Rīgas Stradiņa universitāte

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CHARACTERISTICS OF THE MAIN MOLECULAR PROCESSES IN THE TISSUES OF PLACENTAS OF VARIOUS GESTATIONAL AGES

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ABBREVIATIONS

	English	Latvian
AGA	Appropriate for the gestational age	*Atbilstošs grūtniecības laikam*
BMI	Body mass index	*Ķermeņa masas indekss*
bFGF	Basic Fibroblast growth factor	*Bāziskais Fibroblastu augšanas faktors*
CH	Chorionamnionitis	*Horionamnionīts*
CPAP	Continuous positive airway pressure	*Pastāvīgi pozitīvs spiediens elpceļos*
CS	Cesarean section	*Ķeizargrieziens*
DAB	Diaminobenzidine	*Diaminobenzidīns*
ECM	Extracellular matrix	*Ekstracellulārā matrica*
FGFR	Fibroblast growth factor receptor	*Fibroblastu augšanas faktora receptors*
GBS	Group B Streptococcus	*B grupas streptokoks*
HE	Hematoxylin and eosin	*Hematoksilīns un eozīns*
HGF	Hepatocyte growth factor	*Hepatocītu augšanas faktors*
HIER	Heat induced epitope retrieval	*Karstuma izraisīta epitopu izdalīšana*
HIV	Human immunodeficiency virus	*Cilvēka imūndeficīta vīruss*
IGF1	Insulin-like growth factor	*Insulīnam līdzīgais augšanas factors*
IGF1R	Insulin-like growth factor receptor	*Insulīnam līdzīgā augšanas faktora receptors*
IHC	Immunohistochemistry	*Imunohistoķīmija*
IL	Interleukin	*Interleikīns*
IUGR	Intrauterine growth restriction	*Intrauterīnās augšanas aizture*

	English	**Latvian**
LGA	Large for the gestational age	*Liels grūtniecības laiks*
LPS	Lipopolysaccharide	*Lipopolisaharīds*
MMP	Matrix metaloproteinase	*Matricas metālproteināze*
NICU	Neonatal intensive care unit	*Jaundzimušo intensīvās terapijas nodaļa*
NS	Non-significant	*Nenozīmīgs*
pH	Power of Hydrogen ion H^+	*Ūdeņražu jonu H^+ spēks*
PBS	Phosphate buffered saline	*Fosfātu bufera sāls šķīdums*
pO_2	Partial pressure of oxygen	*Skābekļa parciālais spiediens*
PI	Ponderal index	*Ponderela indekss*
PPROM	Pre-term premature rupture of membranes	*Pirms-termiņa priekšlaicīgs augļa apvalku plīsums*
PROM	Premature rupture of membranes	*Priekšlaicīgs augļa apvalku plīsums*
RNA	Ribonucleic acid	*Ribonukleīnskābe*
ROM	Rupture of membranes	*Augļa apvalku plīsums*
RSU	Riga Stradins university	*Rīgas Stradiņa universitāte*
SGA	Small for the gestational age	*Mazs grūtniecības laiks*
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling	*Terminālās deoksinukleotidiltransferāzes dUTP N gala marķēšana*
TGF	Transforming growth factor	*Transformējošais augšanas faktors*
TNFα	Tumor necrosis factor	*Tumora nekrozes faktors*
VEGF	Vascular endothelial growth factor	*Asinsvadu endotēlija augšanas faktors*

1. RELEVANCE OF THE STUDY

Perinatal and neonatal mortality are important indicators of the development of a country and highly developed ones tend to maintain those figures low, permanently auditing childbirth management related issues (Mancey-Jones et al., 1997; Pattinson et al., 2009) and implementing suggested improvements. In the developing countries causes of mortality quite often appear to be evident and are highly associated with availability of qualified medical care while in the developed ones problems quite often are hidden in the maternal-fetal unit; investigations of this environment and identification of potentially disadvantageous processes can give clues for the achievement of better outcomes. The most commonly seen problems, possibly leading to adverse pregnancy outcomes, are pre-term premature rupture of membranes, leading to pre-term labor (Al-Riyami et al., 2013), fetal growth failure due to various causes (Longo et al., 2013) and events, leading to significant fetal distress and threat to his life (Ribak et al., 2011).

Fetal growth and development is determined by the interaction of mother and fetus by the means of interface placenta throughout the pregnancy; disturbances in regulation of fetal growth and development can result in adverse outcomes for the neonate, and these adverse outcomes may persist into adult life. Placenta ensures circumstances for fetal growth and development (Bauer et al., 1998; Murphy et al., 2006, Jansson et al., 2007) and findings in its tissues reveal processes, having determined the outcome of pregnancy; they have special clinical significance in the cases of high risk pregnancies or unexpected complications. Understanding the physiological and pathological processes in placenta disclose the roads of problem solving with a possibly better perinatal outcome.

Antenatal care as well as post-delivery examination of placenta has become a routine part of the perinatal (obstetrical and neonatal) care for the assessment of pathways, possibly leading or having led to an unwanted outcome. Even routine examination of a post–delivery placenta provides significant information on the fetal environment (Fox and Sebire, 2007; Tomas et al., 2010; Roescher et al., 2011; Roje et al., 2011) having possible impact on the child's health status and maternal wellbeing. Due to the complexity of those processes, routine praxis does not answer many questions. The present thesis show identification of the molecular processes in the post-delivery placentas of different gestational ages for the development of clinically applicable knowledge of molecular processes to create opportunitie11s for the improvement of perinatal outcome in high risk situations.

Pathways of the pathological processes have been researched by different methods; immunohistochemistry (IHC) has been found to be sensitive for the understanding of pathological processes and establishment of accurate clinical diagnosis in more complicated cases (Takizawa et al., 2007). IHC research has been used in the cases of preeclampsia and intrauterine growth restriction (IUGR) (Shen et al., 2011; Cayli et al., 2012; Cozzi et al., 2012), non-immune fetal *hydrops* (Bellini et al., 2010). IHC reveals molecular processes in the placental tissues and possibly could disclose specific targets of clinical management, improving efficiency of the treatment and outcome. A drawback of a practical application of an IHC research is its specificity (Ramos-Vara, 2005); for this reason it would be very useful to identify a scope of reliable markers of the molecular processes in placenta, imperceptible in a routine examination, but possibly having led to one or another outcome.

We were interested in several kinds of placental markers that we expected to be involved in the development of fetus and his environment: growth factors, cytokines, proteins of the basement membrane, tissue degrading

enzymes as well as products of the homeobox genes; were assessed average numbers of apoptotic cells per visual field. From the growth factors and their receptors were chosen the following: one of the most potent growth factors Insulin-like growth factor 1 (IGF1) (Kansra et al., 2012; Becker et al., 2012) and its receptor IGFR1, important factors of fetal growth and promoters of placental development and function (Sferruzzi-Perri et al., 2007; Forbes and Westwood, 2008; Sferruzzi-Perri et al., 2008; Sferruzzi-Perri et al., 2008), Hepatocyte growth factor (HGF), playing a significant role in the development of the placenta (Uehara et al., 1995) and fetus (Bladt et al., 1995; Schmidt et al., 1995; Ebens et al., 1996), Fibroblast growth factor basic (bFGF) and its receptor FGFR1, having been found to be increased in placentas in cases of pathological pregnancies (Hill et al., 1998; Arany et al., 1998; Ozkan et al., 2008). For the research were chosen pro-inflammatory cytokines IL-1α and TNFα as well as anti-inflammatory cytokine IL-10, expecting impact of their interaction and balance on the course of pregnancy (Xie et al., 2011; Chabtini et al., 2012; Chaparro et al., 2012; Kim et al., 2012; Lamarca et al., 2012; Parveen et al., 2012; Taki et al., 2012; Twig et al., 2012; Wu et al., 2012) and its aoutcome (Mitchel et al., 2004; Kobayashi et al., 2010; Brogin Moreli et al., 2012). Collagen IV, one of the constituents of the basement membrane, determinant of the strength of tissues (Kühn, 1995; Nerlich, 1995), participates in the formation of placental barrier (Mori et al., 2007; Jones et al., 2008) and is altered in pathological pregnancies (Hu et al., 2004; van der Velde et al., 1985; Asfhaq et al., 2003; Rath et al., 2011), leading to placental insufficiency (Khozhaĭ et al., 2010). Degradation of extracellular matrix (ECM) is initiated by various enzymes, the most significant of whom are matrix metalloproteinases (MMP) (Reynolds, 1996; Shingleton et al., 1996; Xu et al., 2002; Cawston and Young, 2010); Collagen IV is degraded by MMP2 and MMP9, having impact on the tissue integrity. Coherence of remodeling

processes of ECM can determine the course and outcome of pregnancy (Hopper et al., 2003). Although *MMP9* has already been acknowledged to be a significant player in pregnancy, all the questions have not been answered yet. Development of humen embryo is influenced by a scope of homeobox genes, the most investigated of them are Hox genes. *They were found to determine growth of* anterior-posterior axis of Drosophila melanogaster; further studies confirmed their role in the development of mammal brain (Mc Ginnis and Krumlauf, 1992; Krumlauf, 1994; Schneider-Maunoury et al., 1998; Carapuço et al., 2005; Tümpel et al., 2009) and hemopoesis (Lawrence et al., 1997). Hox gene products – proteins act as trascription factors, influencing transcription of Ribonucleic acid (RNA). Apoptosis, programmed cell death, plays a significant role in morphogenesis and development of placenta (Smith et al., 1997; Huppertz et al., 1998; Mayhew et al., 1999; Mayhew, 2001; Hupperz et al., 2004), promoting growth and development of villi. In pathological cases apoptosis is related with fetal growth restriction (Smith et al., 1997; Axt et al., 1999; Erel et al., 2001; Liu et al., 2002; Burton et al., 2009) or preeclampsia (Huppertz et al., 2003; Kadyrov et al., 2006; Chen et al., 2010; Sharp et al., 2010; Chamley et al., 2011). Assessment of cellular apoptosis in the placental tissues can provide useful insight into its processes.

Research of the presence of mentioned markers in placentas of various gestational ages can improve knowledge regarding gestation related interaction between those processes and their impact on the course and outcome of pregnancy.

2. AIM OF THE STUDY

The aim of the study was to research the main molecular events of cellular growth, regeneration, cell death, tissue degradation, inflammation and gene expression in pre-term and term placentas of different gestational ages, pregnancy risk factors, as well as some anthropometrical and clinical indices of mothers and newborns for the identification of the most important diagnostic and prognostic factors of placental status and fetal well-being.

