or neonatal birthweight less than 2500 grams. Preterm delivery was effected either by spontaneous uterine activity, or premature rupture of the membranes. Cases in which preterm delivery was due to either multiple pregnancies, fetal or chromosomal developmental disorders, anatomical malformations of the genital tract, placental adhesion, implantation disorders or induced deliveries were excluded.

Delivery was either vaginal or by cesarean section based on the clinical situation. In the final analysis, no distinction was made with respect to the type of delivery.

Placental tissue samples were taken in a uniform manner with approximate dimensions of 2x2x2 cm (8 cm^3), which were then kept at -70 ℃ for genetic expression testing. Maternal demographics and relevant clinical data during pregnancy or the perinatal period were collected including maternal and paternal age, obstetric history, genetic history, general medical history, maternal birthweight, gestational age at the time of delivery, fetal gender, weight gain and BMI increase during pregnancy, pregestational BMI, pregnancy-related pathology including disorders of carbohydrate metabolism, neonatal birthweight, Apgar score, smoking history and Group B Streptococcal screening. Consents were obtained in each case from the mother (signatures on file).

Whole placental RNA content was isolated with Quick RNA microprep kit (Zymo Research). RNA concentration was determined using NanoDrop spectophotometer (NanoDrop). Reverse transcription was performed in 20 μl target

volume using 5μg whole RNS, 75 pmol random hexamer primer, 10 mM dNTP (Invitrogen), 20 U M-MuLV Reverse Transciptase enzyme (MBI Fermentas) and 1x-es buffer (MBI Fermentas). The reaction mix was incubated for 2 hours at 42°C. Subsequently, the enzyme was inactivated at 70°C for 15 minutes.

The reverse transcriptase reaction solution was diluted three-fold with nuclease-free water. For the real-time PCR assay, 1 μl diluted cDNS (approximately15 ng RNA-equivalent) and 1 x SYBR Green Master Mixet (Applied Biosystems) were used. Primers were designed using Primer Express Software (Applied Biosystems). Primer sequences are detailed in Table 2. Real-time PCR was performed in 20 μl target volume using 1 μl cDNA, 1 pmol, gene-specific Forward and Reverse primer and 1 x SYBR Green PCR Master mix. All real-time PCR were performed using the MX3000 Real-time PCR (Stratagen) system with the following settings: 40 cycles at 95°C, denaturing process for 15 seconds, annealing at 60 °C, chain elongation and detection for 60 seconds. For each gene, relative expression was normalized using the human *β-actin* gene as standard.

For gene expression studies two-sample t-test was used with 95% confidence interval. Determination of degree of freedom was performed using the Welch-Satterthwaite correction. Values of gene expression testing were interpreted in the following manner: (1) overexpression= Ln value >1, $p<0.05$; (2) underexpression= Ln value <-1, $p<0.05$; (3) normal expression= Ln value $<1,>-1$, $p<0.05$. GraphPad Prism 3.0 (GraphPad Software Inc) software was used in all statistical analytic procedures.

Demographics and clinical data were analysed with SPSS software. Logistic regression was used for dichotomous outcomes with multiple independent variables. For continuous outcomes, analysis of variance (ANOVA) and linear regression were used as appropriate. P value of <0.05 was accepted for statistical significance.

RESULTS

Median maternal age in the preterm delivery group was 30.7±5.20 years, in the control group median age was 31.4±3.12 years (NS).

Through distribution of maternal age by age groups within the preterm delivery group, there was no significant difference in the occurrence of preterm delivery between the different age groups. Most common maternal age was between 31-35 years (n=49, 47.1%).

Median gestational age in the preterm delivery group was 32.8±3.7. As expected, in the preterm delivery group gestational weight gain was found to be significantly lower than controls by logistic regression (11.6±4.6 kg vs. 14.7±2.6 kg). Pre-gestational BMI that had been hypothesized to be a predictor of preterm delivery did not, in fact, predict preterm delivery ($BMI_{preterm\ delivery}$ 21.2±3.72 vs. $BMI_{control}$ 23.3±2.92; NS).

Preterm delivery began with premature rupture of membranes in 70.2% and spontaneous uterine activity 29.8%. In the preterm delivery group, distribution of fetal gender was as follows: 49 males, 55 females (M:F 0.89); in the control group 73 males, 67 females (M:F 1.09), (NS).

