Genetic polymorphisms of selectin proteins and estrogen-receptors in perinatal morbidities and in preeclampsia.

László Derzbach M.D.

Tutor: Barna Vásárhelyi M.D. Ph.D.
1. Introduction

The estimated incidence of preterm birth in Hungary is 10 per cent. It presents an enormous burden on families and health-care systems as the majority of morbidities and deaths occurs among preterm babies. Although the cause of preterm birth is usually unknown, amounting data suggest the contribution of ascending vaginal infection in the initiation of preterm labour and perinatal complications. As a result of infection, acute inflammatory response and increased cytokine production develop in the fetus and newborn and harms tissues such as lungs, kidneys, bowels and central nervous system. Preeclampsia is a maternal complication leading to preterm birth. Beside genetic factors, placental ischemia and oxidative stress, inflammation plays a central role in the patomechanism of preeclampsia. The aim of my PhD work was to elucidate the contribution of some genetic polymorphisms to preterm birth and perinatal morbidities.

Selectins mediate the rolling and capture of leukocytes to endothelial cells. This process is the first step of the adhesion cascade, which results in the extravasation of leukocytes to the sites of inflammation, infection or damage.

There are three main types of selectins. E-selectin is exclusively expressed on endothelial cells following inflammatory stimulation. P-selectin is stored in platelets and in endothelial cells, and is translocated to the cell surface upon activation. L-selectin is expressed on leukocytes. Several data support the significance of adhesion molecules in normal pregnancy. At the maternal-fetal interface, adhesion molecules including E-, P- and L-selectins are highly expressed. Adequate expression of selectin molecules is indispensable for implantation and normal development of placenta. Other data support the role of selectins in perinatal complications. Decreased P-selectin expression on the surface of endothelial cells in preterm infants has been shown to contribute to delayed neutrophil transmigration and increased susceptibility to infection. Inflammatory processes affecting the lung are also mediated partly by selectins. Selectins also play a central role in preeclampsia.

Inflammation is influenced by a number of factors including infection, therapy and physiological changes. These include the alteration of hormonal milieu (i.e premature cessation of in utero high estrogen levels) that may also exacerbate inflammatory processes.

There are several polymorphisms of genes encoding selectins and estrogen-receptor, which may have an impact on the function of the affected protein and hereby on the inflammatory process and, indirectly, on the susceptibility of perinatal complications. The Ser128Arg polymorphism in the E-selectin gene is associated with increased levels of soluble E-selectin, the severity of atherosclerotic arterial disease and altered adhesion of leukocytes to
endothelial cells. The Thr715Pro polymorphism in the P-selectin gene is associated with reduced levels of serum soluble P-selectin and decreased risk of myocardial infarction. The functional consequence of the L-selectin Pro213Ser polymorphism has not been strictly established, some data suggest that it may have an impact on leukocyte–endothelial interactions. The most investigated polymorphisms of estrogen receptor-α gene are the PvuII and the XbaI polymorphisms. Carrier state of the „P” allele of the PvuII polymorphism is associated with reduced ER-α expression, which is verified by increased risk of osteoporosis and myocardial infarction. The XbaI polymorphism was also related to estrogen-mediated diseases.

The perinatal inflammatory process could be therapeutically influenced by immunomodulatory drugs. In my field of research I have collected preliminary data about the direct effect of phosphodiesterase enzyme inhibitor pentoxifyline and estradiol on monocyte functions. We have investigated the impact of these drugs on selectin expression and phagocyte function.
2. Aims

1. Is there any association between the polymorphisms of the selectin genes and the risk of preterm birth and perinatal complications?
2. Is there any association between the polymorphisms of the selectin genes and the risk of preeclampsia?
3. Is there any association between the PvuII polymorphism of the estrogen receptor-\( \alpha \) gene and perinatal complications?
4. Is there any association between the PvuII and XbaI polymorphism of the estrogen receptor-\( \alpha \) gene and the risk of preeclampsia?
5. Is there any immunomodulatory effect of pentoxifylline and 17\( \beta \)-estradiol in our in vitro system?
6. Is there any association between the polymorphisms of the P- and L-selectin genes and the P- and L-selectin expression?
3. Participants

**Polymorphisms of the selectin genes**

and preterm birth, perinatal morbidity: We enrolled 125 low birth weight infants (≤1500 g) and 156 healthy term infants in our study. We recorded the presence of major perinatal morbidities (respiratory distress syndrome, persisting ductus Botalli, perinatal shock, early postnatal sepsis, acute renal failure, necrotising enterocolitis, bronchopulmonary dysplasia and intraventricular hemorrhage.

and preeclampsia: In this study 126 pregnant women with severe preeclampsia and 106 women with uncomplicated pregnancy participated. Medical history, maternal age, pre-pregnancy body mass index (BMI) and smoking habits were recorded.