3. STUDY TASKS

For achievement of the aim were defined the following tasks:

1. To provide statistical analysis of anthropological and basic clinical data of the study patients (women and babies);
2. To assess appearance and distribution of the following molecular factors in placentas of various gestational ages: IGF1, IGFR1, HGF, bFGF, FGFR1, IL-1α, TNFα, IL-10, Collagen IV, MMP9;
3. To evaluate amount and distribution of apoptotic cells in the tissues of placentas of various gestational ages;
4. To assess appearance and distribution of the products of homeobox gene *HoxB3* in placentas of various gestational ages;
5. To look for correlations between the factors of molecular processes in the placentas and gestational age, anthropometrical parameters and main clinical indices of the mothers and newborns.

4. NOVELTY OF THE STUDY

The study researched appearance of different molecular factors in placentas of various gestational ages *in situ* and looked for correlations of the factor positive cells with gestational age, anthropological parameters of the mother and neonate as well as main clinical findings; similar studies have not been described in the literature and can provide information regarding possible impact of molecular processes on the course and outcome of pregnancy.

5. MATERIALS AND METHODS

On the 12th of March, 2009 the study was approved by the Ethics Committee of RSU. 53 HIV negative patients of legal age without systemic diseases, having received sufficient antenatal care and admitted for the delivery care in the Riga Maternity hospital, signed informed consent and were included in the study.

Study groups:

- Group 1 (term): 14 term deliveries with a healthy child from 37 till 41 weeks of pregnancy;
- Group 2 (pre-term): 25 pre-term deliveries with a pre-term child from 22 till 36 weeks of pregnancy;
- Group 3 (distress): 14 deliveries of various gestational ages with an episode of a clinically significant fetal distress before or during the delivery.

Two samples of 1 x 1 cm, taken through all the layers of placenta, were put into fixation of Picric Acid-Formaldehyde (Stefanini et al., 1967) and delivered to the Institute of Anatomy and Anthropology of RSU for further processing. Maternal, placental and neonatal data were obtained from the medical records of Childbirth and Neonatal development of the Riga Maternity hospital and the study survey. Samples underwent the following processing:

- Routine staining with hematoxylin and eosin in accordance with H&E Staining Method and Protocol (Avwioro, 2011; www.ihcworld.com);
- Immunohistochemical (IHC) staining with chosen antibodies (Table 5.1.). Preparation of the samples for IHC processing with mouse and rabbit antibodies was provided in accordance with the Dako REAL™ EnVision Detection System protocol (3rd edition, 2005).

In the cases, where goat antibodies were used, processing was provided in correspondence with the ImmunoCruz goat ABC Staining System protocol sc-2023 (Santa Cruz Biotechnology, Inc., 2011).

- TUNEL was processed by the means of standard In Situ Cell Death Detection kit, POD Cat. No 11684817910, manufactured by Roche Diagnostics (Negoescu et al., 1998).
- Negative and positive controls were provided.

Table 5.1.

Used IHC antibodies

	Antibody	Clone	Species	Company	Dilution
1.	IGF1	56408	mouse	R&D	1 : 50
2.	IGFR1	polyclonal	goat	R&D	1 : 100
3.	HGF	polyclonal	goat	R&D	1 : 300
4.	FGFb	polyclonal	rabbit	Abcam	1 : 200
5.	FGFR1	polyclonal	rabbit	Abcam	1 : 100
6.	IL-10	polyclonal	rabbit	Abcam	1 : 400
7.	IL-1α	B-7	mouse	Santa Cruz	1 : 50
8.	TNFα	polyclonal	rabbit	Abcam	1 : 100
9.	Caspase	EP13254	rabbit	Abcam	1 : 200
10.	Collagen IV	CIV94	mouse	Invitrogen	1 : 30
11.	MMP9	polyclonal	rabbit	Santa Cruz	1 : 250
12.	HoxB3	polyclonal	rabbit	Santa Cruz	1 : 100

IHC findings were evaluated semi–quantitatively by the amount of the indicator positive cells or ECM structures in a visual field (Pilmane et al., 1998): none 0, occasional 0/+, few +, moderate ++, numerous +++ and abundant ++++. Evaluation was done after complete observation of both samples of each placenta. IHC findings were ranked in the ascending order by modified competition ranking method (Pozzi, 2008): correspondingly 0; 0.5; 1; 2; 3; 4; apoptotic cells were counted in 10 visual fields. Evaluations were provided in the following sections:

1. In the whole study, including 53 deliveries;

2. In three study groups: 14 patients term (G1), 25 patients pre-term (G2) and 14 term or pre-term fetal distress patients (G3);
3. In gestation dependent groups: 19 term and 34 pre-term patients;
4. In specific patient groups, determined by the expected impact of the factor.

Descriptive statistics for the whole study group and selected study groups were performed. For cross-sample mean comparison, inferential statistical Student's t-tests were performed. This method was chosen due to the approximate normality of the data analyzed.

Pearson product-moment correlation was used to inspect the correlation of mother and neonate specific data with their respective indicators, as well as between the indicators themselves. This measure was chosen due to the linear relationships observed between the correlated data, the strong tendency of the mother, neonate and indicator data to follow normal distribution, as well as the homoscedastic nature of the data analyzed. Pearson correlation and Mann-Whitney U Test were considered and tested for correlation analysis, and Pearson correlation was chosen due to the normal distribution of the data analyzed.

Data processing software Microsoft Excel 2013 Preview and IBM SPSS 19.0 were used; the statistical significance was set at $p < 0.05$.

6. RESULTS

6.1. Maternal parameters

The study included 53 delivery patients with gestational time at delivery from 22 till 40 weeks; 30 (57%) were vaginal and 23 (43%) were by Cesarean section (CS), 9 of them (20%) were emergency CS due to fetal distress; maternal anthropological parameters are shown in the Table 6.1.

Table 6.1.

Maternal data

	Min	Max	Mean ± SD
Maternal age	18	39	29.79 ± 5.6
Number of pregnancies	1	7	2.59 ± 1.64
Number of deliveries	1	6	1.75 ± 0.98
Weeks of pregnancy	22	40	33.02 ± 5.18
Body mass before actual pregnancy (kg)	46	109	65.82 ± 12.75
Body height (cm)	150	182	166.33 ± 7.025
BMI prior to pregnancy (kg/m^2)	17.20	42.60	23.79 ± 4.74
Increase of the body mass in pregnancy (kg)	0	26	10.90 ± 5.81

Maternal age presented statistically significant positive correlations with the numbers of pregnancies and deliveries (Table 6.2.).

Table 6.2.

Correlations between maternal parameters

		Maternal age	Pregnancies	Deliveries
Maternal age	Pearson Corr.	1	0.527**	0.425**
	Sig. (2-tailed)		0.000	0.002
Pregnancies	Pearson Corr.	0.527**	1	0.733**
	Sig. (2-tailed)	0.000		0.000
Deliveries	Pearson Corr.	0.425**	0.733**	1
	Sig. (2-tailed)	0.002	0.000	

** Statistically significant strong correlation ($p < 0.01$)

Maternal parameters differed between the study groups G1 (term) and G2 (pre-term); mean value of the number of pregnancies was statistically significantly higher in the study group G2 (pre-term) than in the group G1

(term) (Table 6.3.); mean time of ruptured membranes (ROM) also was longer in the study group G2 (pre-term).

Table 6.3.

Differences of the maternal parameters between the study groups G1 and G2

		Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
Pregnancy	G1 (term) G2 (pre-term)	1.86 2.91	0.949 1.730	0.254 0.361	0.022*
Length of ROM	G1 (term) G2 (pre-term)	4.29 52.27	4.687 84.392	1.253 17.597	0.012*

* Correlation is significant at the 0.05 level

6.2. Neonatal parameters

The mean gestational age of neonates was 33.06 ± 5.09 weeks: 34 were premature, born in 22-35 weeks of pregnancy; 19 were mature, born in 37-40 weeks of pregnancy; anthropomtrical data are shown in the Table 6.4.

Table 6.4.

Neonatal anthropometric parameters

	Minimum	Maximum	Mean ± SD
Birth weight	540	4630	2367.15 ± 1122.59
Length	28	59	45.51 ± 7.433
Ponderal index	1.74	3.13	2.33 ± 0.31
Head	22	39	31.15 ± 4.34
Chest	20	37	29.19 ± 4.93

39 of the neonates were appropriate for the gestational age (AGA), weight of 5 neonates was too small for the gestational age (SGA) and 9 were large for the gestational age (LGA). 4 of SGA and 3 of LGA neonates were premature.

In the whole sample anthropometric parameters: body weight, body length, head and chest circumference were directly proportional to the gestational age and between themselves (Table 6.5.).

Table 6.5.

Correlation of the anthropometric parameters of the neonates

		Gestation	Weight	Length	Head	Chest
Gestation	Pearson corr.	1	0.906**	0.900**	0.887**	0.905**
	Sig. (2-tailed)		0.000	0.000	0.000	0.000
Weight	Pearson corr.	0.906**	1	0.938**	0.936**	0.961**
	Sig. (2-tailed)	0.000		0.000	0.000	0.000
Length	Pearson corr.	0.900**	0.938**	1	0.936**	0.934**
	Sig. (2-tailed)	0.000	0.000		0.000	0.000
Head	Pearson corr.	0.887**	0.936**	0.936**	1	0.951**
	Sig. (2-tailed)	0.000	0.000	0.000		0.000
Chest	Pearson corr.	0.905**	0.961**	0.934**	0.951**	1
	Sig. (2-tailed)	0.000	0.000	0.000	0.000	

** Correlation is significant at the 0.01 level (2-tailed)

Criteria for inclusion in the study groups determined statistically significant differences of neonatal anthropometrical parameters (Table 6.6.).

Table 6.6.

Differences between neonatal anthropometrical parameters in the study groups

Parameter	Group	Mean	SD	Std. Error Mean	p*
Body mass	G1 (term)	3591.43	503.019	134.438	0.000**
	G2 (pre-term)	1721.28	578.325	115.665	
	G1 (term)	3591.43	503.019	134.438	0.002**
	G3 (distress)	2296.21	1322.32	353.406	
Body length	G1 (term)	52.93	2.495	0.667	0.000**
	G2 (pre-term)	41.48	5.665	1.133	
	G1 (term)	52.93	2.495	0.667	0.002**
	G3 (distress)	45.29	8.062	2.155	
Head circumference	G1 (term)	35.50	1.019	0.272	0.000**
	G2 (pre-term)	28.96	3.691	0.738	
	G1 (term)	35.50	1.019	0.272	0.001**
	G3 (distress)	30.71	4.410	1.179	
Chest circumference	G1 (term)	34.71	1.490	0.398	0.000**
	G2 (pre-term)	26.52	3.537	0.707	
	G1 (term)	34.71	1.490	0.398	0.000**
	G3 (distress)	28.43	4.957	1.325	

* Sig. (t-test for Equality of Means)

** Statistically significant strong correlation ($p < 0.01$)

Study group **G1 (term)** included 14 term deliveries in 37-40 weeks of pregnancy with healthy children (7 girls; 7 boys) with body mass from 2740 g till 4410 g (Table 6.7.).

