ORIGINAL ARTICLE

Association Between Maternal and Fetal TLR4 (896A>G, 1196C>T) Gene Polymorphisms and the Risk of Pre-term Birth in the Polish Population

Adam Bitner[1], Wojciech Sobala[2], Jarosław Kalinka[1]

[1]Department of Perinatology, First Chair of Gynecology and Obstetrics, Medical University of Lodz, Poland;
[2]Department of Environmental Epidemiology, Nofer Institute of Occupational Medicine, Lodz, Poland

Keywords
Polymorphism, pre-term delivery, TLR4 1196C>T, TLR4 896A>G, Toll-like receptor 4

Correspondence
Adam Bitner, MD, PhD, Department of Perinatology, First Chair of Gynecology and Obstetrics, Medical University of Lodz, 37 Wilenska St, 94-029 Lodz, Poland.
E-mail: adambit@wp.pl

Submission August 27, 2012;
accepted December 5, 2012.

Citation
Bitner A, Sobala W, Kalinka J. Association between maternal and fetal TLR4 (896A>G, 1196C>T) gene polymorphisms and the risk of pre-term birth in the polish population. Am J Reprod Immunol 2013; 69: 272–280

doi:10.1111/aji.12068

Problem
Bacteria activates Toll-like receptor 4 on immune system cells leading to preterm birth (PTB). The aim of study was to evaluate the impact of maternal and fetal carriage of TLR4 gene polymorphisms on the risk of PTB.

Method of Study
Frequency of TLR4 (896A>G, 1196C>T) variants was determined in 121 women who delivered preterm, 152 women who delivered at term, 49 term newborns and 35 preterm newborns with the use of PCR-RFLP.

Results
We found no associations between frequency of TLR4 polymorphic alleles in mothers and fetuses and the risk of delivery before 37th week of pregnancy. However significantly lower frequency of TLR4 1196T allele was observed in subgroup of mothers who delivered before 33rd week comparing with those who delivered later (4,4% versus 14,2%, OR = 0,28 (95%CI: 0,04–0,99).

Conclusions
Maternal carriage of TLR4 1196T allele may be associated with reduced risk of PTB before 33rd week of gestation in Polish population.

Introduction

Pre-term birth (PTB) is a worldwide heath problem responsible for the majority of newborns mortality and morbidity.[1] In 2005, about 13 million births were pre-term, which presented more than 9.5% of all births worldwide.[2] Intra-uterine infection is believed to be a significant cause of prematurity that may account for 25–40% of pre-term births.[3] Bacterial and viral infections gain access to the placenta and the uterus through one of three major routes by ascending from the lower reproductive tracts through maternal circulation and by descending into the uterus from peritoneal cavity. Recently, the mechanism in which intra-uterine infection leads to pre-term delivery has been widely investigated. It has been suggested that Toll-like receptors (TLRs) expressed in various gestational-associated tissues play a role in the onset and/or maintenance of labor in response to microbial stimuli.[4] The TLR family is the class of mammalian pattern recognition receptors (PRRs) which detect a limited set of conserved molecular patterns (PAMPs – pathogen-associated molecular patterns) that are unique to the microbial world. Recognition of PAMPs signals to the host, the presence of infection.[5] To date, 11 mammalian TLRs have been identified (TLR1 – TLR11), however, TLR11 has not been demonstrated as functional in

humans.[4,6] TLR3, TLR7, TLR8, and TLR9 are localized in cytoplasm and recognize intracellular signals, especially those of viral orgin.[7] On the contrary, TLR1, TLR2, TLR4, TLR5, and TLR6 are localized in the cellular membrane and recognize external signals that are present on the outer surface of bacteria, fungi, and parasites.[7] Collectively, the family of TLR recognize a wide range of PAMPs associated with micro-organisms. Activation of TLR results in an inflammatory immune response which is characterized by the production of cytokines, interferons, chemokines, and other antimicrobial factors.[8]

In our present study, we focused on toll-like receptor 4 (TLR4) which is the first toll-like receptor identified in humans and the one most widely examined.[9] TLR4 is a transmembrane protein with extracellular domains containing leucine-rich motifs which play a role in the recognition of PAMPs. A receptor also consists of short transmembrane domain and a cytoplasmic portion known as Toll/IL-1 receptor (TIR) domain, as it shares about 200 amino acids with IL-1 receptor.[7] Ligand for TLR4 has been identified as lipopolysaccharide (LPS), which is a component of the cell wall of Gram-negative bacteria.[10] After ligand recognition, TIR domain of TLR4 recruit the intracellular signaling adapter protein – MyD88 (Myeloid differentiation factor 88), and a subsequent kinase cascade triggers activation of a key transcription factor, NF-κβ (nuclear factor-κβ).[11] Activated NF-κβ can enter the cellular nucleus and bind to the promoter region within DNA encoding for various inflammatory proteins, promoting DNA transcription and production of key inflammatory cytokines among them IL-1β, IL-6, IL-8, and TNF-α.[3] These cytokines enhance the production of matrix metalloproteinase (MMP) in uterine cervix and fetal membranes and increase the production of prostaglandin PGE_2 by the myometrium leading to cervical ripening, membrane rupture, and myometrial contractility and finally to pre-term delivery.[12–14]

