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The significance of IL-1β +3953C>T, IL-6 -174G>C and -596G>A, TNF-α -308G>A gene polymorphisms and 86 bp variable number tandem repeat polymorphism of IL-1RN in bronchopulmonary dysplasia in infants born before 32 weeks of gestation

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Abstract

Introduction: *Bronchopulmonary dysplasia (BPD) is a chronic lung disease that affects primarily preterm infants. Genetic factors are also taken into consideration in the pathogenesis of BPD. Genetic predispositions to higher production of inflammation mediators seem to be crucial.*

Material and methods: *The aim of this study was to evaluate the possible relationship between polymorphisms: interleukin-1β +3953 C>T, interleukin-6 -174 G>C and -596 G>A, tumour necrosis factor -308 G>A and interleukin-1RN VNTR 86bp and the occurrence of BPD in a population of 100 preterm infants born from singleton pregnancy, before 32+0 weeks of gestation, exposed to antenatal steroids therapy, and without congenital abnormalities.*

Results: *In the study population BPD was diagnosed in 36 (36%) newborns. Among the studied polymorphisms we found the higher prevalence for BPD developing of the following genotypes: 1/2 (OR 1.842 [0.673-5.025] and 2/2 IL-1RN (OR 1.75 [0.418-6.908] 86bpVNTR; GC (2.222 [0.658-8.706]) and CC IL-6 -174G>C (1.6 [0.315-8.314]) and GA (2.753 [0.828-10.64]) and AA (1.5 [0.275-8.067] IL-6 -596G>A), GA 1.509 (0.515-4.301) TNF-α -308G>A. However, these finding were not statistically significant.*

Conclusions: *Genetic factors are undeniably involved in the pathogenesis of BPD. In the times of individualised therapy finding genes responsible for BPD might allow the development of new treatment strategies. A new way of specific therapy could ensure the reduction of complications connected with BPD and treatment costs.*

Key words: *gene, polymorphism, bronchopulmonary dysplasia, preterm newborn.*

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Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that primarily affects preterm infants. It was described for the first time by Northway *et al*. in 1967 [1]. BPD occurs mostly in preterm infants, especially those born before the 28th week of gestation, whose lungs are at a late canalicular stage of development [2]. According to the present data, BPD affects as many as 50% of infants with very low birth weight and concerns about 68% of infants born before the 28th week of gestational age [3, 4]. The pathogenesis of this disease is connected with a chronic state of inflammation, which disrupts the growth and

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alveolarisation of lungs and leads to abnormal angiogenesis. Impaired angiogenesis can lead to pulmonary hypertension, which is a severe complication of BPD [5]. There are many different risk factors of BPD. The most pertinent risk factors are low gestational age and lung immaturity; both require prolonged ventilation, which can be associated with a toxic effect of oxygen [6]. The other risk factors are: pre- (chorioamnionitis) and postnatal infections, patent ductus arteriosus (PDA), lack of antenatal steroid therapy, and insufficiency of surfactant [7]. Nowadays, genetic factors are also taken into consideration in pathogenesis of BPD. Genetic predispositions to higher production of inflammation mediators appear to be crucial. Large multicentre research performed on 450 pairs of twins proved that BPD tends to be more frequent among monozygotic twins. This result confirmed the role of genetic factors in BPD pathogenesis [8]. It was estimated that the occurrence of BPD is 50-80% hereditary [9]. Thus far, effective treatment of diagnosed BPD has not been described, so it is imperative to prevent further development of this disease.

The aim of this study was to evaluate the possible relationship between five polymorphisms in genes encoding Interleukin 1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor α (TNF-α), and the interleukin-1 receptor antagonist (IL1RN) and the occurrence of BPD in a population of preterm newborns. In the era of individualised therapy, finding genes responsible for BPD may allow the development of new treatment strategies. An innovative way of targeted therapy could ensure a reduction of complications connected with BPD and its treatment costs.