Table 6.7.

Study group G1 (term)

	Materna age (years)	Pregnacy (number)	Delivery (number)	Maternal BMI (kg/m^2) before pregnancy	Weeks of pregnancy	Gender of the neonate	Neonatal body mass (g)	Neonatal body length (cm)	1' Apgar score	5' Apgar score
1.	27	1	1	42.6	37	G*	3020	49	8	8
2.	37	3	3	20.4	37	G	3200	50	7	9
3.	25	2	2	23.8	37	G	3270	50	7	8
4.	27	2	2	26.7	38	B**	4290	57	8	9
5.	28	2	2	26.4	39	G	2740	50	7	8
6.	39	1	1	20.4	39	B	3230	53	8	9
7.	23	1	1	25.5	39	G	3540	52	7	8
8.	19	2	1	21.9	39	B	3670	52	8	9
9.	32	2	1	22.9	39	G	3810	55	8	9
10.	30	3	3	20.3	39	B	4000	54	8	9
11.	25	1	1	26.0	40	B	3250	54	7	8
12.	21	1	1	20.9	40	G	3670	54	6	8
13.	21	1	1	20.4	40	B	4180	55	8	9
14.	37	4	2	30.5	39	B	4410	56	7	8

* G – girl
** B – boy

Study group **G2 (pre-term)** included 25 pre-term deliveries in 22-35 weeks of gestation with liveborn premature neonates (16 girls; 9 boys) with body mass from 540 g till 2580 g (Table 6.8.).

Table 6.8.

Study group G2 (pre-term)

	Materna age (years)	Pregnacy (number)	Delivery (number)	Maternal BMI (kg/m^2) before pregnancy	Weeks of pregnancy	Gender of the neonate	Neonatal body mass (g)	Neonatal body length (cm)	1' Apgar score	5' Apgar score
1.	20	1	1	24.6	22	G*	540	30	6	6
2.	36	2	2	21.0	23	Z**	650	28	1	2
3.	30	4	2	20.7	24	G	720	32	3	5
4.	18	1	1	28.0	28	G	1130	36	4	6
5.	34	6	3	32.3	28	G	1190	38	6	7
6.	34	1	1	21.0	28	G	1290	37	7	8
7.	37	2	2	25.6	28	G	1410	41	7	7
8.	34	2	2	23.8	29	B	1476	39	7	7
9.	32	4	4	22.9	30	G	1580	41	5	7
10.	36	2	2	22.9	30	B	1710	42	7	8
11.	25	2	2	19.7	30	B	1750	44	6	7
12.	29	4	1	19.9	31	G	1370	38	7	7
13.	28	4	1	20.0	31	G	1780	45	7	7
14.	34	2	2	19.4	31	B	1888	40	7	7
15.	35	1	1	36.6	31	B	2060	46	7	8
16.	23	1	1	19.6	32	B	1940	46	7	7
17.	30	7	6	24.4	32	G	2258	49	7	7
18.	35	4	3	26.8	33	G	1948	46	7	7
19.	21	1	1	18.7	33	G	1992	45	7	7
20.	37	4	2	29.8	33	B	2300	45	7	8
21.	27	2	1	18.6	34	G	2310	48	7	8
22.	29	3	2	26.6	34	G	2320	42	7	8
23.	29	3	2	26.6	34	G	2390	47	7	7
24.	27	2	1	19.9	34	B	2450	48	7	8
25.	29	2	1	30.1	34	G	2580	44	7	7

* G – girl

** B – boy

Study group **G3 (distress)** included 14 term and pre-term deliveries in 23-40 weeks of pregnancy with liveborn or stillborn neonates (7 girls; 7 boys) with body mass from 905 g till 4630 g (Table 6.9.).

Table 6.9.

Study group G3 (distress)

	Materna age (years)	Pregnacy (number)	Delivery (number)	Maternal BMI (kg/m^2) before pregnancy	Weeks of pregnancy	Gender of the neonate	Neonatal body mass (g)	Neonatal body length (cm)	1' Apgar score	5' Apgar score
1.	28	1	1	19.9	23	B*	905	34	0	0
2.	32	6	4	22.9	28	B	1090	35	6	7
3.	36	2	2	21.0	28	G**	1360	41	6	7
4.	25	1	1	19.7	29	G	1114	40	6	7
5.	28	4	1	19.9	30	B	1860	41	7	7
6.	34	6	2	19.4	31	B	1896	44	7	8
7.	36	2	2	22.9	32	G	1088	38	7	7
8.	35	2	2	36.6	33	G	1888	43	7	7
9.	34	1	1	23.8	35	B	1920	45	7	7
10.	37	3	3	25.6	37	G	2770	49	0	0
11.	23	5	2	19.6	38	G	2786	54	0	0
12.	34	4	3	21.0	38	B	4510	55	0	2
13.	21	1	1	18.7	40	B	4330	56	1	2
14.	18	3	1	28.0	40	G	4630	59	0	0

* B – boy
** G – girl

6.3. Placental parameters

Placental weight was 220 g – 930 g with a mean value of 448.95 ± 157.503 g, mean values of the placental weight in the study groups are shown in the Table 6.10.

Table 6.10.

Placental weight in the study groups

Study group	Range (g)	Mean (g)
G1 (terms)	480-750	663.33 ± 102.632
G2 (pre-term)	220-510	421.10 ± 189.968
G3 (distress)	240-930	516.07 ± 216.157

Placental weights of the study group G2 (pre-term) was significantly lower than those one of the study group G1 (term) (Table 6.11.). Similar difference was found between term and pre-term placentas of the whole study.

Table 6.11.

Differences between placental weight in the study groups

		Mean	SD	Std. Error Mean	p*
Placental weight	G1 (term)	663.33	102.63	59.255	0.007**
	G2 (pre-term)	403.39	145.39	30.432	

* *Sig. (t-test for Equality of Means)*
** Statistically significant strong correlation ($p < 0.01$)

In the whole study appeared statistcally significant positive correlations between the placentas weight and the following **maternal** parameters (Table 6.12.): body mass before pregnancy, gestation (also in the study group G2 pre-term) as well as increase of the body mass during pregnancy (also in term cases of the whole study).

Table 6.12.

Correlations of the placental mass with maternal parameters

Maternal parameter		Placental mass
Gestation	Pearson corr.	0.537**
	Sig. (2-tailed)	0.001
Body mass before pregnancy	Pearson corr.	0.342*
	Sig. (2-tailed)	0.036
Increase of body mass in pregnancy	Pearson corr.	0.342*
	Sig. (2-tailed)	0.036

* Statistically significant correlation ($p < 0.05$)
** Statistically significant strong correlation ($p < 0.01$)

In the whole study placental masses presented statistically significant positive correlation with all the main anthropometrical parameters of the neonates (Table 6.13.).

Table 6.13.

Correlations of the placental mass with neonatal parameters

Neonatal parameter		Placental mass
All study and study group G2 (pre-term)		
Body mass	Pearson corr.	0.749**
	Sig. (2-tailed)	0.000
Body length	Pearson corr.	0.692**
	Sig. (2-tailed)	0.044

Tabele 6.13. continued p.23

Table 6.13. (End)

Neonatal parameter		Placental mass
Head circumference	Pearson corr. Sig. (2-tailed)	0.725** 0.000
Chest circumference	Pearson corr. Sig. (2-tailed)	0.788* 0.000
Study group G1 (term)		
Chest circumference	Pearson corr. Sig. (2-tailed)	0.801* 0.030
Ponderal index	Pearson corr. Sig. (2-tailed)	- 0.975** 0.001
Study group G3 (distress)		
Ponderal index	Pearson corr. Sig. (2-tailed)	0.554* 0.040

* Statistically significant correlation ($p < 0.05$)
** Statistically significant strong correlation ($p < 0.01$)

Similar correlations were seen in the study group G2 (pre-term) and pre-term placentas of the whole study; in the study group G1 (term) appeared positive correlations of the placentas messes with neonatal chest circumferences and negative with their ponderal indices. In the study group G3 (distress) appeared a positive correlation between placentasl masses ans ponderal indices of neonates.

6.4. Routine microscopy of placental preparations

Staining with hematoxylin and eosin (HE) revealed differences in morphological maturity of placental preparations: second trimester placentas were young (Figure 6.1.), placentas of early third trimester transitional (Figure 6.2.) and those of late third trimester presented signs of ageing (Figure 6.3.).

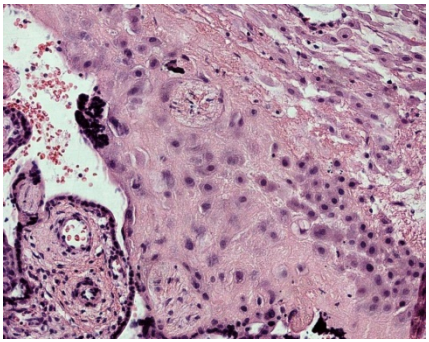

Figure 6.1. **Maternal part and tertiary villi of a young 23 weeks placenta**
HE, X 250

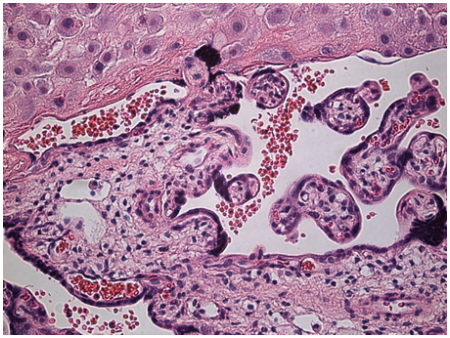

Figure 6.2**. Maternal part and teriary villi of a transitional 28 weeks placenta**
HE, X 250

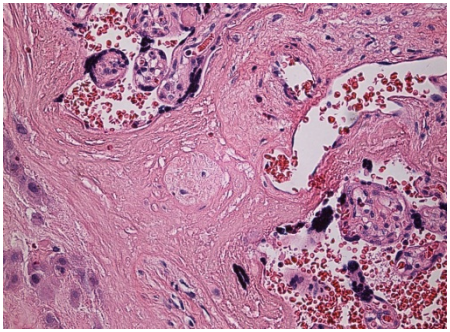

Figure 6.3**. Maternal part and tertiary villi of a 40 weeks placenta; signs of ageing**
HE, X 250

6.5. IHC research of placental samples

Growth factors and receptors: IGF1, IGFR1, HGF, bFGF un FGFR1

Amount of **IGF1** positive cells in the placental tissues did not correlate with their gestational ages and were found from 0 (none) till abundance (++++) in a visual field (Figures 6.4. and 6.5.). By the means of the modified competition ranking method (Pozzi, 2008) were estimated ranks with further calculation of the mean rank values of the findings (Table 6.14.).