In the preterm delivery group, 15 out of 104 mothers had a positive history for at least 1 preterm delivery (14.4%). This was significantly higher than in the control group (6 out of 140; 4.3%; p<0.05) suggesting that positive history of preterm delivery significantly increases the risk for subsequent preterm delivery.

In the preterm delivery group, we had data for either gestational age or birth weight of the mother in only 41 cases. In 7 out of 41 cases (17.2%) the mother herself had been preterm by either criteria. In the preterm delivery group, Group B streptococcal screening was performed during prenatal care in 34 cases. It was found to be positive in 14.7% (5 out of 34), and negative in 85.3% (29 out of 34). This was not significantly different (p>0.05) from results in the control group where, out of a total of 92, 17 were positive (18.5%) and 75 negative (81.5%) (NS).

The occurrence of smoking during pregnancy was significantly higher in the preterm delivery group (28 out of 104; 26.9%), than in the control group (10 out of 140; 7.1%; p<0.05) suggesting that smoking during pregnancy is associated with a significant increase in the risk for preterm delivery.

On comparing IGF-I, IGF-II and IGFBP-3 gene expression between the preterm delivery and normal pregnancy groups, we observed a 1.57-fold decrease in the gene expression of IGF-I (p<0.04). In contrast, no significant difference could be identified between the groups in either IGF-II or IGFBP-3 gene expression. The underexpression of IGF-I gene in the preterm delivery group proved to be independent of gestational age. Similarly, the expression of IGF-II and IGFBP-3 was unchanged and did not seem to correlate with gestational age. With respect to fetal gender, we could not find an association between IGF-I gene expression and fetal gender; however, both IGF-II and IGFBP-3 gene expression were associated with fetal gender distribution: a 2.04-fold overexpression of both genes were seen in male as compared to female fetuses (IGF-II p<0.04; IGFBP-3 p<0.03).

There was no difference in the preterm delivery vs. normal pregnancy placental samples regarding the expression of the antiapoptotic Bcl-2 gene. In contrast, the proapoptotic Bax gene was 1.35-fold overexpressed in the preterm delivery group compared to controls (p<0.04). Bcl-2 gene expression did not seem to depend on gestational age at the time of delivery: it remained unchanged and similar in the 24-28, 28-32 and 32-36 week gestational age subgroups. However, Bax gene expression differed between these subgroups: it was overexpressed in the 28-32 week subgroup (1.56-fold overexpression; p<0.04) and in the 32-36 week subgroup (1.41-fold overexpression; p<0.04), while in the 24-28 week subgroup gene activity was not different from the control group. Within the preterm delivery group, there was no fetal gender-dependent difference in placental Bax or Bcl-2 gene expression.

Placental expression of the 11β-HSD2 gene was significantly decreased in the preterm delivery as compared to the normal pregnancy group (1.87-fold underexpression; p<0.04). Similar to the apoptotic genes, gene expression for the 11β-HSD2 gene was also dependent on gestational age at time of delivery. In the 24-28 gestational week subgroup, 11β-HSD2 was unchanged compared to the control group. However, in the 28-32 week subgroup there was a 2.23-fold decrease in gene activity

(p<0.04) while in the 32-36 week subgroup the decrease in gene activity was 1.89-fold (p<0.04). No fetal gender-dependent differences could be identified within the preterm delivery group.

CONCLUSIONS

In our study no significant difference was seen in median maternal age between the preterm and normal pregnancy groups. Similarly, pre-gestational BMI was not different between the groups.

Both history of preterm delivery and history of preterm birth of the mother were significantly more common in women who delivered preterm.

Although we did not see a significant difference in the rate of vaginal colonization by Group B Streptococcus between the preterm and normal delivery groups, the number of cases were simply too low to arrive at a definitive conclusion in this respect. The most important environmental factor increasing the risk for preterm delivery in our study was smoking during pregnancy.

Placental expression of the IGF-I gene showed a significant decrease in the preterm delivery group compared to the normal pregnancy group. No such difference could be identified with respect to either IGF-II or IGFBP-3 gene expression in the placenta.