TLR4s are present in many gestational-associated tissues. Their expression has been found in decidua (in decidual cells and in infiltrating macrophages and neutrophils), placenta (in syncytiotrophoblast, cytotrophoblast, endothelial cells and villous Hofbauer cells), and in amnion.[15–20] Such localization allows the recognition of Gram-negative bacteria invading the uterus regardless of the route of invasion. In case of an ascending infection, hematogenous dissemination, and a descending infection from peritoneal cavity, bacteria faces TLR4 present in decidua, syncytiotrofoblast, and amniotic epithelium, respectively, initiating the cytokine production. What is interesting is syncytiotrofoblast which covers the surface of placental villi and has the potential to release cytokines directly to maternal blood.

Arbour et al. demonstrated that common polymorphisms of TLR4 gene (896A>G, 1196C>T) are associated with hyporesponse to LPS challenge.[21] These polymorphisms result in the replacement of aspartic acid with glycine at amino acid 299 (rs 4986790) and threonine with an isoleucine at amino acid 399 (rs 4986791) respectively, which affects extracellular domain of toll-like receptor 4. Authors revealed that NF-κβ activity following LPS stimulation and LPS-stimulated release of IL-1α were significantly lower for cells containing polymorphic alleles compared with wild-type cells. Hyporesponsiveness of polymorphic TLR4 might be associated with defective transport of this receptor to the cell membrane or impaired ligand binding. Arbour et al. have proven that cells containing polymorphic TLR4 alleles express less receptors on their surface, which supports the theory of impaired transport to cellular membrane.[21]

Based on the observations that gram-negative bacteria may cause pre-term delivery acting through TLR4 and that common TLR4 gene polymorphisms (rs 4986790, rs 4986791) impaired tissue expression of these receptors and lower NF-κβ activity and cytokine production following LPS stimulation, we hypothesized that carriage of these two common polymorphisms may be associated with decreased risk of pre-term delivery. As mentioned before, TLR4 are present in both maternal and fetal tissues (decidua, chorion, and amnion). It seems probable that both maternal and fetal carriage of these polymorphisms can influence the risk of prematurity in the same way. However, as bacteria gain access to the uterus through ascending route from lower reproductive tract, facing TLR4 receptors in decidua, which is of maternal origin in the majority of cases, we decided to focus on the impact of maternal carriage of examined polymorphism on the risk of PTB.

To address our hypothesis, we examined the association between maternal carriage of two TLR4 gene polymorphisms (896A>G and 1196C>T) and the risk of delivery before 37 weeks of gestation in the population of Polish women. Given that infections are much more often responsible for very pre-term birth than for pre-term birth close to the term, we decided to perform additional analysis of an impact of these

two polymorphisms on the duration of pregnancy in a subgroup of women who gave birth to a child before 33rd week of gestation.[22] We hypothesized that in such a subgroup of women the protective impact of TLR4 polymorphism on the duration of pregnancy might be more visible. Additionally we analyzed the impact of fetal carriage of TLR4 variants on the risk of pre-term birth. Finally, we determined the frequency of rs 4986790, rs 4986791 TLR4 gene polymorphisms in the population of Polish women who delivered at term, which in our opinion represents the frequency of these two polymorphisms in the general Polish population.

Materials and methods

The study was approved by the Committee for Bioethics of Medical University of Lodz (number RNN/108/07/KE). Written informed consent was obtained from all patients.

In order to examine the influence of maternal carriage of two analyzed polymorphisms on the risk of PTD before the 37th week of gestation we enrolled into the case – control study 273 Caucasian women who delivered in the Department of Perinatology, First Chair of Gynecology and Obstetrics, Medical University of Lodz between 2008 and 2011. The women were divided into two groups. 121 women who delivered a child before completing 37 weeks of gestation constituted a case group. Control subjects ($n = 152$) were defined as women who delivered after 37 weeks gestation. Because of the fact that very pre-term deliveries are in up to 80% associated with intra-uterine infection and taking under consideration reports of distinct influence of TLR4 polymorphic alleles on the risk of prematurity depending on gestational age we distinguished the subgroup of 43 mothers who delivered before the end of the 33rd week of gestation. Finally, in order to check the contribution of fetal carriage of TLR4 variants to the delivery before term we enrolled into the study 84 newborns of whom 35 were born before the 37th week of gestation (cases subjects). In all women and newborns gestational age was determined by the date of the last period and confirmed by the ultrasound evaluation, which was performed between 11 and 13.6 weeks of pregnancy. Mothers with incompetent cervix, congenital anomalies of uterus, fetal malformations and iatrogenic pre-term delivery, as well as, mothers enrolled in our previous studies, were excluded from the study. Clinical and demographic information was collected by filling out the questionnaire and patient's self-reports.