Table 6.14.

The rank values of IGF1 positive cells in different divisions of the study

	Group	Mean ± SD
1.	The whole study	1.58 ± 0.86
2.	Study group G1 (healthy term)	1.32 ± 0.75
3.	Study group G2 (normal pre-term)	1.74 ± 0.99
4.	Study group G3 (distress)	1.54 ± 0.69
5.	Term placentas of the whole study	1.40 ± 0.70
6.	Pre-term placentas of the whole study	1.68 ± 0.94

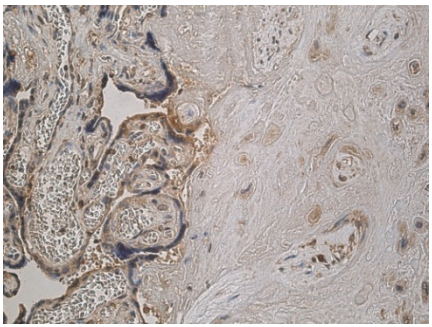

Figure 6.4**. Few (+) IGF1 positive cells in a 40 gestational weeks placenta**
IGF1 IHC, X 250

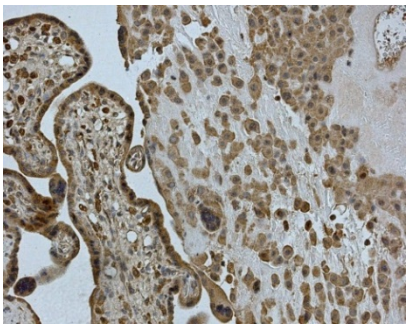

Figure 6.5. **Abundance (++++) of IGF1 positive cells in a 22 weeks placenta**
IGF1 IHC, X 250

Amount of **IGFR1** positive cells significantly decreased with ongoing pregnancy; they were in almost all placentas from 0 (none) till abundance

(++++) (Figures 6.6. and 6.7.). By the means of the modified competition ranking method (Pozzi, 2008) were estimated ranks with further calculation of the mean rank values of the findings (Table 6.15.).

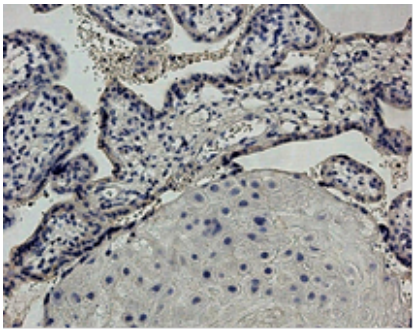

Figure 6.6. **Occasional (0/+) IGFR1 positive cells in a 23 weeks placenta**
IGFR1 IHC, X 250

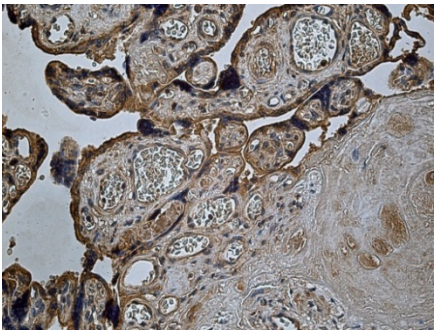

Figure 6.7**. Numerous (+++) IGFR1 positive cells in a 40 weeks placenta**
IGFR1 IHC, X 250

Table 6.15.

The rank values of IGFR1 positive cells in different divisions of the study

	Group	Mean ± SD
1.	The whole study	2.06 ± 1.12
2.	Study group G1 (healthy term)	1.25 ± 0.83
3.	Study group G2 (normal pre-term)	2.38 ± 1.24
4.	Study group G3 (distress)	2.29 ± 0.73
5.	Term placentas of the whole study	1.55 ± 0.91
6.	Pre-term placentas of the whole study	2.34 ± 1.14

In pre-term placentas appeared statistically significant higher rank values of IGFR1 positive cells in comparison with term placentas (Table 6.16.).

Table 6.16.

Differences of the rank values of IGFR1 positive cells in the study groups

	Mean	SD	Std. Error Mean	p*
G1 (term) G2 (pre-term)	1.250 2.380	0.8263 1.2440	0.2208 0.2488	0.002**
G1 (term) G3 (distress)	1.250 2.286	0.8263 0.7263	0.2208 0.1941	0.002**

Tabele 6.16. continued p.27

Table 6.16. (End)

	Mean	SD	Std. Error Mean	p*
Term of the whole study	1.553	0.9113	0.2091	0.009**
Pre-term of the whole study	2.338	1.1396	0.1954	

* *Sig. (t-test for Equality of Means)*
** Statistically significant strong correlation ($p < 0.01$)

Appeared a statistically significant difference between placentas of SGA and LGA neonates (Table 6.17.).

Table 6.17.

Differences between rank values of IGFR1 positive cells in *AGA* and *LGA* placentas

Parameter		N	Mean	SD	Std. Error Mean	p*
IGFR1	*SGA*	5	16.00	5.477	2.449	0.024*
	LGA	9	26.67	10.00	3.333	

* *Sig. (t-test for Equality of Means)*
** Statistically significant correlation ($p < 0.05$)

Amount of **HGF** positive cells in placentas did not correlate with their gestational ages and were from 0 (none) till abundance (++++) in a visual field (Figures 6.8. and 6.9.). By the means of the modified competition ranking method (Pozzi, 2008) were estimated ranks with further calculation of the mean rank values of the findings (Table 6.18.).

Table 6.18.

The rank values of HGF positive cells in different divisions of the study

	Group	Mean ± SD
1.	The whole study	1.61 ± 0.94
2.	Study group G1 (healthy term)	1.17 ± 0.80
3.	Study group G2 (normal pre-term)	1.74 ± 0.98
4.	Study group G3 (distress)	1.82 ± 0.91
5.	Term placentas of the whole study	1.45 ± 0.91
6.	Pre-term placentas of the whole study	1.62 ± 0.97

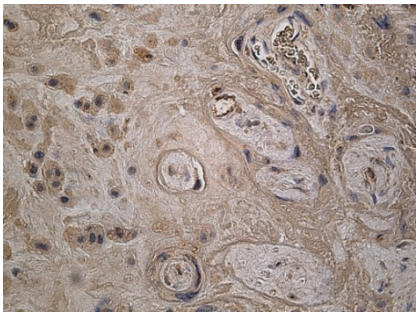

Figure 6.8**. Moderate amount (++) of HGF positive cells in a 32 weeks placenta**
HGF IHC, X 250

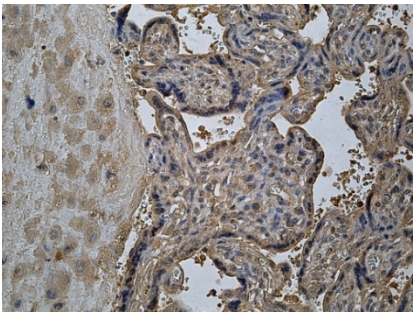

Figure 6.9**. Abundance (++++) of HGF positive cells in a 29 weeks placenta**
HGF IHC, X 250

Basic FGF (**bFGF**) presented weak immunoreactivity and was visually identified only in few preparations (Figures 6.10. and 6.11.), therefore was excluded from further research.

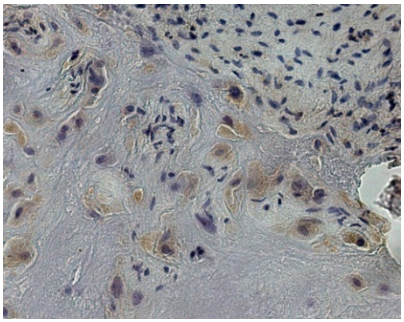

Figure 6.10. **Moderate amount (++) of bFGF positive cells in a 40 weeks placenta**
bFGF IHC, X 250

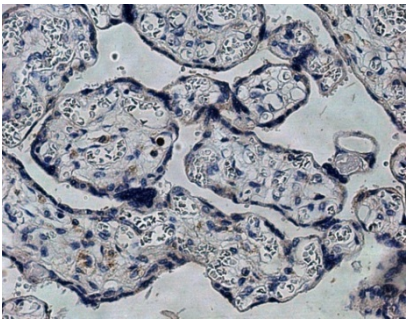

Figure 6.11. **Occasional (0/+) bFGF positive cells in a 38 weeks placenta**
bFGF IHC, X 250

The rank values of **FGFR1** positive cells in a visual field of a placenta did not correlate with its gestational age; they were found in all the placental samples from occasional (0/+) till abundance (++++) in a visual field (Figures

6.12. and 6.13.). By the means of the modified competition ranking method (Pozzi, 2008) were estimated ranks with further calculation of the mean rank values of the findings (Table 6.19.).

Table 6.19.

