Rīgas Stradiņa universitāte

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

Doctoral Thesis

Speciality – Neonatology

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ANNOTATION

The aim of the research was assessment of molecular events in the third trimester placentas of different gestational ages and clinical scenarious, based on immunohistochemical findings in the placental tissues. As the appearance of various factors correlate placental tissue changes *in situ* with gestational age, anthropological, morphological and basic clinical parameters, they are suggested to be original. In the samples from 53 post-delivery placentas of 22 till 40 weeks of gestation were evaluated amounts of cells or extracellular structures, containing the following factors of the molecular processes: growth factors and their receptors IGF1, IGFR1, HGF, bFGF and FGFR1, cytokines IL-10, IL-1α, IL-6 and TNFα, proteins of the basement membrane Collagen IV and Laminin, tissue degrading enzymes MMP2 and MMP9 and products of the homeobox gene HoxB3. Findings were evaluated semi-quantitively and ranked in the ascending order by the modified competition ranking method. Rank values were statistically correlated among themselves, as well as with the gestational age and weight of placenta, maternal and neonatal anthropological data and basic clinical parameters. Visually detectable amounts of bFGF, IL-6 and MMP2 positive cells and Laminin containing structures were not found; all the other factor positive cells or structure of extracellular matrix were seen in various amounts. Amount of IGFR1 and IL-10 positive cells significantly decresed with advanced gestation, suggesting decrease of their impact approaching the term. Significantly higher amount of apoptotic cells in pre-term placentas suggested switch to other types of cellular disposal in later gestation. Positive structures of Collagen IV were significantly more in term placentas. Correlations of the other molecular factors with gestational age were not significant. There were found several correlations among the molecular factors, providing insight into interactions between processes in the tissues and suggesting their impact on the course and outcome of pregnancy.

ANOTĀCIJA

Pētījuma mērķis bija ar imūnhistoķīmisku metožu palīdzību izzināt molekulāros notikumus trešā grūtniecības trimestra dažāda gestācijas vecuma un dažādas klīniskās gaitas pēcdzemdību placentās. Pētījums ir orģināls ar to, ka tajā dažādu imūnhistoķīmisko marķieru parādīšanās *in situ* placentas audos tiek sasaistītas ar gestācijas vecumu, antropoloģiskām, morfoloģiskām īpatnībām un pamata klīniskajiem rādītājiem. Preparātos no 53 pēcdzemdību placentām no 22 līdz 40 gestācijas nedēļām tika izvērtēts sekojošus marķierus saturošu šūnu vai ekstracellulārās matricas struktūru daudzums: augšanas faktorus un receptorus IGF1, IGFR1, HGF, bFGF un FGFR1, citokīnus IL-10, IL-1α, IL-6 un TNFα, bāzes membrānas olbaltumus Kollagēnu IV un Laminīnu, audu noārdīšanas enzīmus MMP2 un MMP9, kā arī *homeobox* gēna HoxB3 produktus. Atradnes tika novērtētas puskvantitatīvi, piešķirot rangu ascendējošā kārtībā izmantojot modificēto konkurences ranga piešķiršanas metodi. Ranga vērtības tika korelētas savstarpēji, kā arī ar gestācijas laiku, placentas masu, mātes un jaundzimušā antropoloģiskajiem parametriem un pamata klīniskajiem rādītājiem. Bāziskā FGF, IL-6 un MMP2 pozitīvu šūnu, kā arī laminīnu saturošu struktūru vizuāli netika atrasts; citus marķierus saturošas šūnas vai ekstracellulārās matricas struktūras bija redzamas dažādos daudzumos. IGFR1 un IL-10 pozitīvo šūnu daudzums nozīmīgi samazinājās ar gestācijas vecuma palielināšanos, liekot domāt par to iedarbības mazināšanos grūtniecības beigās. Statistiski nozīmīgi lielāks apoptotisko šūnu skaits priekšlaicīgu dzemdību placentās like domāt par pārslēgšanos uz citu šūnu likvidēšanas veidu vēlīnākā grūtniecības laikā. Kolagēna IV pozitīvu struktūru bija vairāk iznēsātu dzemdību placentās. Citu marķieru korelācija ar gestācijas laiku nebija statistiski nozīmīga. Tika identificētas vairākas korelācijas starp molekulārajiem marķieriem, sniedzot ieskatu audu procesos un izvirzot jautājumu par to ietekmi uz grūtniecības gaitu un iznākumu.