Maternal and fetal genomic DNA was isolated according to Higuchi from white cells of peripheral blood and umbilical cord blood respectively.[23] The polymerase chain reaction (PCR) was carried out with the use of GeneAmp PCR System 2007 and GeneAmp PCR system 2400 (Applied Biosystems, USA) following the manufacturer's recommendations. To detect two evaluated polymorphisms we used methods previously described by Lorenz et al.[24]

In order to analyze TLR4 896 A>G polymorphism, the region that contain *Nco*I restriction site was amplified with following primers: 5′ – GATTAGCATACTTAGACTACTACCTCCATG – 3′ and 5′ – GATCAACTTCTGAAAAAAGCATTCCCAC – 3′. PCR conditions comprised of a denaturating step at 95°C for 4 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. 4 μL of PCR product was digested overnight with *Nco*I (Fermentas, Canada) and fractionated on a 10% agarose gel. The size of digestion products was 249 bp for wild allele and 226 bp for polymorphic one.

Oligonucleotides 5′ – GGTTGCTGTTCTCAAAGTGA TTTTGGGAGAA – 3′ and 5′ – ACCTGAAGACTGGAG AGTGAGTTAAATGCT – 3′ were used as PCR primers in order to amplify a region containing TLR4 (1196 C>T) polymorphic site. After initial denaturation at 95°C for 4 min, PCR was performed for 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension for 30 s at 72°C. 4 μL of the resulting PCR products were digested overnight with *Hinf*I restriction enzyme (Fermentas, Canada) and run out on a 10% agarose gel. Digestion gave 406 bp bands for wild – type allele and 377 bp band for polymorphic one (Fig. 1).

Genotype frequencies in the studied and control groups were compared using an exact logistic regres-

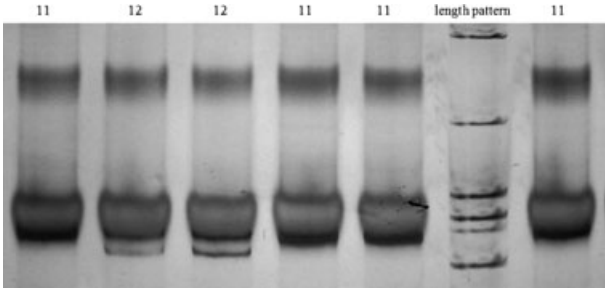

Fig. 1 Gel electrophoresis of *Hinf*I digest for TLR4 1196 C>T RFLP assay. Genotype assignment are indicated above each line (1 for wild type allele, 2 for mutant allele).

sion model. Analysis was used to estimate the OR and its 95% confidence intervals. Analysis was performed with the statistical analysis package R version 2.7.2 (http://www.r-project.org). Accordance with Hardy – Weinberg equilibrium was estimated using exact test for Hardy-Weinberg equilibrium.[25]

Results

The characteristics of examined mothers and newborns is presented in Table I and II. In mothers, we found no differences in age, weight at delivery and percentage of cesarean sections performed. Mothers who delivered pre-term significantly more often smoked cigarettes during their pregnancy and had a history of pre-term birth. As we expected there were significant differences in percentage of PROM, gestational age at delivery and newborn's birth weight. In newborns significant differences concern gestational age at the delivery and birth weight. No differences were found according to maternal age and maternal weight at the delivery.

In our study we have determined the frequency of two TLR4 polymorphic alleles in general Polish population (in mothers who delivered after the 37th week of pregnancy). In analysis of TLR4 896G allele frequency successful DNA isolation and PCR amplification was performed in 127 cases. Among them we stated 111 (87.4%) AA genotypes, 15 (11.8%) heterozygous AG genotypes and 1 (0.8%) homozygous GG genotype. Calculated frequency of TLR4 896G allele was 0.067. Successful PCR amplification was performed in 125 cases in the analysis of TLR4 1196T allele occurrence. Among them in 107 (85.6%) cases we found wild CC genotype, in 18 (14.4%) cases we found heterozygous CT genotype. No homozygous TT genotype was found. The frequency of TLR4 1196T allele was 0.072. We stated strong linkage disequilibrium between 896AA and 1196CC TLR4 genotypes. Kappa rate was 0.75 for mothers and 0.85 for newborns respectively. The distribution of genotypes did not deviate from the Hardy – Weinberg equilibrium in any of the studied populations (*P*-value for TLR4 896 genotype for mothers and newborns were 0.59 and 0.44 and for TLR4 1196 genotype 1.0 and 0.34 respectively).

Analyzing the associations between maternal frequency of TLR4 1196 C>T and TLR4 896 A>G polymorphic alleles and the risk of pre-term delivery before the 37th week of gestation, we found no statistically significant relationship (Table III).

Analyzing the impact of maternal carriage of examined polymorphisms in TLR4 gene on the risk of very pre-term birth before the 33rd week of gestation we found statistically significant differences in the frequency of TLR4 1196C>T polymorphism between mothers who delivered before 33 weeks gestation and those who delivered later. Polymorphic CT genotype was found in 4.4% of women who gave birth to a child before the 33rd week of gestation and in 14.2% of women delivering after 33 weeks of pregnancy OR = 0.28 (95% CI: 0.04–0.99). We found no statistically significant association between frequency of maternal carriage of TLR4 896A>G polymorphism and the risk of delivery

Table I Characteristics of examined mothers

	Term delivery	PTD < 37 weeks	PTD < 33 HBD
n	152	121	43
Average age at the delivery (years)	28.6	29.9	29.9
Average weight at the delivery (kg)	73.9	69.3	67.9
Smoking during the pregnancy (%)	13	23[a]	25[a]
History of pre-term delivery (%)	6	17[a]	21[a]
PROM (%)	19.7	55[a]	54[a]
Percentage of cesarean sections (%)	32.8	38	37.5
Gestational age at the delivery (weeks)	39.2	32.5[a]	29.1[a, b]
Newborn's birth weight	3370	1972[a]	1360 [a, b]

[a]$P < 0.05$ versus term delivery.
[b]$P < 0.05$ versus PTD < 37 weeks.