Material and methods

Study population

Our study included 100 of 428 (23.4%) Caucasian inborn infants from singleton pregnancy, with antenatal steroids therapy (AST), delivered from 24+0 to 32+0 weeks of gestation, between the June 1st, 2014 and August 15th, 2016 in the Clinical Hospital of Gynaecology and Obstetrics at Poznan University of Medical Sciences. These neonates were then admitted to the Neonatal Intensive Care Unit. The following exclusion criteria were used: neonates born before 24+0 and after 32+0 weeks of pregnancy, outborn infants, lack of antenatal steroid therapy, multiple pregnancy births, pregnancies complicated by death of one of the foetuses, chromosomal abnormalities or TORCH infections (toxoplasmosis, other, rubella, cytomegalovirus, herpes), and inherited errors of metabolism.

Clinical features

We explored the relationship between the occurrence of BPD and the following prenatal and perinatal factors: gender, gestational age (GA; weeks), birth weight (BW, grams), small for gestational age (SGA, defined as birth weight under 10th percentile), APGAR score; type of delivery (vaginal birth vs. cesarean section), birth asphyxia (defined as APGAR score less than 6 at 10 minutes and ph < 7.0 or blood base excess (BE) ≤ 12 mmol/l in cord blood), intrauterine infection (defined as a positive culture in originally sterile environment accompanied by clinical symptoms), therapy with surfactant (according to recommendations of European consensus guidelines on the management of neonatal respiratory distress syndrome in preterm infants published in 2013 [10], type of ventilation support (non-invasive vs. conventional), duration of ventilation support (cut-off point – mean days of ventilation support), and therapy with inhaled nitric oxide (iNO, in patients with diagnosed pulmonary hypertension based on recommendations of the Committee on Foetus and Newborn; American Academy of Paediatrics [11]).

BPD diagnosis

BPD was diagnosed based on the National Institutes of Health Consensus definition of bronchopulmonary dysplasia [12].

BPD prophylaxis

BPD prophylaxis was provided based on local standards. Low-dose hydrocortisone therapy (1-2 mg/kg per day for 10 consecutive days) was given after the seventh day of life in infants requiring conventional mechanical ventilation.

Studied polymorphisms

The criteria for selection of candidate genes in the present study were their potential involvement in the pathogenesis of BPD and their individualised response to inflammation. We studied five single nucleotide polymorphisms: IL-1β *+3953C>T*, IL-6 *-174G>C* and *-596 G>A*, TNF-α *-308 G>A* and *IL-1RN VNTR 86bp*.

Samples of blood were taken after delivery and banked. Genomic DNA was extracted from blood leukocytes using QIAamp DNA Blood Mini Kit (QIAGEN Inc., Germany). Genotyping was performed using polymerase chain reaction (PCR) procedures. For detection of the IL-1β *+3953C>T* (rs 1143634) mutation, PCR was amplified with starters: F 5`- gTTgTC ATC Aga CTT TgA CC - 3`; R 5`- TTC AgT TCA TAT ggA CCA gA - 3` (PCR product 251 bp long) and hydrolysed with *Taq*I restriction enzyme (Thermo Scientific). The following genotypes were obtained: CC (137, 114bp), CT (251,137,114 bp), and TT (251bp).

For detection of the *-174G>C* (rs1800795) mutation, PCR was amplified using the starters: F 5`- ACA TgC CAA gTgCTgAgT CA - 3`, R 5`- AAT CTT TgTTggAg-ggTg Ag - 3' (PCR product 214 bp long) and hydrolysed with *LweI* restriction enzyme (Thermo Scientific). The following genotypes were obtained: GG (114, 100 bp), GC (214, 114, 100 bp), and CC (214 bp). The following start-

ers were used for detection of the *-596G>A* (rs1800797) IL-6 mutation: F 5`- ggAgTC ACA CAC TCC ACC Tg - 3` and R 5`- AAgCAg AAC CAC TCT TCC TTT ACT T - 3'. The PCR products (420 bp long) were hydrolysed with *BseGI (BtsCI)* restriction enzyme (Thermo Scientific) and yielded the following genotypes: GG (420 bp), GA (420, 354, 66 bp), and AA (354, 66 bp).

The *-308G>A* TNF-α (rs1800629) polymorphism was detected using the following starters: 5` - AAA TggAgg CAA Tag gTTTTgAggggCTTg – 3' and 5` - TAC CCC TCA CAC TCC CCA TCC TCC CTg ATC – 3' (TIBMolBiol). The PCR product (131 bp) was hydrolysed with *FaqI (BsmFI)* restriction enzyme, and the following genotypes were found: GG (86, 45 bp), GA (131, 86, 45 bp), and AA (131 bp).