TABLE OF CONTENTS

ABBREVIATIONS

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

	Abbreviation	English	Latvian
21.	LPS	Lipopolysaccharide	*Lipopolisaharīds*
22.	MMP	Matrix metaloproteinase	*Matricas metālproteināze*
23.	NICU	Neonatal intensive care unit	*Jaundzimušo intensīvās terapijas nodaļa*
24.	PBS	Phosphate buffered saline	*Fosfātu bufera sāls šķīdums*
25.	pO_2	Partial pressure of oxygen	*Skābekļa parciālais spiediens*
26.	PI	Ponderal index	*Ponderela indekss*
27.	PPROM	Pre-term premature rupture of membranes	*Pirms-termiņa priekšlaicīgs augļa apvalku plīsums*
28.	PROM	Premature rupture of membranes	*Priekšlaicīgs augļa apvalku plīsums*
29.	ROM	Rupture of membranes	*Augļa apvalku plīsums*
30.	SGA	Small for the gestational age	*Mazs grūtniecības laiks*
31.	TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling	*Terminālās deoksinukleotidiltransferāzes dUTP N gala marķēšana*
32.	TGF	Transforming growth factor	*Transformējošais augšanas faktors*
33.	TNFα	Tumor necrosis factor	*Tumora nekrozes faktors*
34.	VEGF	Vascular endothelial growth factor	*Asinsvadu endotēlija augšanas faktors*
35.	VLBW	Very low birth weight	*Ļoti mazs dzimšanas svars*

INTRODUCTION

Perinatal and neonatal mortality are important indicators of the development of a country and highly developed ones tend to maintain those figers 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 oftern 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. Transport of all the essential nutrients is provided by the placental blood flow; placenta produces and transports hormones, promoting fetal growth. 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 outcome for the fetus, leading to long–term health benefits for the human child.

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 to 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 opportunities for the improvement of perinatal outcome in high risk situations.

1. LITERATURE REVIEW

Perinatal medicine is one of the most challenging specialties of human health care, necessarily facing challenges related to our genetic capabilities, reproductive health issues as well as problems of the perinatal period, when physiological processes of fetal development and childbirth meet the strongest audit of quality assurance and natural selection. Today our expectations differ from those ones from the previous centuries therefore we are working towards improvement of survival of fetuses and neonates at least those ones with potentials of appropriately high quality of life. For this reason there have been performed a lot of studies on the factors, having impact on the processes of placentation, progression of pregnancy and its termination. 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), like pre-eclampsia 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 as well as the outcome in severe cases, like caused by human immunodeficiency virus (HIV) (Shuetz et al., 2011). A drawback of a practical application of an IHC research is its specificity (Ramos-Vara, 2005); each study reveals targeted examination of the tissues. For this reason it would be very useful to identify a scope of reliable factors 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 factors that we expected to be involved in the development of fetus and his environment. Choice of the factors was based on our understanding of the processes from conception till termination of pregnancy that could possibly be reflected in the maternal-fetal interface – placental tissues.

1.1. Growth factors and receptors

Growth factors have been proven to play a significant role in the placental and

fetal development throughout pregnancy as they face significant physical and developmental growth throughout this period of time. Growth factors are substances, produced by the variety of cells and modulating proliferation and bio-activities of non-immune cells.

1.1.1. Insulin-like growth factor 1 (IGF1) and its receptor (IGF1R)

IGF1 is one of the most potent growth factors, produced by different cell types and having impact on the body size and stature (Kansra et al., 2012; Becker et al., 2012), presenting altered expression in the cases of different pathologies: up-regulated in neonates with insufficient lung development (Chetty et al., 2004) and IUGR (Muhlhausler et al., 2009); it can increase the risk for childhood leukemia (Chokkalingam et al., 2012) and Alzheimer's disease in adulthood (Wang et al., 2012). It can also have an impact on human longevity (Mari, 2011).

IGF1 in its molecular structure is similar to insulin in an aqueous solution (Figure 1.1.). The structure is characterized by the presence of three helical rods corresponding to the sequence regions, Ala8-Cys18, Gly42-Cys48 and Leu54-Cys61.