Table II Characteristics of examined newborns

	Term delivery	PTD
n	49	35
Average maternal age at the delivery (years)	27.8	30.8
Average maternal weight at the delivery (kg)	73.3	69.2
Gestational age at the delivery (weeks)	38.8	34.3[a]
Birth weight	3333	2441[a]

[a]$P < 0.05$.

Table III Impact of maternal TLR4 gene variants on the risk of pre-term delivery before the 37th week of pregnancy

		Genotype frequencies (*n*)		
	Genotype	PTB < 37	Term delivery	Odds ratio
TLR4 1196C>T	CC	90% (99)	85.6% (107)	Reference group
	CT	10% (11)	14.4% (18)	OR = 0.66 (95% CI: 0.29–1.45)
		100% (110)[a]	100%(125)[a]	
TLR4 896A>G	AA	89.6% (95)	87.4% (111)	Reference group
	AG, GG[b]	10.4% (11)	12.6% (16)	OR = 0.8 (95% CI: 0.35–1.80)
		100% (106)[a]	100%(127)[a]	

n, Number of genotypes.
[a]Total number of analyzed mothers differs from the number of mothers enrolled into the study, because of either DNA isolation or PCR amplification failure.
[b]Only one homozygous TLR4 896GG genotype was found in a group of women who delivered after the 37th week of pregnancy.

Table IV Impact of maternal TLR4 gene variants on the risk of pre-term delivery before the 33rd week of pregnancy

		Genotype frequencies (*n*)		
	Genotype	PTB < 33	Term delivery	Odds ratio
TLR4 1196C>T	CC	95.6% (43)	85.8% (163)	Reference group
	CT	4.4% (2)	14.2% (27)	OR = 0.28 (95% CI: 0.04–0.99)
		100% (45)[a]	100%(190)[a]	
TLR4 896A>G	AA	95.3% (41)	86.8% (165)	Reference group
	AG	4.7% (2)	13.2% (25)	OR = 0.32 (95% CI: 0.05–1.14)
		100% (43)[a]	100%(190)[a]	

n, number of genotypes.
[a]Total number of analyzed mothers differs from number of mothers enrolled into study because of either DNA isolation or PCR amplification failure.

before the 33rd week of gestation. This data is shown in Table IV.

In a group of women who delivered before the 33rd week of gestation we performed an additional analysis to find out, if the combination of examined polymorphisms in maternal TLR4 gene influence the risk of PTB. We revealed that the number of polymorphisms inside TLR4 gene influences the risk of birth before the 33rd week of gestation. Maternal carriage of only one of analyzed polymorphisms inside TLR4 gene (either 896G or 1196T) does not significantly affect the risk of prematurity (OR = 0.76; 95% CI: 0.11–3.03) while simultaneous carriage of both examined polymorphisms (896G and 1196T) was associated with significant reduction in risk of birth before the 33rd week of gestation OR = 0.18 (95% CI: 0.01–0.91).

We haven't found any statistically significant associations between frequencies of analyzed TLR4 polymorphisms in fetuses and the risk of pre-term delivery, neither before the 37th nor the 33rd week of pregnancy (Table V and VI). However, fetuses born before the 37th week of gestation tended to have lower TLR4 1196T allele frequency then term fetuses OR = 0.27 (95% CI: 0.04–1.14). Because of the small sample size of newborns enrolled into the study, we weren't able to examine the influence of various combinations of these two polymorphisms in TLR4 gene on the risk of prematurity.

Discussion

According to our knowledge, this is the first study in which the frequency of rs 4986790 and rs 4986791 polymorphisms has been determined in the Polish population.

The frequency of polymorphic TLR4 896G and 1196T alleles was 0.067 and 0.072 respectively and in both cases did not differ from one reported by investigators from other countries (Table VII and VIII).

This is also the first study in which an association between the frequency of maternal and fetal carriage of two TLR4 gene polymorphisms (896A>G, 1196C>T) and the risk of pre-term delivery has been evaluated in a homogenous Caucasian Polish population. In our research we have found a significantly lower frequency of TLR4 1196T allele in mothers who delivered before the 33rd week of gestation compared to mothers who delivered later (two carriers in a total of 45 PTBs < 33rd week of pregnancy – 4.4% versus 27 carriers in a total of 190

Table V Impact of fetal TLR4 gene variants on the risk of pre-term delivery before the 37th week of pregnancy

		Genotype frequencies (*n*)		
	Genotype	PTB < 37	Term delivery	Odds ratio
TLR4 1196C>T	CC	94% (33)	82% (40)	Reference group
	CT	6% (2)	18% (9)	OR = 0.27 (95% CI: 0.04–1.14)
		100% (35)	100%(49)	
TLR4 896A>G	AA	90% (27)	79.5% (35)	Reference group
	AG	10% (3)	20.5% (9)	OR = 0.43 (95% CI: 0.09–1.61)
		100% (30)[a]	100%(44)[a]	

n, Number of genotypes.
[a]Total number of analyzed newborns differ from number of newborns enrolled into study because of either DNA isolation or PCR amplification failure.