It has been recognized that in the majority of cases the primary factor inducing preterm delivery is intrauterine infection. An increased production of inflammatory mediators due to intrauterine infection leads to decreased activity of the IGF-I system. This inhibition of the IGF system by an infectious process does not operate in term pregnancies. When gene expression of IGF-I is decreased due to intrauterine infection, a decreased fetal capacity to handle distress likely ensues. In our study, IGF-I was significantly underexpressed in placenta samples in the preterm delivery group while IGF-II and IGFBP-3 remained unchanged. This underexpression of IGF-I in preterm delivery was not fetal gender-dependent. On the other hand, an overexpression of IGF-

II and IGFBP-3 genes was present when fetal gender was male, suggesting that IGF-II and IGFBP-3 may generally participate in the development of male phenotype.

In our studies, we found no evidence for altered gene expression of the antiapoptotic Bcl-2 gene in the preterm delivery group. In contrast, there was a clear overexpression of the proapoptotic Bax gene in placental samples obtained from preterm delivery as compared to normal pregnancy samples. We propose that it is increased activity of the proapoptotic Bax gene but not decreased of the activity of the antiapoptotic Bcl-2 gene that mediates the influence of apoptotic regulatory genes in the induction of preterm delivery. Correspondingly, there was no change in Bcl-2 gene activity in any of the gestational age subgroups (24-28 weeks, 28-32 weeks and 32-36 weeks). In contrast, we found an increased gene activity of the Bax gene in both the 28-32 week and 32-36 week subgroups, although not in the 24-28 week subgroup. We conclude that the role of apoptosis in preterm delivery occurring between gestational weeks 24-28 is less pronounced than in those after gestational week 28. It is probable that when preterm delivery occurs before gestational week 28, other risk factors may be more prominent. These may include age and nutritional status of the mother, smoking during pregnancy and history of previous preterm delivery. Our findings indicate that neither Bax nor Bcl-2 gene expression is dependent on fetal gender.

We found a diminished placental expression of the 11β-HSD2 gene in preterm delivery. During the later stages of term pregnancies, placental production of 11β-HSD2 increases substantially. The significance of this phenomenon lies in the protection of the fetus against the effects of maternal glucocorticoids. In the case of impending preterm delivery, a decrease in 11β-HSD2 gene activity results in impaired fetal protection against maternal glucocorticoids through an inadequate placental functional barrier. The enhanced maternal steroid exposure due to decreased 11β-HSD2 activity may lead to increased risk for intrauterine infections. In addition, augmented maternal glucocorticoid exposure also affects birth weight that is already reduced in premature birth due to a shorter gestational period. When decreased activity of the 11β-HSD2 enzyme is due to genetic mutation, the risk for intrauterine growth restriction and perhaps also for preterm delivery may increase. If positive history for either intrauterine growth restriction or preterm delivery is identified, a closer follow-up of the pregnant

woman or, depending on the clinical situation, hospitalization may be warranted due to increased risk for preterm delivery. We speculate that the abnormal feto-maternal glucocorticoid metabolism associated with alteration in 11β-HSD2 gene activity may play a role in the development of "fetal programming" (according to the theory of "fetal programming" the predisposition to some adult diseases is acquired during the intrauterine development). Genetic expression of the 11β-HSD2 gene in the placenta appears to be dependent on gestational age at the time of delivery. When preterm delivery occurs between gestational weeks 24-28, there is no alteration in 11β-HSD2 gene activity. On the other hand, in preterm deliveries occurring between gestational weeks 28-32 or 32-36 a decreased 11β-HSD2 activity is present. We conclude that increased maternal glucocorticoid exposure becomes an important factor in the development of preterm delivery after gestational week 28. In the time period between gestational weeks 24-28 abnormal fetomaternal glucocorticoid metabolism appears to be much less important. Placental expression of the 11β-HSD2 gene does not appear to be dependent on fetal gender.

LIST OF PUBLICATIONS (IF: 17.51)

PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

1. **Demendi C**, Börzsönyi B, Nagy ZB, Rigó J Jr, Pajor A, Joó JG. Gene
    expression patterns of insulin-like growth factor 1, 2 (IGF-1, IGF-2) and insulin-
    like growth factor binding protein 3 (IGFBP-3) in human placenta from preterm
    deliveries: influence of additional factors. Eur J Obstet Gynecol Reprod Biol,
    2012; 160: 40-44. **IF: 1.764**

2. **Demendi C**, Börzsönyi B, Végh V, Nagy ZB, Rigó J Jr, Pajor A, Joó JG. Gene
    expression patterns of the Bcl-2 and Bax genes in preterm birth. Acta Obstet
    Gynecol Scand (in press), doi: 10.1111/j.1600-0412.2012.01428.x. **IF: 1.860**

3. Joó JG, **Demendi C**, Börzsönyi B, Csanád M, Pajor A, Rigó J Jr, Nagy ZB. A
    fetomaternalis glükokortikoid-anyagcsere egyensúlyzavarának kóroki szerepe a
    koraszülés hátterében; a lepényi 11β-hidroxiszteroid dehidrogénáz 2 enzim
    génjének expressziós mintázata. Magy Nőorv L, 2012; 75: 14-21.