The rank values of GFGR1 positive cells in different divisions of the study

	Group	*Mean* ± *SD*
1.	The whole study	2.31 ± 0.95
2.	Study group G1 (healthy term)	2.46 ± 0.88
3.	Study group G2 (normal pre-term)	2.32 ± 1.02
4.	Study group G3 (distress)	2.18 ± 0.95
5.	Term placentas of the whole study	2.42 ± 0.97
6.	Pre-term placentas of the whole study	2.24 ± 0.98

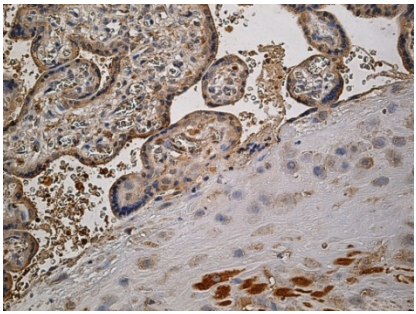

Figure 6.12. **Moderate amount (++) FGFR1 positive cells in a 31 weeks placenta** FGFR1 IHC, X 250

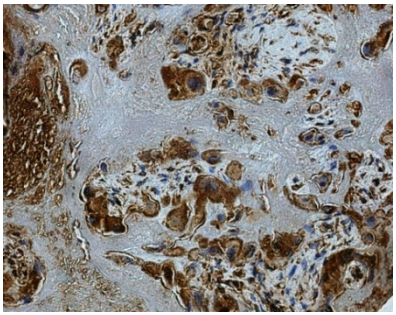

Figure 6.13. **Numerous (+++) FGFR1 positive cells in a 40 weeks placenta** FGFR1 IHC, X 250

Correlations with anthropological and clinical parameters in the study group G1 (term) revealed positive correlations of the rank values of HGF with maternal BMI prior to actual pregnancy and the rank values of FGFR1 showed negative correlations with maternal weight before actual pregnancy and neonatal glucose level (Table 6.20.). In the study group G2 (pre-term) the rank values of IGF1 positively correlated with the neonatal glucose and the rank values of IGFR1 – with the first neonatal blood pH. In the study group G3

(distress) the rank values of HGF presented negative correlations with the number of maternal pregnancies.

Table 6.20.

Correlations of the rank values of growth factors/receptors with anthropological and basic clinical parameters

Parameter		IGF1	IGFR1	HGF	FGFR1
Study group G1 (term)					
Maternal BMI prior to pregnancy	Pearson cor. Sig.	NS	NS	0.601* 0.023	NS
Maternal body mass prior to pregnancy	Pearson cor. Sig.	NS	NS	NS	-0.644* 0.017
Neonatal blood glucose	Pearson cor. Sig.	NS	NS	NS	-0.638* 0.026
Study group G2 (pre-term)					
Neonatal blood pH	Pearson cor. Sig.	NS	0.428* 0.033	NS	NS
Neonatal first blood glucose	Pearson cor. Sig.	0.444* 0.026	NS	NS	NS
Study group G3 (distress)					
Number of pregnancies	Pearson cor. Sig.	NS	NS	-0.538* 0.047	NS

* Statistically significant correlation ($p < 0.05$)
NS – Statistically non-significant correlation

Correlations between the rank values of researched growth factor/ receptor positive cells and other maternal or neonatal parameters were not statistically significant.

Cytokines: pro-inflammatory IL-1α, TNFα and anti-inflammatory IL-10

Amount of pro-inflammatory cytokine **IL-1α** positive cells was from 0 (none) till numerous (+++) in a visual field of placental preparation (Figures 6.14. and 6.15.); their amount did not correlate with the gestational age By the means of the modified competition ranking method (Pozzi, 2008) were estimated ranks with further calculation of the mean rank values of the findings (Table 6.21.).

Table 6.21.

The rank values of IL-1α positive cells in different divisions of the study

	Group	Mean ± SD
1.	The whole study	0.811 ± 0.7353
2.	Study group G1 (healthy term)	1.75 ± 0.85
3.	Study group G2 (normal pre-term)	1.40 ± 0.88
4.	Study group G3 (distress)	1.39 ± 0.79
5.	Term placentas of the whole study	1.53 ± 0.89
6.	Pre-term placentas of the whole study	1.47 ± 0.83

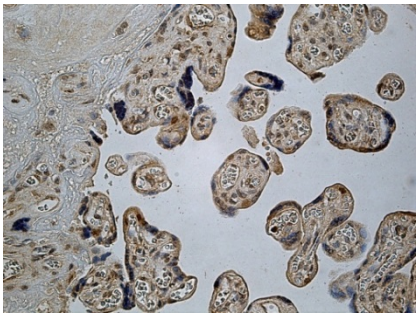

Figure 6.14. **Few (+) IL-1α positive cells in a 39 weeks placenta**
IL-1α IHC, X 250

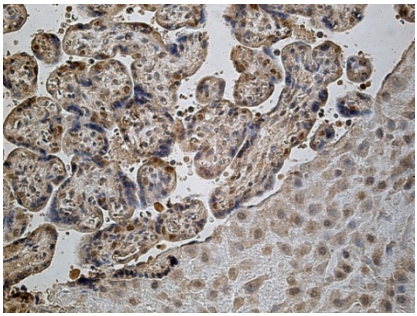

Figure 6.15. **Numerous (+++) IL-1α positive cells in a 31 weeks placenta**
IL-1α IHC, X 250

Amount of pro-inflammatory cytokine **TNFα** positive Höfbauer cells were from 0 (none) till numerous (+++) in a visual field (Figures 6.16. and 6.17.). By the means of the modified competition ranking method (Pozzi, 2008) were estimated ranks with further calculation of the mean rank values of the findings (Table 6.22.).

Table 6.22

The rank values of TNFα positive cells in different divisions of the study

	Group	Mean ± SD
1.	The whole study	2.311 ± 0.9468
2.	Study group G1 (healthy term)	1.18 ± 0.96
3.	Study group G2 (normal pre-term)	1.06 ± 0.76
4.	Study group G3 (distress)	0.71 ± 0.51
5.	Term placentas of the whole study	1.09 ± 0.88
6.	Pre-term placentas of the whole study	0.94 ± 0.69

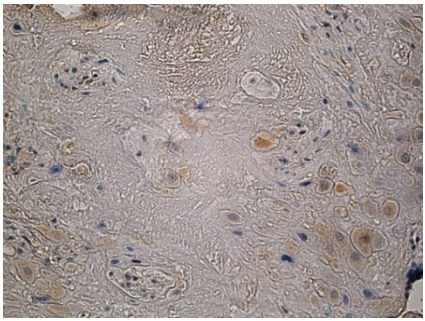

Figure 6.16. **Few (+) TNFα positive cells in a 37 weeks placenta**
TNFα IHC, X 250

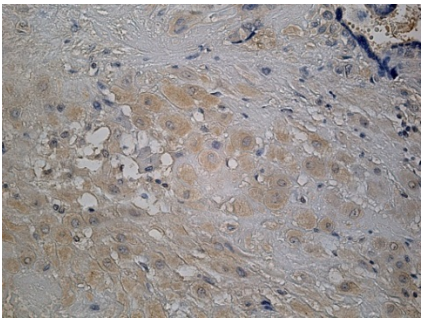

Figure 6.17. **Numerous (+++) TNFα positive cells in a 34 weeks placenta**
TNFα IHC, X 250

Amount of anti-inflammatory cytokine **IL-10** positive cells were seen in all the preparations from few (+) till abundance (++++) in a visual field; their number decreased with advanced gestation (Figures 6.18. and 6.19.). By the means of the modified competition ranking method (Pozzi, 2008) were estimated ranks with further calculation of the mean rank values of the findings (Table 6.23.).

Table 6.23.

The rank values of IL-10 positive cells in different divisions of the study

	Group	Mean ± SD
1.	The whole study	3.019 ± 0.9505
2.	Study group G1 (healthy term)	2.71 ± 0.91
3.	Study group G2 (normal pre-term)	3.20 ± 1.00
4.	Study group G3 (distress)	3.00 ± 0.88
5.	Term placentas of the whole study	2.79 ± 0.98
6.	Pre-term placentas of the whole study	3.15 ± 0.93

Differences between the study groups were not statistically significant; appeared a statistically significant correlation between the rank values of IL-10 in appropriate for the gestational age (AGA) and large for the gestational age (LGA) neonate placentas (Table 6.24.).

Table 6.24.

Differences between the rank values of IL-10 positive cells in placentas of AGA and LGA neonates

Parametrs		N	Mean	SD	Std. Error Mean	p*
IL-10	*AGA*	39	28.72	10.047	1.609	0.024**
	LGA	9	34.44	5.270	1.757	

* Sig. (t-test for Equality of Means)
** Statistically significant correlation ($p < 0.05$)

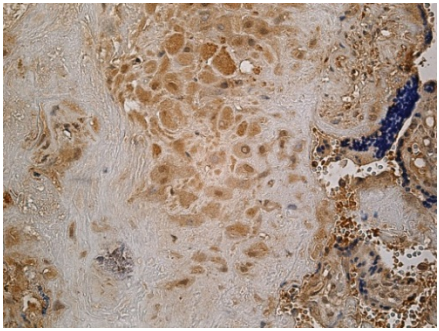

Figure 6.18. **Moderate amount (++) of IL-10 positive cells in a 34 weeks placenta**
IL-10 IHC, X 250

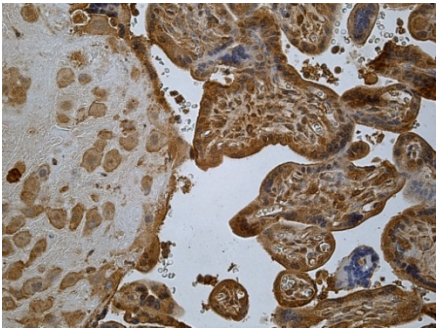

Figure 6.19. **Abundance (++++) of IL-10 positive cells in a 22 weeks placenta**
IL-10 IHC, X 250

In the **whole study** appeared a statistically significant positive correlation between the rank values of TNFα and IL-10 positive cells; similar relation was seen also in the study group G1 (term). In term placentas of the whole study and in the study group G1 (term) appeared a statistically significant positive correlation between the rank values of both pro-inflammatory cytokines (TNFα and IL-1α).

In the **study groups** appeared statistically significant correlations between the rank values of cytokines and anthropological/ clinical data (Table 6.25.): in the study group G1 (term) were found correlations between the rank values of a pro-inflammatory cytokine TNFα and maternal age, numbers of

pregnancies and deliveries. In the study group G2 (pre-term) rank values of IL-10 negatively correlated with the gestation, rank values of IL-1α presented positive correlations with ROM and negative – with initial blood pH and glucose level. In the study group G3 (distress) the rank values of IL-1α negatively correlated with gestation and TNFα with neonatal blood pH.

Table 6.25.