Table VI Impact of fetal TLR4 gene variants on the risk of pre-term delivery before the 33rd week of pregnancy

		Genotype frequencies (*n*)		
	Genotype	PTB < 33	Term delivery	Odds ratio
TLR4 1196C>T	CC	80% (4)	87% (69)	Reference group
	CT	20% (1)	13% (10)	OR = 1.73 (95% CI: 0.08–13.23)
		100% (5)	100% (79)[a]	
TLR4 896A>G	AA	60% (3)	85.5% (59)	Reference group
	AG	40% (2)	14.5% (10)	OR = 3.93 (95% CI: 0.47–26.79)
		100% (5)	100% (69)[a]	

n, Number of genotypes.
[a]Total number of analyzed fetuses differ from number of fetuses enrolled into study because of either DNA isolation or PCR amplification failure.

Table VII Frequencies of TLR4 896G allele in different population

Country	Frequency of TLR 896G allele	N
USA[21]	0.066	155
Uruguay[26]	0.05	250
Finland[27]	0.083	351
African-America[28]	0.066	218
Poland (present study)	0.067	254[a]

[a]Number of alleles is twice as much as the number of mothers in whom successful amplification was performed because each genotype contains two alleles.

Table VIII Frequencies of TLR4 1196T allele in different population

Country	Frequency of TLR 1196T allele	N
India[29]	0.09	500
Greece[30]	0.067	490
Ethiopia[31]	0.077	494
Malaysia[32]	0.048	500
Poland (present study)	0.072	250[a]

[a]Number of alleles is twice as much as number of mothers in which successful amplification was performed because each genotype contains two alleles.

mothers $\geq$34th week– 14.2%; odds ratio – 0.28; 95% confidential interval 0.04–0.99). We have determined no statistically significant association between maternal frequency of TLR4 1196T allele and the incidence of pre-term delivery after the 33rd week of pregnancy and also no correlation between maternal carriage of TLR4 896G allele and PTB regardless of the time of delivery.

We have also evaluated fetal frequencies of two above mentioned polymorphisms among the cases and controls, finding no statistically important differences. However, fetuses born before the 37th week of pregnancy tended to have lower frequency of TLR4 1196T allele comparing with the term newborns (two carriers in a total of 35 PTB – 6% versus nine carriers in a total of 49 term newborns – 18%; OR = 0.27; 95% CI: 0.04–1.14 – statistically not significant). Probably because of small sample size, we were unable to define the fetal TLR4 variants as a factor influencing risk of prematurity.

Our results seem to confirm our hypothesis that carriage of TLR4 receptor polymorphism might be associated with the lower risk of pre-term delivery. Here we show for the first time that maternal carriage of TLR4 1196T allele might protect against delivery before the 33rd week of gestation. This pro-

tection is probably caused by a change in extracellular domain of TLR4 in carriers of polymorphic 1196T allele, which impair transport of this receptor to membrane cell, resulting in lower expression of polymorphic receptor on cellular surface.[21] This lower receptor expression reduces detection of Gram negative bacteria and causes lower NF-κβ activity and lower release of IL-1α following LPS stimulation.[21] This impair response to LPS stimulation and depress inflammatory response to Gram-negative bacteria seem to result in lower production of MMP and prostaglandins and thus reduce cervical ripening and uterine contractility, decreasing the risk of pre-term delivery. The theory of impact of TLR4 malfunction on the PTB risk reduction seems to be supported by murine models. Wang et al. have shown that a strain of mice that has a spontaneous mutation of TLR4 is less likely to deliver pre-term after intra-uterine inoculation of heat killed Gram–negative bacteria than wild-type mice.[33] Moreover, in mice models Sulfasalazine which blocks activation of NF-κβ reduced rates of pre-term delivery after intra-uterine injection of *Escherichia coli*.[34] We hypothesized that the fetal carriage of TLR4 polymorphic alleles would have the same influence on the risk of prematurity as the maternal one, however, probably because of the small sample we detected no statistically significant associations.

In our study we have also examined the impact of simultaneous maternal carriage of two different polymorphisms (896 A>G and 1196C>T) among TLR4 gene on the risk of delivery before 33rd week of gestation. What is interesting, the reduction of the risk of very pre-term delivery caused by concurrent presence of these two polymorphic alleles (896A and 1196C) was higher than caused by the presence of TLR4 1196T allele alone suggesting that superimposing changes inside a single gene can further alter the genes impact on the eventual phenotype.