**PvuII polymorphism of the estrogen receptor-α gene**

and perinatal morbidity: We enrolled 141 low birth weight infants (≤1500 g) and 167 healthy term infants in our study. We recorded the presence of perinatal complications during the first postnatal week. We enrolled low birth weight preterm infants with retinopathy of prematurity (ROP) requiring cryotherapy/laser therapy (n = 105) and in those without ROP (n = 117).

and preeclampsia: In our retrospective, case control study 119 pregnant women with severe preeclampsia were enrolled. One hundred and three control patients with uncomplicated pregnancies were randomly selected. Medical history, maternal age, pre-pregnancy body mass index (BMI) and smoking habits were recorded.

**Functional studies**

Association of polymorphisms of the P- and L-selectin genes with selectin expression

We enrolled 54 healthy people in our study. We used remnant blood sample from diagnostic blood test.

The in vitro immunomodulatory effect of pentoxifylline and 17β-estradiol

We enrolled 5 healthy men in our study.
4. Methods

**Polymorphisms study**

DNA was extracted from dried blood or whole blood specimens. The PCRs were carried out with positive and negative controls and sequence specific primers, then PCR products were digested with restriction enzymes specified below.

**Investigating of the polymorphisms of the selectin and ER-α genes with PCR-RFLP**

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Restriction enzyme</th>
<th>Lenght of the amplified product (basepair)</th>
<th>Lenght of the digested product (basepair)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-selectin Ser128Arg</td>
<td>Pst I</td>
<td>193</td>
<td>109 / 84</td>
</tr>
<tr>
<td>P-selectin Thr715Pro</td>
<td>BstE II</td>
<td>256</td>
<td>129 / 127</td>
</tr>
<tr>
<td>L-selectin Pro213Ser</td>
<td>Hph I</td>
<td>230</td>
<td>131 / 99</td>
</tr>
<tr>
<td>ER-a PvuII</td>
<td>PvuII</td>
<td>255</td>
<td>155 / 100</td>
</tr>
<tr>
<td>ER-a XbaI</td>
<td>XbaI</td>
<td>255</td>
<td>143 / 112</td>
</tr>
</tbody>
</table>

**Flow cytometric studies**

*Investigating the phagocyte function and burst function of the neutrophil phagocytes and monocytes* We tested the phagocyte function with Phagotest kit® (Orpegen Pharma, Heidelberg, Germany) and the burst function with Phagoburst® kit (Orpegen Pharma, Heidelberg, Germany).

*The measure of activated status of thrombocyte, neutrophil granulocyte and monocyte*

The activated status of thrombocytes was investigated with the changes of the P-selectin (CD62P) expression. The activating agent was ADP. The activated status of neutrophil phagocytes and monocytes was investigated with the changes of the CD11b and L-selectin expression. The activating agent was fMLP.

**Statistical analysis**

*Polymorphisms in perinatal morbidity and preeclampsia*

In case of non-parametric distribution, the continuous data were compared with Mann-Whitney U test, in case of parametric data, the continuous data were compared with t-test. We compared the categorical data with Chi-square test or Fischer-exact test. The genotype distribution and allele frequencies between the case and control population and between the subgroups were compared with Chi-square or Fischer-exact test. We determined the Hardy-Weiberg equilibrium in all cases. Logistic regression analysis was used to determine the
independent effects of the investigated polymorphisms on risk of the disease. The associations were adjusted for the known risk factors. Stepwise linear regression analysis was used to evaluate the independent effects of clinical characteristics and the investigated polymorphisms on the length of oxygen requirement.

*The in vitro immunemodulatory effect of pentoxifylline and 17β-estradiol* The effect of the various concentrations of tested drugs was investigated with Friedman - test.