Correlations of the rank values of cytokines with maternal and neonatal data

Parameter		IL-10	IL-1α	TNFα
Study group G1 (term)				
Maternal age	Pearson Correlation Sig. (2-tailed)	NS	NS	0.614* 0.044
Number of pregnancies	Pearson Correlation Sig. (2-tailed)	NS	NS	0.628* 0.039
Number of deliveries	Pearson Correlation Sig. (2-tailed)	NS	NS	0.622* 0.041
Study group G2 (pre-term)				
Gestation	Pearson Correlation Sig. (2-tailed)	-0.440* 0.028	NS	NS
ROM	Pearson Correlation Sig. (2-tailed)	NS	0.536** 0.008	NS
Neonatal blood pH	Pearson Correlation Sig. (2-tailed)	NS	-0.559** 0.004	NS
1' APGAR	Pearson Correlation Sig. (2-tailed)	NS	-0.526** 0.007	NS
Study group G3 (distress)				
Gestation	Pearson Correlation Sig. (2-tailed)	NS	-0.613* 0.020	NS
Neonatal blood pH	Pearson Correlation Sig. (2-tailed)	NS	NS	-0.855** 0.002
Neonatal blood glucose	Pearson Correlation Sig. (2-tailed)	NS	NS	0.768** 0.009

* Statistically significant correlation ($p < 0.05$)
** Statistically significant strong correlation ($p < 0.01$)
NS – Statistically non-significant correlation

Correlations between the rank values of researched cytokine positive cells and other maternal or neonatal parameters were not statistically significant.

Number of apoptotic cells in the placental tissues

The mean amount of apoptotic cells in the placental tissues was from 0.91 ± 0.83 till 75.91 ± 14.42 in a visual field, decreasing with advanced gestation (Figures 6.20. and 6.21.). The mean values of apoptotic cells in various sections of the study are shown in the Table 6.26.

Table 6.26.

The mean numbers of apoptotic cells in different divisions of the study

	Group	Mean ± SD
1.	The whole study	22.15 ± 17.82
2.	Study group G1 (healthy term)	11.85 ± 11.718
3.	Study group G2 (normal pre-term)	27.16 ± 17.899
4.	Study group G3 (distress)	22.79 ± 19.292
5.	Term placentas of the whole study	13.00 ± 13.052
6.	Pre-term placentas of the whole study	27.00 ± 18.246

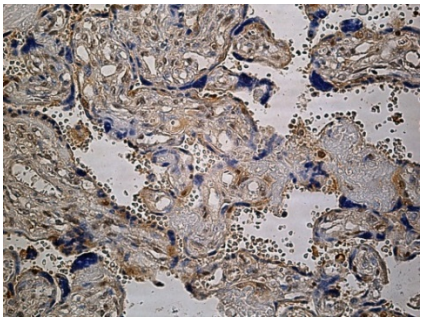

Figure 6.20. **8.0 ± 3.32 apoptotic cells in a 34 weeks placenta**
Caspase IHC, X 250

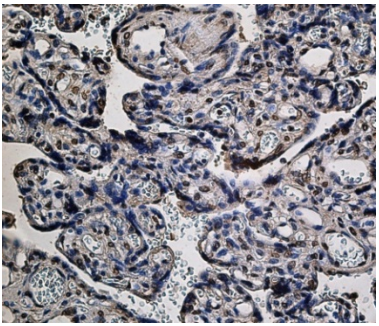

Figure 6.21. **43.91 ± 6.76 apoptotic cells in a 40 weeks placenta**
TUNEL, X 250

In the pre-term placentas apoptotic cells were significantly more (Table 6.27.).

Table 6.27.

Differences between the numbers of apoptotic cells

	Mean	SD	Std. Error Mean	p*
G1 (term)	11.85	11.718	3.250	0.003**
G2 (pre-term)	27.16	17.899	3.580	
Term of the whole study	13.00	13.052	3.076	0.003**
Pre-term of the whole study	27.00	18.246	3.129	

* Sig. (t-test for Equality of Means)
** Statistically significant strong correlation ($p < 0.01$)

Looking for correlations between the number of apoptotic cells in placentas and clinical findings appeared significantly higher amount of apoptotic cells in term placentas of mothers with smaller history of pregnancies; correlation was valid both in the study group G1 (term) and term placentas of the whole study (Table 6.28.).

Table 6.28.

Correlation of the number of apoptotic cells in placenta with clinical data

Parameter		Apoptosis
Study group G1 (term)		
Number of pregnancies	Pearson Correlation	-0.586*
	Sig. (2-tailed)	0.035

** Statistically significant correlation ($p < 0.05$)

Protein of the basement membrane Collagen IV

Collagen IV positive structures were found in almost all the preparations from 0 (none) till abundance (++++) in a visual field (Figures 6.22. and 6.23.); structures were more prominenet in term placentas. By the means of the modified competition ranking method (Pozzi, 2008) were estimated ranks with further calculation of the mean rank values of the findings (Table 6.29.).

Table 6.29.

The rank values of Collagen IV structures in different divisions of the study

	Group	Mean ± SD
1.	The whole study	3.019 ± 0.9505
2.	Study group G1 (healthy term)	2.71 ± 0.91
3.	Study group G2 (normal pre-term)	3.20 ± 1.00
4.	Study group G3 (distress)	3.00 ± 0.88
5.	Term placentas of the whole study	2.79 ± 0.98
6.	Pre-term placentas of the whole study	3.15 ± 0.93

Larger amount of Collagen IV structures in term placentas was statistically significant (Table 6.30.).

Table 6.30.

Differences of Collagen IV structures in term and pre-term placentas

	Mean	SD	Std. Error Mean	p*
Term of the whole study	2.14	1.28	3.020	0.040*
Pre-term of the whole study	1.39	1.16	2.015	

* Sig. (t-test for Equality of Means)
** Statistically significant correlation ($p < 0.05$)

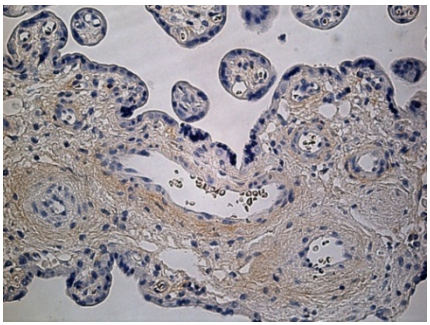

Figure 6.22. **Few (+) Collagen IV positive structures in a 23 weeks placenta**
Collagen IV IHC, X 250

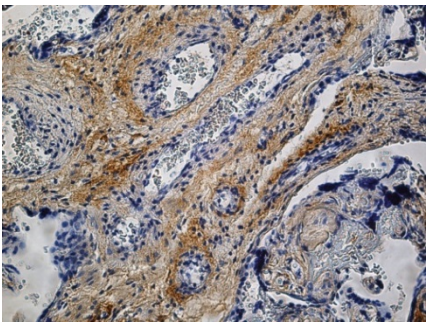

Figure 6.23**. Abundance (++++) of Collagen IV structures in a 40 weeks placenta**
Collagen IV IHC, X 250

Looking for correlations between the amount of Collagen IV positive structures and clinical findings appeared a statistically significant positive

correlation in term placentas of the whole study with maternal number of pregnancies and a negative correlation in pre-term placentas of the whole study with maternal weight before the actual pregnancy (Table 6.31.).

Table 6.31.

Correlation of Collagen IV rank values with clinical data

Parameter		Collagen IV
Term placentas of the whole study		
Number of pregnancies	Pearson Correlation	0.494*
	Sig. (2-tailed)	0.037
Pre-term placentas of the whole study		
Maternal body mass prior to pregnancy	Pearson Correlation	-0.363*
	Sig. (2-tailed)	0.038

* Statistically significant correlation ($p < 0.05$)

Tissue degrading enzyme MMP9

MMP9 positive cells were seen in almost all the placental preparations from 0 (none) till abundance (++++) in a visual field (Figures 6.24. and 6.25.); findings did not correlate with the gestational age. By the means of the modified competition ranking method (Pozzi, 2008) were estimated ranks with further calculation of the mean rank values of the findings (Table 6.32.).

Table 6.32.

The rank values of MMP9 positive cells in different divisions of the study

	Group	Mean ± SD
1.	The whole study	1.44 ± 1.20
2.	Study group G1 (healthy term)	0.86 ± 0.74
3.	Study group G2 (normal pre-term)	1.42 ± 1.12
4.	Study group G3 (distress)	2.07 ± 1.44
5.	Term placentas of the whole study	1.11 ± 1.14
6.	Pre-term placentas of the whole study	1.63 ± 1.21

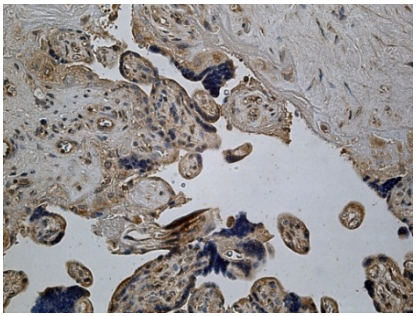

Figure 6.24. **Moderate amount (++) of MMP9 positive cells in a 33 weeks placenta**
MMP9 IHC, X 250

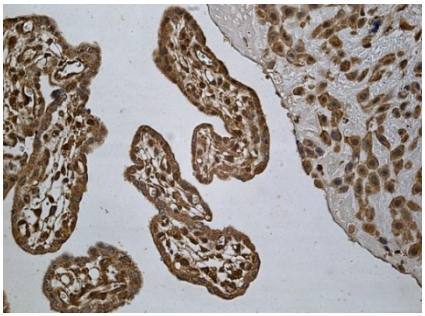

Figure 6.25. **Abundance (++++) of MMP9 positive cells in a 23 weeks placenta**
MMP9 IHC, X 250

Comparison of MMP9 rank values in study groups appeared a statistically significant difference between the study groups G1 (term) and G3 (distress) (Table 6.33.).

Table 6.33.

Differences of MMP9 rank values between the study groups

	Mean	SD	Std. Error Mean	p*
G1 (term)	0.857	0.745	0.199	0.009**
G3 (distress)	2.071	1.439	0.384	

* Sig. (t-test for Equality of Means)
** Statistically significant correlation ($p < 0.05$)

Looking for correlations between the rank values of MMP9 positive cells and clinical data appeared a few statistically significant ones: in the study group G1 (term) – positive correlations with neonatal body mass and chest circumference, in the study group G2 (pre-term) a positive correlations with neonatal blood pH and in the study group G3 (distress) – a negative correlation with neonatal blood glucose (Table 6.34.).

Table 6.34.

Correlations between the rank values of MMP9 and clinical data

Parameter		MMP9
Study group G1 (term)		
Neonatal body mass	Pearson Correlation Sig. (2-tailed)	0.622* 0.018
Chest circumference	Pearson Correlation Sig. (2-tailed)	0.550* 0.042
Study group G2 (pre-term)		
Neonatal blood pH	Pearson Correlation Sig. (2-tailed)	0.464* 0.020
Study group G3 (distress)		
Neonatal blood glucose	Pearson Correlation Sig. (2-tailed)	-0.713* 0.021

* Statistically significant correlation ($p < 0.05$)

Correlations between the rank value of MMP9 positive cells and other maternal or neonatal parameters were not statistically significant.