In our study we did not detect an influence of maternal carriage of TLR4 variants on the risk of pre-term delivery after the 33rd week of gestation. Distinct influence of TLR4 gene variants on the risk of pre-term delivery regarding gestational age does not seem to be surprising. Infections have been reported responsible for about 40% of all pre-term labor cases.[35] However, in very early premature births (before 30 weeks of gestation) ascending infections or chorioamnionitis occur in up to 80%.[35] Therefore, before the 33rd week of gestation TLR4 variants which blunt inflammatory response might be beneficial for the duration of pregnancy. In more advanced pregnancies other causes of pre-term delivery such as: uteroplacental ischemia, uterine overdistension, allergic phenomena and hormonal disorders may become more important making a protective impact of TLR4 1196T allele less significant.[3] Our data showing an association between maternal TLR4 1196T allele carriage and reduction of the risk of birth <33rd week of pregnancy emphasize the differences in susceptibility to the inflammatory process among pre-term neonates.

In another study conducted in Poland authors have analyzed the impact of maternal carriage of TLR4 1196 C>T polymorphism on the risk of PPROM.[36] Although small sample size including 33 cases with PPROM and 60 controls authors have observed a tendency towards protection against PPROM in women carrying TLR4 1196T variant (9.1% of carriers in case group versus 16.7% in controls – data statistically not significant).

Results of these two studies emerging from Poland suggest that in Central Europe carriage of TLR4 1196 C>T polymorphism might be associated with reduction of risk of prematurity. This reduction seems to effect very early pregnancies before the 33rd week of gestation. In few researches conducted in other countries authors obtained different results. In Finland fetal, as well as, maternal carriage of TLR4 variants were associated with increased risk of premature birth.[24] In this study the frequency of the polymorphic alleles had no detectable influence on the degree of prematurity (<32 and $\geq$ 32 week). Rey el al. in a research conducted among Uruguayan newborns have shown an increased risk of prematurity and PPROM in individuals carrying 896 A>G substitution in TLR4 gene.[26] This increase was however restricted only to very premature infants before the 33rd week of gestation.

On the contrary to mentioned studies Ferrand et al. in Philadelphia (USA) have demonstrated no association between frequency of TLR4 896 A>G polymorphism and PPROM.[28]

The fact of distinct influence of TLR4 gene variants on the risk of prematurity in different countries does not seem to be surprising. It emphasizes disparities in the genetic background of the populations. It has been estimated that there are about 3 million single nucleotide polymorphisms (SNP) in the human genome. For the last several years research considering the impact of maternal and fetal poly-

morphisms of different genes on the risk of prematurity has been conducted around the world. These studies concentrate mainly on genes associated with inflammatory mechanism of pre-term delivery.[3] Results of these studies generally, seem to indicate that the impact of gene polymorphisms on the risk of pre-term delivery depends on ethnic disparities. For example Genç et al. in a research conducted in a population of New York newborns have shown an increased risk of PTD in fetuses carrying polymorphic alleles of IL-1β gene (IL1β+3953C>T).[37] This risk was, however, restricted to newborns of Afro-American origin only. On the contrary, in Florida Edwards et al. did not detect an impact of fetal carriage of this polymorphism on the risk of prematurity.[38] IL1RN gene encoding for IL-1ra (Interleukin-1 receptor antagonist) seems to be exceptional, because in majority of studies both maternal and fetal carriage of the second allele of this gene (IL1RN*2) increases the risk of pre-term delivery, regardless the population studied.[37,39,40] Other than inflammatory mechanisms of pre-term delivery have also been investigated. Polymorphisms of genes accounting for uteroplacental ischaemia, another cause of PTD according to Romero, have become a point of interest.[3] Valez et al. have shown, that maternal and fetal carriage of polymorphic alleles encoding for tPA (tissue-type plasminogen activator) and coagulation factors V and VII (F5, F7) may increase the risk of prematurity.[41] Considering aforementioned data it seems that individual polymorphisms presented in specific population are interacting with each other and they are together responsible for the final phenotype (gene-gene interaction). In our study we have shown that even interactions between specific polymorphisms inside one gene can alter the final phenotype. Moreover, interactions between genes and environment have also been described. Macones et al. have found that women presenting both bacterial vaginosis (BV) and TNFα polymorphism were at synergistically increased risk of PTB compared with women with only gene polymorphism or BV alone.[42] Nukui et al. have shown that carriers of polymorphic allele of GSTT1 gene (glutathione S-transferase theta-1 – enzyme taking part in metabolism of mutagens and carcinogens present in tobacco smoke) are at increased risk of preterm delivery in case of exposure to tobacco smoke.[43]

The impact of TLR4 genetic variants on the duration of pregnancy is not clear. It seems to depend on geographical region, race and probably on interactions with environmental factors. In our study we have shown that it may also depend on the gestational age. Further studies conducted in different populations and which would take environmental factors into account, are necessary to elucidate association between maternal and fetal TLR4 gene variants and the risk of prematurity.

Conclusions

The impact of TLR4 896 A>G and TLR4 1196C>T gene polymorphisms on the duration of pregnancy is geographically disparate. In Poland maternal carriage of TLR 1196T variant is associated with reduction in risk of pre-term delivery before the 33rd week of gestation. There is a need for further worldwide studies concerning associations between fetal TLR4 variants and the risk of prematurity.