The 86 bp variable number tandem repeat polymorphism of *IL-1RN* (rs2234663) was analysed with PCR using the following starters: F 5`- CTC AgC AAC ACT CCT AT - 3` and R 5`- TCC Tgg TCT gCAggT AA – 3` (TiBMolBiol). It was possible to obtain products with lengths of: 154 bp (IL1RN*0), 410 bp (IL1RN*1), 240 bp (IL1RN*2), 500 bp (IL1RN*3), 325 bp (IL1RN*4), and 595 bp (IL1RN*5).

Informed consent was obtained from all parents. The study was approved by the Bioethics Committee of Poznan University of Medical Sciences (no. 66/14 and 799/16).

The results are presented as a percentage for categorical variables, or median (range) for non-normally distributed continuous variables as tested by the Shapiro-Wilk test. A *p*-value of less than 0.05 was considered significant. The Fisher exact probability test, the χ^2 test, Fisher Freeman Halton and χ^2 test with Yates correction were all used to evaluate the association between BPD and categorical studied variables. Logistic regression analysis was used to compute ORs and their 95% confidence intervals (CI) for patients without BPD and BPD combined with different genotypes and alleles. The expected genotype frequencies were calculated from allele frequencies with the Hardy-Weinberg equation. Statistical analysis was performed using CytelStudio version 10.0, created January 16, 2013 (CytelStudio Software Corporation, Cambridge, Massachusetts, United States), and Statistica version 10, 2011 (Stat Soft, Inc., Tulsa, Oklahoma, United States).

Results

In our study population, BPD was diagnosed in 36 (36%) newborns. BPD was diagnosed more often in infants born from 24+0 to 28+0 weeks of gestation (29/54 [53.7%] vs. 7/36 [19.4%]; $p = 0.0006$), with lower Apgar score at the first (median 4.5 [range 1-7] vs. median 6 [range 1-10]; $p = 0.005$) and fifth minutes (median 7 [range 1-9] vs. median 8 [range 5-10]; $p = 0001$). Surfactant therapy (29/50 [58%] vs. 7/48 [14.58%]; $p = 0.0001$), conventional ventilation vs. non-invasive ventilation support (27/48 [56.25%] vs. 9/52 [17.3%]; $p = 0.00005$) and ventilation longer than 29 days (25/35 [71.42%] vs. 3/54 [5.55%]; $p < 0.0001$) were used more often in BPD patients. The risk of BPD influenced by other factors was not significant. Patients' clinical characteristics are shown in Table 1.

The IL-1β *+3953C>T*, IL-6 *-174G>C* and *-596G>A*, TNF-α *-308G>A* and *IL-1RN VNTR 86 bp* gene polymorphisms in the BPD and non-BPD groups are described

Table 1. Demographic and clinical characteristic of enrolled infants

Parameter	Group without BPD (*n* = 64; %)	Group with BPD (*n* = 36; %)	*P* value
Gender			0.854a
Male	35 (54.69)	19 (52.78)	
Female	29 (45.31)	17 (47.22)	
Gestational age (weeks)			0.00006[a]
24-28	25 (39.06)	29 (80.56)	
29-32	39 (60.94)	7 (19.44)	
Birth weight (grams)			0.0004[a]
< 750	5 (7.81)	9 (25.00)	
750-1000	12 (18.75)	15 (41.67)	
> 1000	47 (73.44)	12 (33.33)	
IUGR			0.833[b]
Yes	11 (17.19)	6 (16.67)	
No	53 (82.81)	30 (83.33)	
Apgar score (median and range)			
1st minute	6 (1-10)	4.5 (1-7)	0.005[c]
5th minute	8 (5-10)	7 (1-9)	0.0001[c]
Mode of delivery			0.428[c]
Vaginal	25	16	
Caesarean section	39	20	
Asphyxia (pH lower than 7.0 or BE lower than -12)			0.581[b]
Yes	1 (1.59)	2 (5.88)	
No	62 (98.41)	32 (94.12)	
Intrauterine infection			0.615[a]
Yes	34 (53.13)	21 (58.33)	
No	30 (46.88)	15 (41.67)	
Surfactant therapy			0.0001[a]
Yes	21 (33.87)	29 (80.56)	
No	41 (66.13)	7 (19.44)	
Ventilation support			0.00005[a]
Non-invasive	43 (67.19)	9 (25.00)	
Conventional	21 (32.81)	27 (75.00)	
Ventilation support			< 0.0001[a]
≤ 29 days	51 (83.61)	3 (10.71)	
> 29 days	10 (16.39)	25 (89.29)	
iNO			0.529[b]
Yes	4 (6.56)	4 (12.90)	
No	57 (93.44)	27 (87.10)	
Deaths			0.056[d]
Yes	10 (15.87)	0 (0.00)	
No	53 (84.13)	25 (100.0)	