HoxB3 gene products

HoxB3 gene product positive cells were found in all the placental preparations from occasional (0/+) till numerous (+++) in a visual field (Figures 6.26. and 6.27.); their amount did not correlate with the gestational age. By the means of the modified competition ranking method (Pozzi, 2008) were estimated ranks with further calculation of the mean rank values of the findings (Table 6.35.).

Table 6.35.

The rank values of HoxB3 product positive cells in different divisions of the study

	Group	Mean ± SD
1.	The whole study	1.44 ± 1.20
2.	Study group G1 (healthy term)	1.25 ± 0.27
3.	Study group G2 (normal pre-term)	1.36 ± 0.71
4.	Study group G3 (distress)	1.39 ± 0.76
5.	Term placentas of the whole study	1.16 ± 0.71
6.	Pre-term placentas of the whole study	1.44 ± 0.72

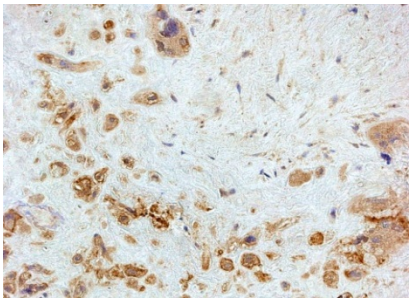

Figure 6.26. **Moderate amount (++) of HoxB3 product positive cells in a 32 weeks placenta**
HoxB3 IHC, X 250

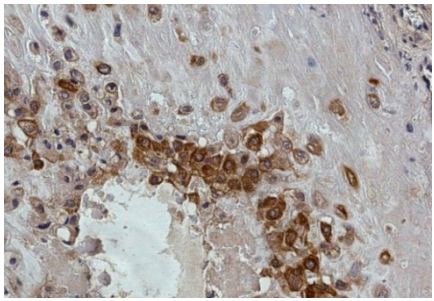

Figure 6.27. **Numerous (+++) HoxB3 product positive cells in a 40 weeks placenta**
HoxB3 IHC, X 250

Looking for correlations between the rank values of HoxB3 product positive cells and clinical data appeared a few statistically significant correlations with longitudinal measurements of maternal and fetal bodies (Table 6.36.).

Table 6.36.

Correlations between the rank values of HoxB3 product positive cells and anthropological data

Parameter		HoxB3 products
The whole study		
Ponderal index	Pearson Correlation	-0.323*
	Sig. (2-tailed)	0.018
G1 (laicīgas)		
Neonatal body length	Pearson Correlation	0.541*
	Sig. (2-tailed)	0.046
G3 (distresa)		
Maternal body height	Pearson Correlation	0.573*
	Sig. (2-tailed)	0.032
Visas priekšlaicīgu dzemdību placentas		
Ponderal index	Pearson Correlation	-0.461**
	Sig. (2-tailed)	0.008

* Statistically significant correlation ($p < 0,05$)
** Statistically significant strong correlation ($p < 0,01$)

Correlations between the rank value of HoxB3 positive cells and other maternal or neonatal parameters were not statistically significant.

7. DISCUSSION

7.1. Anthropology

Growth factors/ receptors: positive cells in general varied depending on the getstaional age although statistical significance characterised only decrease of **IGFR1** positive cells with advanced gestation. Our data suggested more significant role of the placental growth factors IGF1 and HGF before the term, having direct impact on the placental growth, therefore influencing fetal development. In the literature there are described some studies correlating gestation related expression of **IGF1** and having found its decrease with ongoing pregnancy (Kumar et al., 2006; Kumar et al., 2012), unchanged (Han et al., 1996) or even increase in the maternal blood serum (Tennekoon et al., 2007); neonatal blood also has shown higher level of IGF1 in older gestational age (Smith et al., 1997). Our findings suggest not just larger amount of IGF1 and IGFR1 positive cells in pre-term placentas, but their impact on viability and health status of a premature child. In the placentas of our studu group G2 (pre-term) as well as the pre-term placentas of the whole study the rank of IGFR1 positively correlated with the initial blood pH of the neonate, one of the basic witnesses of fetal homeostasis; similar correlations in the literature have not been described. Findings of positive correlations of the circulating level of IGF1 with the growth velocity of very low birth weight (VLBW) infants during 9 weeks of postnatal life (Kajantie et al., 2003) somehow coincide with our results showing positive correlations of the rank values of IGF1 in the placentas of the study group G2 (pre-term). Such a corelation was not found in all the pre-term placentas of the study (including distress ones) therefore we could suggest that a larger number of IGF1 positive cells can testify potentially more promissing health status of a premature neonate and therapeutical application of IGF1 in the treatment of extremely premature neonates is worth considering.

Although decrease of the rank value of **HGF** with advancing gestational age showed only weak significance ($0.05 \leq p \leq 0.1$), statistically significant positive correlations with the rank value of IGFR1 supported this tendency; other studies describe more prominent expression of HGF in placentas of the 2nd trimester in comparison with placentas of the 1st trimester (Somerset et al., 2000) or its decrease in the amniotic membranes with an advanced gestation and older maternal age (Lopez – Valladares et al., 2010). In our study the rank values of HGF did not correlate with the maternal age, but presented negative correlation with the numbers of pregnancies of the mothers, suggesting decreased capabilities of the maternal organism like it could be suggested in both conditions. In the study group G1 (term) the rank value of HGF positive cells in the placentas showed positive correlation with the maternal BMI before actual pregnancy, to some extent matching with studies, describing HGF as a driving force of compensatory hyperinsulinemia in obese or type 2 diabetes patients (Araújo et al., 2012) or pregnant women, who develop hyperglycemia due to pregnancy induced maternal insulin resistance (Ernst et al., 2011). We did not find correlations of the rank values of HGF with the blood glucose levels of the neonates; the described hyperinsulinemia was possibly induced by HGF in the maternal and not neonatal pancreas. **FGFR1** was the only factor of our researched growth factors and receptors, whose rank value in the term placentas was higher than in the pre-term ones coinciding with studies, having stated FGF and FGFR strongly contributing to the growth and development of the placenta and fetus (Marzioni et al., 2005), pronounced at the end of the third trimester.

Cytokines: most of the studies researching pro-inflammatory cytokines in the maternal-fetal unit are focusing on the pre-term parturition and pre-term premature rupture of membranes (PPROM) (Huleihel et al., 2004; Holcberg et al.. 2008) and we expected high amount of **IL-1α** and **TNFα** positive cells in

extremely pre-term placentas; our assumption was not supported, suggesting non-infectious causes of pre-term deliveries in our cases. Only in the study group G3 (distress) rank values of placental IL-1α showed negative correlation with the gestational age, coinciding with studies, offering detection of IL-1 for an indicator of inflammation and termination of pregnancy due to threat to fetal health (Girard et al., 2010; Girard et al., 2012). In pre-term placentas we found significant reduction of the rank value of an anti-inflammatory cytokine **IL-10** with advanced gestation, supported by negative correlations with anthropometrical parameters of the neonates and suggesting decrease of actuality of maternal immunological tolerance against fetus with advanced gestation; other studies have not related IL-10 in placental tissues with gestation (Dembinski et al., 2003).

Apoptosis: programmed cellular death is a process that is present in placenta throughout the pregnancy, providing turnover of its cells. We found more apoptotic cells in the pre-term placentas of the study group G2 (healthy pre-term) in comparison with the term ones in the study group G1 (healthy term). Our data were somehow controversial with other studies, having described increased cellular apoptosis with the course of pregnancy (Smith et al., 1997); their suggestions were based on a comparison of apoptotic cells in the third trimester with the first trimester while we researched placentas from the end of the second trimester till term; līdz iznēsātībai; possibly in full-term placentas changing the balance between apoptotic and necrotic paths of cellular death is changing towards necrosis (Huppertz et al., 2003; Huppertz et al., 2004). Additionally in our study numbers of apoptotic cells positively correlated with HGF rank values, suggesting regenerative capacity of those placentas.

Collagen IV: amount of this basement membrane constituent positive structures in placental tissues increased with gestational age, matching with

suggestions in the literature regarding indicative role of Collagen IV in fetal maturation (Papadopuolos et al., 2001); in our study group G3 (distress) the rank values of Collagen IV positive structures showed positive correlations with neonatal body mass and chest circumference, suggesting undetected prolongation of pregnancy as the promoter of fetal distress. Our results showing increased amount of Collagen IV positive structures in placentas of women with more pregnancies somehow coincide with findings in the other studies, having described more structures of Collagen IV in placentas of less favourable courses of pregnancies: inflammation (Kumar et al., 2006) or hypoxia (Chen and Aplin, 2003; Chen et al., 2005), including due to maternal smoking (Jalali et al., 2010).

Tissue degrading enzyme MMP9 in our study did not show significant correlations with the gestational age, mode of delivery or time of ROM; other studies describe more prominent expression of MMP9 in cases of pre-term spontaneous vaginal deliveries (Xu et al., 2002; Sundrani et al., 2012). We suggest manifestation of MMP9 in maternal-fetal unit with expression in certain placental cells, coinciding with some other studies (Demir-Weusten et al., 2007). In our study group G1 (term) the rank values of placental MMP9 positively correlated with neonatal anthropometrical values, suggesting role of MMP9 in the initiation of term delivery.

7.2. Appropriateness for the gestation

In our study we found a significantly higher mean rank value of **IGFR1** in LGA placentas in comparison with SGA ones; finding coincides with studies, having found higher expression of IGFR1 in the placentas of macrosomic neonates (Jiang et al., 2009) or higher concentration of IGF1 in the cord blood of LGA neonates (Akcakus et al., 2006) and also with the studies

having found lower expression of IGF1 in the placentas (Regnault et al., 2005; Koutsaki et al., 2010) or in the blood serum (Orbak et al.,2001; Akcakus et al., 2006; Lee et al., 2010) of SGA neonates. In the literature have been described opposite results with higher IGF1 expression in the placentas of SGA neonates in comparison with the placentas of AGA or LGA ones (Iniguez et al., 2010; Ahram et al., 2011) as well as negative correlations of IGF1 in the cord blood with the birth weight (Pathmaperuma et al., 2007). IGF1 and IGFR1 both in our study and majority of literature sources play a significant positive role in fetal growth. Placentas of LGA neonates presented significantly higher rank values of **IL-10** than placentas of SGA or AGA neonates; this finding agrees with studies having found lower expression of IL-10 in placentas of SGA neonates (Hahn-Zoric et al., 2002); other authors propose deficiency of IL-10 to be causative for IUGR (Raghupathy et al., 2012). Although the other studies have found increase of apoptotic cells in placentas of SGA neonates (Athapathu et al., 2003; Vogt Isaksen et al., 2004; Roje et al., 2011; Longtine et al., 2012), we did not find such correlations; negative correlations between the numbers of apoptotic cells in placental tissues with neonatal anthropometrical parameters in our study is probably associated with gestation.