Acknowledgments

The study was supported by a grant 'Epidemiology of risk affecting procreation in Poland, multicenter, prospective cohort study' nr PBZ-MEiN-/8/2/2006 financed by Polish Ministry of Science and High Education, task nr K 140/P01/2007/1.3.1.2.

References

1 Esplin MS: Preterm birth: a review of genetic factors and future directions for genetic study. *Obstet Gynecol Surv* 2006; 61:800–806.
2 Beck S, Wojdyla D, Say L, Betran AP, Merialdi M, Requejo JH, Rubens C, Menon R, Van Look PF: The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bull World Health Organ* 2010; 88:31–38.
3 Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, Chaiworapongsa T, Mazor M: The preterm parturition syndrome. *BJOG* 2006; 113(Suppl 3):17–42.
4 Koga K, Aldo PB, Mor G: Toll-like receptors and pregnancy: trophoblast as modulators of the immune response. *J Obstet Gynaecol Res* 2009; 35:191–202.
5 Janeway CA Jr, Medzhitov R: Innate immune recognition. *Annu Rev Immunol* 2002; 20:197–216.
6 Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh S: A toll-like receptor that prevents infection by uropathogenic bacteria. *Science* 2004; 303:1522–1526.
7 Patni S, Flynn P, Wynen LP, Seager AL, Morgan G, White JO, Thornton CA: An introduction to Toll-like receptors and their possible role in the initiation of labour. *BJOG* 2007; 114:1326–1334.
8 Medzhitov R, Janeway CA Jr: Innate immunity: impact on the adaptive immune response. *Curr Opin Immunol* 1997; 9:4–9.
9 Medzhitov R, Preston-Hurlburt P, Janeway CA Jr: A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997; 388:394–397.