*Polymorphisms of the P- and L-selectin genes and selectin expression* The P-selectin expression was compared in the subgroups depend on the P-selectin genotype with Mann-Whitney U test before and after ADP stimulation. The L-selectin expression was compared in the subgroups depend on the L-selectin genotype with Mann-Whitney U test before and after fMLP stimulation.
5. Results

Polymorphisms study

Polymorphisms of the selectin genes and preterm birth, perinatal morbidity

The genotype distribution of investigated polymorphisms of the E- and P-selectin gene did not differ between the LBW group and control group. The carrier state of the mutant L-selectin allele was more common in the LBW group than in the group of healthy term infants. There was no association between the L-selectin polymorphism and risk of sepsis. There was also no association between the length of oxygen requirement and the L-selectin genotype, but carriers of the L-selectin Ser213 allele were at greater risk for BPD after adjustment for known risk factors. We found no association between the E-selectin and P-selectin polymorphisms and premature birth, sepsis or BPD in LBW infants.

Polymorphisms of the selectin genes and preeclampsia

Dissimilar allele frequencies in patients with severe preeclampsia and in healthy pregnant women were not detectable. Within the group of severe preeclampsia, the onset of hypertension was associated with carrier state of the mutant (715Pro) P-selectin allele (hypertension occurred 2.05 week earlier), maternal age, smoking and pre-pregnancy BMI.

PvuII polymorphism of the estrogen receptor-α gene and perinatal morbidity

ER-α PvuII genotype distribution was similar in preterm and term infants. Regression analysis showed no association between neonatal morbidity and genotype in girls. However, boys carrying “p” allele were at lower risk for necrotizing enterocolitis and patent ductus arteriosus. The carrier state of the “p” allele was associated with 34-hour shorter period of oxygen supplementation on average. Patients with pp genotype, on the other hand, were at greater risk for intraventricular hemorrhage compared with those carrying either Pp or PP genotypes after adjustment for known risk factors. There was no association between the polymorphism and the risk of ROP.

PvuII and XbaI polymorphisms of the estrogen receptor-α gene and preeclampsia

There were no significant differences in the genotype and allele frequencies of PvuII and XbaI polymorphisms between the case and control groups. However regarding the simultaneous carriage of the two polymorphisms, the „PP” / „XX” genotype combination occurred frequently in severe preeclamptic patients than in healthy pregnant women. According to the haplotype estimation, the homozygous P-X haplotype carriers had an increased risk of severe preeclampsia, which was independent of known risk factors.
The „xx” genotype of the XbaI polymorphism was associated with lower risk for fetal growth restriction in patients with severe preeclampsia.

**Functional studies**

*Polymorphisms of the P- and L-selectin genes and selectin expression*

There was no association between the polymorphisms of the P- and L-selectin gene and the selectin expression even after stimulation with ADP or fMLP.

*The in vitro immunomodulatory effect of pentoxifylline and 17β-estradiol*

In our in vitro study, the PTXf and 17β-estradiol had no impact on the CD11b and L-selectin expression on the surface of neutrophil granulocyte, monocyte and on the phagocyte and burst function.
6. Summary and discussion

Polymorphisms study

Polymorphisms of the selectin genes and preterm birth, perinatal morbidity

In the placenta the adhesion molecules including E-, P- and L-selectins are highly expressed. Adequate expression of selectin molecules is indispensable for implantation. It has been also suggested that abnormal levels of L-selectin may be a contributing factor in unexplained reproductive failure, because cytotrophoblast invasion is deficient in the absence of L-selectin. An association of inadequate L-selectin levels and complicated pregnancies has also been shown. We raised the question, whether the selectin expression is associated with the risk of preterm birth. We demonstrated that carrier state of the mutant L-selectin allele is a risk factor for preterm birth. Several data indicate, that selectins play a role in the pathomechanism of BPD. We demonstrated that those who carry the mutant allele are at increased risk for BPD.

Polymorphisms of the selectin genes and preeclampsia

Our study is the second one that attempts to find a link between selectin genotype and preeclampsia. Freeman et al. detected no association between preeclampsia and carrier state of E-selectin Ser128Arg polymorphism. We verified their results in a larger group of women with severe preeclampsia. We also collected data about the association between P-selectin genotype and preeclampsia. Our results revealed that carrier state of 715Pro mutant allele of P-selectin gene is not protective against the disease. In addition to testing the association between risk of preeclampsia and selectin genotype we also analyzed the impact of genotype on disease progression. Assuming that soluble P-selectin levels are increased in preeclampsia and subjects with 715Pro allele present with low soluble P-selectin levels, we hypothesized that the disease would develop later in carriers of 715Pro allele. Instead, we found that the presence of 715Pro allele was associated with an earlier onset of hypertension.