PUBLICATIONS INDEPENDENT OF THE SUBJECT OF THE THESIS

1. Joó JG, Börzsönyi B, **Demendi C**, Végh V, Pajor A, Rigó J Jr, Nagy ZB. A 11ß-
    hidroxiszteroid dehidrogenáz 2 enzim génjének expressziós mintázata intrauterin
    etardációval járó terhességekből származó lepényszövetekben; a fetomaternalis
    glükokortikoid-anyagcsere egyensúlyzavarának kóroki szerepe. Magy Nőorv L,
    2012; 75: 21-28.

2. Börzsönyi B, **Demendi C**, Pajor A, Rigó J Jr, Marosi K, Ágota A, Nagy ZsB, Joó JG. Gene expression patterns of the 11β-hydroxysteroid dehydrogenase 2 enzyme in human placenta from intrauterine growth restriction: the role of impaired feto-maternal glucocorticoid metabolism. Eur J Obstet Gynecol Reprod Biol, 2012; 161: 12-17. **IF: 1.764**

3. Joó JG, Csatlós É, Börzsönyi B, **Demendi C**, Csaba Á, Rigó J Jr. Non-syndromic malformations of the central nervous system in twin pregnancies: diagnostic and other clinical features of importance. Eur J Obstet Gynecol Reprod Biol, 2011; 154: 27-30. **IF: 1.764**

4. Joó JG, Csatlós É, Börzsönyi B, **Demendi C**, Rigó J Jr. Fetopathological investigations after induced abortions performed in mid-term gemini pregnancies. Pathol Res Pract, 2011; 207: 443-447. **IF: 1.258**

5. **Demendi C**, Németh M, Langmár Z. Congenital disorders. Holoprosencephalia. Orv Hetil, 2011; 152: 2105-2108.

6. **Demendi C**, Langmár Z, Bánhidy F, Börzsönyi B, Csatlós É, Joó JG. Successful operative management of an intact second trimester abdominal pregnancy with additional preoperative selective catheter embolisation and postoperative methotrexate therapy. Med Sci Monit, 2011; 17: 53-55. **IF: 1.699**

7. Börzsönyi B, **Demendi C**, Nagy ZB, Tóth K, Csanád M, Pajor A, Rigó J Jr, Joó JG. Gene expression patterns of insulin-like growth factor 1, insulin-like growth factor 2 and insulin-like growth factor binding protein 3 in human placenta from pregnancies with intrauterine growth restriction. J Perinat Med, 2011; 39: 701-707. **IF: 1.871**

8. Nagy ZB, Csanád M, Tóth K, Börzsönyi B, **Demendi C**, Rigó J Jr, Joó JG. Current concepts in the genetic diagnostics of rheumatoid arthritis. Expert Rev Mol Diagn, 2010; 10: 603-618. **IF: 4.652**

9. Szabó I, Börzsönyi B, **Demendi C**, Langmár Z. Successful laparoscopic management of a non-communicating rudimentary horn pregnancy. Orv Hetil, 2009; 150: 513-515.

10. **Demendi C**, Langmár Z, Bánhidy F, Ács N, Paulin F, Nemes B. Intakt második trimeszterbeli hasűri terhesség műtétes megoldása preoperatív szelektív embolizáció alkalmazásával. Magy Nőorv L, 2008; 71: 91-94.

11. Acs N, Vajo Z, **Demendi C**, Nadasy G, Monos E, Szekacs B. Estrogen improves impaired musculocutaneous vascular adrenergic reactivity in pharmacologically ovariectomized rats: a potential peripheral mechanism for hot flashes? Gynecol Endocrinol, 2001; 15: 68-73. **IF: 0.878**

12. Siklósi G, Ács N, Demendi C, Börzsönyi B, Gimes G, Bakos L, Olajos F, Marcsek F. Luteal function as the main determinant of prengnancy outcome: succesful prevention of spontaneus abortion, prematurity and IUGR. Early Pregnancy, 2001; 5: 22-23.