10 Pasare C, Medzhitov R: Toll-like receptors: linking innate and adaptive immunity. *Microbes Infect* 2004; 6:1382–1387.
11 Akira S, Hoshino K: Myeloid differentiation factor 88-dependent and -independent pathways in toll-like receptor signaling. *J Infect Dis* 2003; 187(Suppl 2):S356–S363.
12 Watari M, Watari H, DiSanto ME, Chacko S, Shi GP, Strauss JF 3rd: Pro-inflammatory cytokines induce expression of matrix-metabolizing enzymes in human cervical smooth muscle cells. *Am J Pathol* 1999; 154:1755–1762.
13 Maymon E, Romero R, Pacora P, Gomez R, Athayde N, Edwin S, Yoon BH: Human neutrophil collagenase (matrix metalloproteinase 8) in parturition, premature rupture of the membranes, and intrauterine infection. *Am J Obstet Gynecol* 2000; 183:94–99.
14 Todd HM, Dundoo VL, Gerber WR, Cwiak CA, Baldassare JJ, Hertelendy F: Effect of cytokines on prostaglandin E2 and prostacyclin production in primary cultures of human myometrial cells. *J Matern Fetal Med* 1996; 5:161–167.
15 Kim YM, Romero R, Chaiworapongsa T, Kim GJ, Kim MR, Kuivaniemi H, Tromp G, Espinoza J, Bujold E, Abrahams VM, Mor G: Toll-like receptor-2 and -4 in the chorioamniotic membranes in spontaneous labor at term and in preterm parturition that are associated with chorioamnionitis. *Am J Obstet Gynecol* 2004; 191:1346–1355.
16 Canavan TP, Simhan HN: Innate immune function of the human decidual cell at the maternal-fetal interface. *J Reprod Immunol* 2007; 74:46–52.
17 Beijar EC, Mallard C, Powell TL: Expression and subcellular localization of TLR-4 in term and first trimester human placenta. *Placenta* 2006; 27:322–326.
18 Ma Y, Krikun G, Abrahams VM, Mor G, Guller S: Cell type-specific expression and function of toll-like receptors 2 and 4 in human placenta: implications in fetal infection. *Placenta* 2007; 28:1024–1031.
19 Kumazaki K, Nakayama M, Yanagihara I, Suehara N, Wada Y: Immunohistochemical distribution of Toll-like receptor 4 in term and preterm human placentas from normal and complicated pregnancy including chorioamnionitis. *Hum Pathol* 2004; 35:47–54.
20 Adams KM, Lucas J, Kapur RP, Stevens AM: LPS induces translocation of TLR4 in amniotic epithelium. *Placenta* 2007; 28:477–481.
21 Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, Frees K, Watt JL, Schwartz DA: TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000; 25:187–191.
22 Goldenberg RL, Hauth JC, Andrews WW: Intrauterine infection and preterm delivery. *N Eng J Med* 2000; 342:1500–1507.
23 Kawasaki ES: Simple preparation of blood, cell and other fluids. In: PCR protocols: A guide to methods and applications, MA Innis, DH Gelfand, JJ Sninsky, TJ White (eds). San Diego, Academic Press, 1990, pp 146–152.
24 Lorenz E, Frees KL, Schwartz DA: Determination of the TLR4 genotype using allele-specific PCR. *Biotechniques* 2001; 31:22–24.
25 Wigginton JE, Cutler DJ, Abecasis GR: A note on exact tests of Hardy-Weinberg equilibrium. *Am J Hum Genet* 2005; 76:887–893.
26 Rey G, Skowronek F, Alciaturi J, Alonso J, Bertoni B, Sapiro R: Toll receptor 4 Asp299Gly polymorphism and its association with preterm birth and premature rupture of membranes in a South American population. *Mol Hum Reprod* 2008; 14:555–559.
27 Lorenz E, Hallman M, Marttila R, Haataja R, Schwartz DA: Association between the Asp299Gly polymorphisms in the Toll-like receptor 4 and premature births in the Finnish population. *Pediatr Res* 2002; 52:373–376.
28 Ferrand PE, Fujimoto T, Chennathukuzhi V, Parry S, Macones GA, Sammel M, Kuivaniemi H, Romero R, Strauss JF 3rd: The CARD15 2936insC mutation and TLR4 896 A>G polymorphism in African Americans and risk of preterm premature rupture of membranes (PPROM). *Mol Hum Reprod* 2002; 8:1031–1034.
29 Najmi N, Kaur G, Sharma SK, Mehra NK: Human Toll-like receptor 4 polymorphisms TLR4 Asp299Gly and Thr399Ile influence susceptibility and severity of pulmonary tuberculosis in the Asian Indian population. *Tissue Antigens* 2010; 76:102–109.
30 Liadaki K, Petinaki E, Skoulakis C, Tsirevelou P, Klapsa D, Germenis AE, Speletas M: Toll-like receptor 4 gene (TLR4), but not TLR2, polymorphisms modify the risk of tonsillar disease due to Streptococcus pyogenes and Haemophilus influenzae. *Clin Vaccine Immunol* 2011; 18:217–222.
31 Bochud PY, Sinsimer D, Aderem A, Siddiqui MR, Saunderson P, Britton S, Abraham I, Tadesse Argaw A, Janer M, Hawn TR, Kaplan G: Polymorphisms in Toll-like receptor 4 (TLR4) are associated with protection against leprosy. *Eur J Clin Microbiol Infect Dis* 2009; 28:1055–1065.
32 Bhuvanendran S, Hussin HM, Meran LP, Anthony AA, Zhang L, Burch LH, Phua KK, Ismail A, Balaram P: Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms and typhoid susceptibility in Asian Malay population in Malaysia. *Microbes Infect* 2011; 13:844–851.
33 Wang H, Hirsch E: Bacterially-induced preterm labor and regulation of prostaglandin-metabolizing enzyme expression in mice: the role of toll-like receptor 4. *Biol Reprod* 2003; 69:1957–1963.
34 Nath CA, Ananth CV, Smulian JC, Peltier MR: Can sulfasalazine prevent infection-mediated pre-term birth in a murine model? *Am J Reprod Immunol* 2010; 63:144–149.
35 Goldenberg RL, Rouse DJ: Prevention of premature birth. *N Engl J Med* 1998; 339:313–320.
36 Łukaszewski T, Barlik M, Seremak-Mrozikiewicz A, Kurzawińska G, Mrozikiewicz PM, Sieroszewski P, Drews K: Polymorphism in the genes of Toll-like receptors type 2 and type 4 (TLR-2 and TLR-4) and the risk of premature rupture of the membranes-preliminary study. *Ginekol Pol* 2009; 80:914–919.
37 Genç MR, Gerber S, Nesin M, Witkin SS: Polymorphism in the interleukin-1 gene complex and spontaneous preterm delivery. *Am J Obstet Gynecol* 2002; 187:157–163.
38 Edwards RK, Ferguson RJ, Duff P: The interleukin-1 beta +3953 single nucleotide polymorphism: cervical protein concentration and preterm delivery risk. *Am J Reprod Immunol* 2006; 55:259–264.
39 Murtha AP, Nieves A, Hauser ER, Swamy GK, Yonish BA, Sinclair TR, Heine RP: Association of maternal IL-1 receptor antagonist intron 2 gene polymorphism and preterm birth. *Am J Obstet Gynecol* 2006; 195:1249–1253.
40 Kalish RB, Vardhana S, Gupta M, Chasen ST, Perni SC, Witkin SS: Interleukin-1 receptor antagonist gene polymorphism and multifetal pregnancy outcome. *Am J Obstet Gynecol* 2003; 189:911–914.
41 Velez DR, Fortunato SJ, Thorsen P, Lombardi SJ, Williams SM, Menon R: Preterm birth in Caucasians is associated with coagulation and inflammation pathway gene variants. *PLoS ONE* 2008; 3:e3283.
42 Macones GA, Parry S, Elkousy M, Clothier B, Ural SH, Strauss JF 3rd: A polymorphism in the promoter region of TNF and bacterial vaginosis: preliminary evidence of gene-environment interaction in the etiology of spontaneous preterm birth. *Am J Obstet Gynecol* 2004; 190:1504–1508.
43 Nukui T, Day RD, Sims CS, Ness RB, Romkes M: Maternal/newborn GSTT1 null genotype contributes to risk of preterm, low birthweight infants. *Pharmacogenetics* 2004; 14:569–576.