[a]χ^2 test, χ^2test with Yates correction, [c]Fisher Freemen Halton test, [d]Fisher's exact test

Table 2. Genotype distribution of polymorphisms in infants without and with BPD

Gene symbol		Group without BPD *n* (%)		Group with BPD *n* (%)	Expected	*P* value	OR (CI)
IL-1β (rs1143634) +3953C>T	Genotypes						
	CC	39 (60.93)	36.75	23 (63.89)	23.36		reference
	CT	19 (29.69)	23.49	12 (33.33)	11.28	1.000	1.071 (0.396-2.829)
	TT	6 (9.38)	3.75	1 (2.78)	1.36	0.447	0.283 (0.006-2.599)
	H-W		0.126		0.701		
	Allele						
	C	97		58		-	references
	T	31		14		0.553	0.755 (0.342-1.609)
IL-1RN (rs2234663) 86 bp VNTR	Genotypes						
	1/1	35 (54.69)	31.94	15 (41.67)	14.06		reference
	1/2	19 (29.69)	25.12	15 (41.67)	16.88	0.274	1.842 (0.673-5.025)
	1/3	2 (3.13)		0 (0.00)		1.000	0.000 (0.000-13.31)
	2/2	8 (12.50)	4.94	6 (16.67)	5.06	0.551	1.750 (0.418-6.908)
	2/3	0 (0.00)		0 (0.00)		–	–
	H-W		0.055		0.505		
	Allele						
	1	91		45		–	references
	2	35		27		0.209	1.56 (0.801-3.016)
	3	2		0		0.905	0.000 (0.00-11.04)
IL-6 (rs1800795) -174G>C	Genotypes						
	GG	16 (25.00)	18.06	5 (13.89)	8.51		reference
	GC	36 (56.25)	31.88	25 (69.44)	17.99	0.250	2.222 (0.658-8.706)
	CC	12 (18.75)	14.06	6 (16.67)	9.51	0.761	1.600 (0.315-8.314)
	H-W		0.301		0.019		
	Allele						
	G	68		35		–	references
	C	60		37		0.641	1.198 (0.645-2.225)
IL-6 (rs1800797) -596G>A	Genotypes						
	GG	18 (28.13)	19.14	5 (13.89)	9.00		reference
	GA	34 (53.13)	31.72	26 (72.22)	18.00	0.112	2.753 (0.828-10.64)
	AA	12 (18.75)	13.14	5 (13.89)	9.00	0.847	1.500 (0.275-8.067)
	H-W		0.565		0.007		
	Allele						
	G	70		36		–	references
	A	58		36		0.624	1.207 (6.50-2.241)
TNF–α (rs1800629) -308G>A	Genotypes						
	GG	51 (79.69)	51.66	26 (72.22)	26.69		reference
	GA	13 (20.31)	11.68	10 (27.78)	8.61	0.541	1.509 (0.515-4.301)
	AA	0 (0.00)	0.66	0 (0.00)	0.69	–	–
	H-W		0.366		0.333		
	Allele						
	G	115		62		–	references
	A	13		10		0.566	1.427 (0.526-3.75)

N – observed; Expected – genotype frequencies calculated from allele frequencies with the Hardy-Weinberg (H-W) equation

in Table 2. Among the studied polymorphisms we found a higher prevalence for BPD developing of the following genotypes: 1/2 (OR 1.842 [0.673-5.025] and 2/2 *IL-1RN* (OR 1.75 [0.418-6.908]) *86bp VNTR*, GC (2.222 [0.658-8.706] and CC IL-6 *-174G>C* (1.6 [0.315-8.314]) and GA (2.753 [0.828-10.64]) and AA (1.5 [0.275-8.067] IL-6 *-596G>A*; GA 1.509 (0.515-4.301) TNF-α *-308G>A*. However, these finding were not statistically significant.