PvuII polymorphism of the estrogen receptor-α gene and perinatal morbidity

Data show that male neonates are at higher risk for morbidity than female ones. The reason could be the differences in the level of sexual steroids in the male and female neonates. In our study, the PvuII polymorphism was associated with perinatal complication only in male neonates. Theoretically, the PvuII polymorphism could be clinically significant only in male neonates, who have lower estrogen levels. This could be one of the explanation for the sex dependency of the perinatal complication. We demonstrated that heterozygote carriers are the
most protected individuals. The “pP” genotype could have the most balanced estrogen effect. There is need to verify this hypothesis with functional studies.

We separately investigated the PvuII polymorphism in patients with ROP. ER-α can be found in various tissues, among others on the vessels of retina too. The risk of retinopathy decreased in an animal study, where the mice were treated with estrogen. For this reason we investigated the importance of the PvuII polymorphism in ROP. Our results show, that this polymorphism does not associate with the risk of ROP in our population.

PvuII and XbaI polymorphisms of the estrogen receptor-α gene and preeclampsia

Cardiovascular diseases in familiar history increase the risk of preeclampsia and preeclampsia itself increases the risk of cardiovascular diseases. Increasing data suggest that polymorphisms of the ER-α gene play role in cardiovascular disorders. For this reason came up to investigate the PvuII and XbaI polymorphism of the ER-α gene in preeclampsia.

We found, that the P-X haplotype increases the risk of severe preeclampsia. In the background could stand, that in case of the „P” allele, the binding site of the B-myb transcription factor is eliminated, therefore the expression of the ER-α decreases. The relative estrogen deficit increases the resistency of the uterine vessels, which is part of the patomechanism of preeclampsia.

Functional studies

Polymorphisms of the P- and L-selectin genes and selectin expression

We investigated in our in vitro model, whether pentoxifillyne and 17-β-estradiol have an impact on the CD11b and L-selectin expression, phagocyte and burst function of neutrophil granulocyte and monocyte. Literally data and our polymorphism studies came out, that these play an important role in perinatal complication. Pentoxifylline is already used in perinatology and estrogens have important immunomodulatory effect in preterm infants. We simultaneously investigated effects in three cell type with more pentoxifylline and estrogen doses. Interestingly we could not find any effect of these drugs on the investigated functions. The reason for this is not known, there is need to make more study to verify the perinatal immunomodulatory effect of these drugs.

Polymorphisms of the P- and L-selectin genes and selectin expression

There are some data, that Thr715Pro polymorphism of the P-selectin gene is associated with soluble P-selectin levels. We could not detect any association between this polymorphism and P-selectin expression on thrombocytes. Similarly there was no connection
between L-selectin expression on neutrophil granulocyte and monocyte and the Pro213Ser polymorphism of the L-selectin gene. So far the explanation for this is not known. Although it is known, that Thr715Pro polymorphism is in the sequence of the membrane binding domain of the protein and this changes the force of binding to the membrane and the shedding of the protein. This phenomenon can not be investigated on cellular level.
7. Acknowledgement

I express my thanks to Tivadar Tulassay, who established this intellectual environment where I spent three years as PhD student and where I could acquire the modern research methodology, which is essential for medical research on the highest level.

My tutor, Barna Vásárhelyi was the leader my PhD work. He enrolled me as a research fellow into the current scientific research project and provided me independent tasks. I have learned who to define the goals of a research study, to collect data, to analyze and publish the results.

Ádám Vannay and Gergő Kozma provided technical notes and helped me to establish the PCR techniques. András Treszl collected clinical data of the patients and contributed to statistical analysis.

I would like to express my thanks to Ilona Banyasz, Krisztina Rusai, Bea Szebeni, Ádám Balogh for their help. I am grateful to Maria Bernáth for the excellent technical support. I am indebted to Géza Bokodi, who helped me a lot, particularly in flow cytometric works and statistical analysis of data.

The research team works within the frames of the 1st. Department of Pediatrics. This gives a unique opportunity to analyze the results with the help of the clinicians. On regular clinician-researcher meetings, the clinicians’ particularly Andrea Fekete, Anna Körner, András Arató, György Reusz, András Szabó, Attila Szabó, Miklós Szabó practical remarks put our results in a different complexion.

The investigations could not happened without the strong collaboration between other institutions and research teams. András Nobilis and his co-workers from the 2nd Department of Obstetrics and Gynecology provided samples from preterm and newborn. Ágnes Schuler from Budai Children Hospital helped to recruit remnant blood samples. János Rigó gave the preeclamptic samples and helped me to formulate the correct question and to publicate our results. I started my PhD work with some technical skill that I gained as a medical student in the research laboratory of Péter Lakatos from the 1st. Department of Internal Medicine.
8. Publications