7.3 Morphology

Our study disclosed several statistically significant correlations between the rank values of different factors. Rank values of **IGF1** and **IGFR1** in pre-term placentas showed positive correlations with the rank values of IL-10 suggesting the role of maternal immune tolerance not just in fetal allograft, but in fetal growth and development. Such correlation was not found in term placentas, suggesting decrease of the significance of the maternal immune tolerance at term; in literature similar correlations have not been described. In

our study group G2 (pre-term) rank values of IGF1 presented negative correlations with the rank values of pro-inflammatory cytokine IL-1α, to a certain extent supporting described negative impact of pro-inflammatory cytokines on IGF axis (Hsiao et al., 2011), while the rank values of another pro-inflammatory cytokine of our study TNFα positively correlated in pre-term placentas with the rank values of IGFR1 and in term placentas with the rank values of IGF1, suggesting specific impact of placental pro-inflammatory cytokines on fetal growth. In the literature there is described increased level of TNFα in children with growth hormone deficiency without correlations with level of IGF1 (Andiran and Yordam, 2007); findings are somehow controversial to our results. In pre-term placentas appeared statistically significant positive correlations of the rank values of **HGF** with the rank values of TNFα and the mean amount of apoptotic cells in a visual field, somehow coinciding with the role of HGF in tissue regeneration (Matsumoto et al., 2001; Nayeri et al., 2002; Urbanek et al., 2005; Mizuno et al., 2008; Dai et al., 2010) and influence on inflammatory processes (Nakamura et al., 2011). Our findings do not match to studies, having found HGF to inhibit apoptosis in pathological pregnancies (Dash et al., 2005). Regenerative function of HGF in our study is supported by positive correlations of its rank values with the rank values of Collagen IV structures in term placentas; lack of HGF probably indicates decompensation of placental regenerative capabilities. One of pregnancy success factors is balance of pro- and anti-inflammatory cytokines in the maternal-fetal unit (Bowen et al., 2002; Szukiewich, 2012). In our study statistically significant correlations appeared only in term placentas: in term placentas of the whole study (including term distress) **TNFα** positively correlated only with **IL-1α** while in the study group G1 (term) besides this one appeared another positive correlation between TNFα and **IL-10**. Simultaneous increase of IL-10 and TNFα in cases of infectious stimuli (Barrera et al., 2012;

Mitchell et al., 2012) can possibly be referable to our findings in G1 (term) as increase of TNFα and IL-10 during microbial colonization at delivery. In our study group G3 (distress) was found a statistically significant positive correlation between the rank values of IL-10 and the rank values of tissue degrading enzyme MMP9; in the literature there are *in vitro* data, describing IL-10 to decrease MMP2 and MMP9 and suggesting its therapeutic application to arrest pre-term delivery (Fortunato et al., 2001). Taking into account that immediate termination of pregnancies were the best tactics for particular clinical situations, results should not be percieved as controversial, especially as in those placentas the rank values of IL-10 positively correlated also with the rank values of **Collagen IV**, suggesting discrepancy of maintenence and interruption processes of pregnancy. Looking for correlations between the rank values of Collagen IV and other molecular factors in the study group G1 (term) we found positive correlations with the rank values of HGF and IGFR1, while in the study groups G2 (pre-term) and G3 (distress) appeared positive correlations with the rank values of FGFR1, suggesting presence of Collagen IV in term placentas indicating placental growth and regeneration while in pre-term and distress placentas – with fibrosis and possible decrease of functionality. Data agree with described placental fibrosis in cases of increased expression of Collagen IV (Chen and Aplin, 2003; Chen et al., 2005). Statistically significant positive correlation between the rank values of MMP9 and IL-10 suggests the role of MMP9 in the initiation of labor in cases with sustaining maternal tolerance against fetal allograft, coinciding studies on the role of MMP9 in initiation of pre-term labor (Romero et al., 2002; Weiss et al., 2007; Karthikeyan et al., 2012). We recognized MMP9 as a significant molecular factor of the maternal-fetal unit, playing a significant role in the initiation of labor to achieve better perinatal result. Rank values of the products of a homeobox gene **HoxB3** in the study group G1 (term) significantly

correlated with the rank values of IGFR1 and Collagen IV; in the study group G3 (distress) they showed positive correlations with the rank values of MMP9, allowing us to assume role of HoxB3 in sustaining of tissue integrity. Such correlations have not been described in the literature; on the other hand we did not find described negative correlations between Hox gene products and pro-inflammatory cytokines (Sarno et al., 2006; Sarno et al., 2009). To our mind role of Hox genes and presence of their products in the maternal-fetal unit is a worthwhile subject for further research, possibly disclosing view of fetal growth.

7.4. Clinical data

In the study group G1 (term) and in the whole study were found negative correlations between the rank values of **FGFR1** and neonatal blood glucose. The study group G1 (term) included normal term deliveries with healthy children and no hypoglycaemia, therefore finding rather agree with studies, that have found more pronounced expression of FGFR in placentas of macrosomic neonates of diabetic mothers (Grissa et al., 2010) and suggest possible cases of undetected glucose intolerance. In the study group G2 (pre-term) we found positive correlations between the rank values of pro-inflammatory cytokine **IL-1α** and ROM; the rank values of **TNFα** did not show such correlations, supporting our suggestion regarding specific reactions of pro-inflammatory cytokines in placental tissues. Rank values of a pro-inflammatory cytokine IL-1α in pre-term placentas showed significant negative correlation with neonatal pH and 1st minute Apgar score coinciding with studies, having found increase of IL-1 and IL-6 in maternal and fetal blood in acidotic circumstances (Prout et al., 2010). In the study group G3 (distress) appeared statistically significant negative correlation of the rank values of TNFα and

neonatal blood pH, possibly coinciding studies, having found higher level of TNFα in children with problems of neurologic and motoric development (Carpentier et al., 2011). Our results suggest gestation related pathways of inflammation. In the study group G3 (distress) we found a significant positive correlation of the rank values of placental TNFα and neonatal blood glucose, matching results of other studies, having found increased production of TNFα in placentas of patients with a higher blood glucose (Moreli et al., 2012) and possibly indicate undiagnosed glucose intolerance in pregnancy. The highest mean rank value of a tissue degrading enzyme **MMP9** appeared in the study group G3 (distress); difference with the mean rank value of the study group G1 (term) was statistically significant. Assessed and not found deviations of MMP9 expression in vitro in cases of oxygen deprivation (Merchant et al., 2004) is not controversial to our results and should be undertaken as an affirmation of compensatory potential of the placenta. Evaluating correlations of the rank values of HoxB3 products with clinical parameters in the study group G3 (distress) we found a positive correlation with maternal body height, that could be undertaken as a coincidence if having been find alone; previously described correlations with neonatal anthropometrical parameters suggest substantial impact. These data are unique and indicate factors of fetal longitudinal growth and not just impact of Hox genes on placental growth and development (Zhang et al., 2002; Amesse et al., 2003).

8. CONCLUSIONS

1. Patients of pre-term deliveries previously had more pregnancies (not deliveries), suggesting termination of pregnancy as a risk factor for consecutive pre-term delivery; maternal anthropological and social parameters did not differ. Weight of a pre-term placenta directly proportional to the gestational age and positively correlate with neonatal anthrolometrical data; term placentas do not show such correlations. The main neonatal anthropometrical parameters are directly proportional to the gestational age.
2. Growth factor and receptor IGF1, IGFR1, HGF and FGFR1 positive cells are seen in almost all the placentas from 22 weeks of pregnancy till term, indicating their permanent growth potential. Decrease of IGF1, IGFR1 and HGF positive cells with advanced gestation seems to be related to placental maturation/ ageing. Amount of FGFR1 positive cells increase with advanced gestation, indicating placental maturity, especially close to term.
3. Dissimilar correlations of the rank values of IL-1α and TNFα show placental capability to produce pro-inflammatory cytokines selectively, depending on the process and gestation. Decrease of IL-10 positive cells in placentas of ongoing pregnancy emphasize significance of maternal immune tolerance in late second trimester and early third trimester.
4. Apoptosis is seen in placentas of various gestational ages and decreases with ongoing pregnancy, suggesting switch to another way of cellular disposal. Smaller amount of apoptotic cells was seen in patients with histories of more pregnancies, indicating restricted capabilities of those placentas to provide non-destructive disposal of cells.

5. Component of the basement membrane Collagen IV is seen in placentas of various gestational ages with a significant increase towards term, suggesting development of placental barrier.
6. Tissue degrading enzyme MMP9 positive cells are seen in placentas of various gestational ages; their significant increase in distress circumstances indicate possible role of placental MMP9 in natural termination of pregnancy in different terms.
7. Homeobox gene HoxB3 product positive cells are seen in all the placentas from 22 weeks till term; their amount does not correlate with the gestational age, but with some neonatal anthropometrical parameters, suggesting their role in the fetal longitudinal growth.
8. Normal term placentas regularly present smaller amount of IGF1, IGFR1 and HGF positive cells and larger amount of FGFR1 positive cells. More advanced maternal age, number of pregnancies and deliveries are associated with an increased amount of TNFα positive cells, suggesting idea of the risk for ascending infections in those patients. Smaller number of apoptotic cells in placentas of patients with larger number of pregnancies suggest decrease of programming capabilities in consecutive pregnancies. Increase of MMP9 positive cells in placentas of neonates with larger body masses and chest circumferences indicate increeased tissue strength of those placentas.
9. Pre-term placentas are characterized by a higher amount of IGF1 and IGFR1 positive cells, indicating significant growth capabilities of those placentas. Correlations with the rank values of IL-1α describe their susceptibility to infectious agents; larger number of apoptotic cells suggest active remodeling processes in this gestational time.
10. Placentas after significant fetal distress are characterized by imbalance of pro- and anti-inflammatory cytokines, possibly having led to impairment

of placental and fetal growth and decrease of placental defence. Positive correlations of the rank values of HGF positive cells with FGFR1 suggest placental fibrosis in regeneration processes. Large amount of tissue degrading enzyme MMP9 positive cells in distress placentas suggest its role in natural triggering of labor in circumstances of fetal threat, possibly influencing fetal well-being.

9. REFERENCES

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