Discussion

The inflammatory process plays an indisputable role in the pathogenesis of bronchopulmonary dysplasia. In their research Ambalavanan *et al*. measured the level of 25 cytokines in the blood of 1062 neonates with very low birth weight. In the blood of 606 patients with BPD, levels of IL-1β, IL-6, IL-8, and IL-10 and interferon-γ were significantly increased [13]. Taking into account the fact that both genetic and inflammatory factors are relevant to the pathogenesis of BPD allows us to hypothesise that BPD is connected with individualised inflammatory responses. In our study, we investigated five polymorphisms of genes connected with inflammatory response and their influence on the occurrence of BPD. The following polymorphisms were examined: IL-1β *+3953C>T*; IL-6 *-174G>C* and *-596G>A*; TNF-α *-308G>A* and *IL-1RN VNTR 86bp*. Genes involved in inflammation pathway have not been investigated in a Polish population of preterm newborns. Kwinta *et al*. performed a similar study on a Polish population, but they investigated polymorphisms for other substances: vascular endothelial growth factor (VEGF), transforming growth factor β1 (TGF-β1), insulin-like growth factor (IGF-1), and 5,10-methylenetetrahydrofolate reductase (MTHFR). Their research suggested that VEGF *-460T>C* polymorphism may have an influence on developing BPD [14].

IL-1β 3953 C>T polymorphism

Interleukin-1 is the main pro-inflammatory cytokine, which induces the production of other pro-inflammatory cytokines such as IFN-γ, IL-6, and TNF-α. Interleukin-1 is produced by activated macrophages. The variant IL-1β is responsible for most effects induced by this cytokine. Increased level of IL-1β was frequently reported in the blood of infants with BPD [13, 15]. It was proven in animal models that heightened level of IL-1β disrupts morphogenesis and angiogenesis of the lungs [16, 17]. To the best of our knowledge, IL-1β polymorphisms were not yet investigated in the context of developing BPD. Polymorphism Il-1β *3953C>T*, which was taken into account in our research, consists of replacing cytosine with thymine. It leads to the appearance of a rarer allele 2, which is connected with higher production of IL-1β [18]. Our results did not show any correlation between this polymorphism and the higher risk of BPD among the infants born before the 32nd week of gestational age.

IL-1RN VNTR 86bp

Interleukin-1 receptor antagonist (IL-1 RA) is a protein that blocks Il-1 receptor and inhibits its effect. It is encoded by the IL-1RN gene. It is a protective factor in pathogenesis of BPD, which has been proven on murine models [19, 20]. The quantity of IL-1 RA and IL-1β depends on IL-1RN polymorphism [21]. The main role is played by the amount of repeats of 86-bp sequences within intron 2 of the human Il-1 receptor antagonist gene [22]. The number of repeats is of functional significance because these repeats contain binding sites for transcription factors. It was proven that the occurrence of IL1RN*2 is connected with more severe and prolonged inflammatory response [21]. Cakmak *et al*. explored the connection between BPD and IL-1RN polymorphisms. Their research showed that IL-1RN 2/2 genotype increases the risk of BPD and of IL-1RN 1/1 genotype has protective character [23]. In our research we did not prove any influence of this polymorphism on BPD risk. In the group without BPD 55% of infants had 1/1 genotype, 30% had 1/2 genotype, 12.5% had 2/2 genotype, and 3.5% had 1/3 genotype. In infants with BPD 42% had 1/1 genotype, 42% had 1/2 genotype, and 17% had 2/2 genotype. Our results only partially correspond with previously cited findings of Cakmak *et al*., probably because of the inadequate number of examined infants.

IL-6 *-174 G>C* and *-596 G>A* polymorphism

Iinterleukin 6 is a cytokine with a wide spectrum of effects. It plays both a pro- and anti- inflammatory role in different mechanisms. Its role in the pathogenesis of BPD is ambiguous. Increased levels of IL-6 were described either as a risk factor for BDP [13, 24] or as a protective factor [25]. There are reports about elevated levels of IL-6 in respiratory tracts in response to high concentration of oxygen. Higher levels of IL-6 were also reported in neonates diagnosed with BPD [26]. Huusko *et al*. performed research on 379 preterm infants (114 with BPD, 265 in the control group), investigating 44 single nucleotide polymorphisms (SNPs) in search for their connection with BPD. Among them were the following polymorphisms: five of IL-6, nine of IL6R, and four of IL6ST (IL-6 receptors). Polymorphisms of IL-6: *-1363G>T* (rs2069827), *-597A>G* (rs1800797), *IVS2G>A* (rs2069832), *-1753C>G* (rs2069840), and *-174G>C* (rs1800795) were taken into account. None of the explored polymorphisms were linked with a higher risk of BPD [27]. Usuda *et al.* checked polymorphism IL-6 *-634G>C* in the context of BPD development. There was no statistically significant increase of BPD risk. However, carriers of allele G required longer oxygen therapy than the children with allele C. Similar-

ly, allele G carriers with BPD more often required treatment with steroids [28]. In our research we analysed IL-6 *-174G>C* and *-596G>A* polymorphisms. IL-6 *-174G>C* is connected with the replacement of guanine by cytosine at position -174, and IL-6 *-596G>A* consists of replacing of guanine by adenosine at position -596. Both homozygotes CC of polymorphism IL-6 *-174G>C* and AA of polymorphism IL-6 *-596G>A* result in decreased production of IL-6. In our research there was no significant correlation between these polymorphisms and BPD.

TNF-α *-308G>A* polymorphism

Tumor necrosis factor α is primarily responsible for the cytotoxic effect against neoplasm cells; however, it also plays a role in states of inflammation, such as BPD. Tumor necrosis factor α is a pro-inflammatory cytokine. Increased levels of TNF-α were reported in children with BPD [15, 29]. Tumor necrosis factor α recruits and stimulates inflammatory cells and probably disrupts normal expression of fibroblast growth factor (FGF) [15]. It was described that TNF-α impaired pulmonary endothelial cells [25]. The role of TNF-α polymorphisms in the pathogenesis of BPD is not clearly defined. Strassberg *et al*. performed analysis of the following polymorphisms of TNF-α: *-1031T>C, -863C>A, -857C>T, -308G>A,* and *-238G>A* in a group of 105 infants (89 with BPD). None of the investigated polymorphisms was linked with increased risk of BPD [30]. Elhawary *et al*. investigated the polymorphism TNF-α *-238G>A* in 220 preterm infants (120 with BPD), and they demonstrated that it is connected with twice the risk of BPD. Allele A was also more frequent in children with severe and moderate (accordingly in 39% and 52%) than with mild BPD (9%) [31]. Kazzi *et al.* investigated the same polymorphism in a group of 154 neonates with low birth weight. According to their findings, allele A had a protective influence on BPD and correlated with the milder course of illness [32]. Meta-analysis performed by Chauhan *et al*. consisted of six cumulative research projects (804 infants in total), proving that the polymorphism TNF *-308G>A* is not significantly connected with BPD [33]. In research conducted by Mailaparambil *et al*., investigation of polymorphisms of TNF-α were *-1031T>C* (rs1799964), *-857C>T* (rs1799724), and *-308G>A* (rs1800629). Only one of those (TNF-α *-857C>T*) was connected with the occurrence of BPD [34]. In the previously quoted research by Huusko *et al*. two polymorphisms of TNF-α: *-1031T>C* and *-308G>A* were also taken into account, but they also, similarly to the other polymorphisms analysed in this study, were not involved in BPD occurrence [27]. In our research, we explored the role of polymorphism TNF *-308G>A* in the pathogenesis of BPD. Replacement of guanine by adenosine results in loss of binding place for AP-2 transcription factor. Our results are consistent with the previously performed ones. Polymorphism TNF *-308G>A* does not play a significant role in the pathogenesis of BPD.

Conclusions

Bronchopulmonary dysplasia is a disease with complicated pathogenesis. As well as the well-known risk factors, other factors should be taken into consideration. Among the proven risk factors of BPD the following should be mentioned: low gestational age, prolonged ventilation, occurrence of RDS, and low Apgar score. Genetic factors are undeniably involved in the pathogenesis of BPD. Further studies are necessary to establish which polymorphisms increase the risk of BPD, and which protect against it.

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