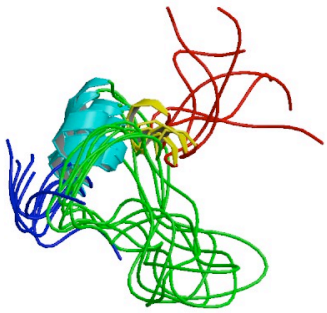

Figure 1.1. **Structure of the human IGF1**
(Adapted from Sato et al., 1993)

IGF1 is synthetized also in the tissues of human placenta (Hill et al., 1993) and influence its growth and endocrine function; it is found in different cell types of mammal placentas from 6 weeks of gestation till term (Coulter and Han, 1996; Han et al., 1996; Dhara et al., 2001). The expression of IGF1 by the placental cells throughout the pregnancy has impact on its course; studies of perinatal research are looking for more correlation of IGF1 with the pregnancy outcomes.

The activity of IGF1 is mediated through the receptor IGF1R, which has also been found in the placental tissues throughout the pregnancy (Hiden et al, 2006). IGF1R is a member of the tyrosine-kinase receptor superfamily. In the Figure 1.2. is presented the structure of three domains of IGFIR (L1-Cys-rich-L2). The three domains surround a central space of sufficient size to accommodate a ligand molecule.

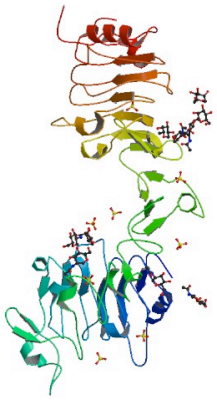

Figure 1.2. **Structure of IGF1R**
(Adapted from Garrett et al., 1998)

IGF1 and IGF1R influence growth, differentiation, survival, and metabolism in various organs (Jones and Clemmons, 1995). They are important factors of fetal growth (Sferruzzi-Perri et al., 2007; Forbes and Westwood, 2008; Sferruzzi-Perri et al., 2008; Sferruzzi-Perri et al., 2008) and promoters of placental development and function. Mutations in the IGF1R gene, leading to the resistance to IGF1, are associated with severe growth failure, both antenatally (IUGR) and after delivery (Gannagé-Yared et al., 2012). Animal studies show positive impact of the treatment with IGF1 in early to mid-pregnancy on the glucose transport in placenta (Sferruzzi-Perri et al., 2007a) with consequently improved fetal growth and viability near term. Although IGF1 is proven to be so important for human growth, that it is used as a therapeutic agent in the cases of growth deficiency, oncologists are working on the possible blockade of IGF1R to avoid the impact of IGF axis on the initiation and progression of cancer (Arnaldez and Helman, 2012). Power and wide spectrum of activity of IGF1 position this growth factor as a significant player of human growth and development with a potentially hazardous nature in the cases of its inappropriate activity. Perinatal period is one of the most active for growth and development and sensitive to the exposure of different

factors; studies on the expression of IGF1 and IGF1R in the maternal-fetal environment can disclose processes during pregnancy, having led to certain extent of fetal size and development.

1.1.2. Hepatocyte growth factor (HGF)

HGF is an activating ligand of the Met receptor tyrosine kinase, essential for tissue development and organ regeneration; its abnormal activation is implicated in growth, invasion, and metastasis of many types of tumors (Weidner et al., 1990). HGF has two natural splice variants, NK1 and NK2: NK1 is Met agonist and NK2 (Figure 1.3.) is a Met antagonist, having down-regulating impact on the activity of HGF (Tolbert et al., 2010).

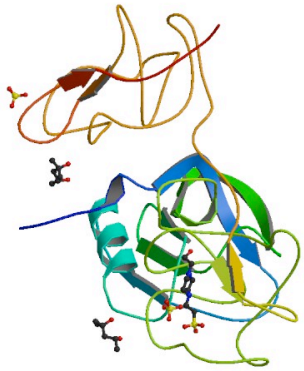

Figure 1.3. **Crystal structure of the NK2 fragment (28-289) of human HGF**
(Adapted from Tolbert et al., 2010).

HGF is a multi-functional growth factor, having been identified in 1991 from the research studies in various unrelated fields (Nakamura et al., 2011). It has a large variety of target cells, including cytotrophoblast of placenta. HGF seems to play a significant role in the development of the placenta (Uehara et al., 1995), fetal liver (Schmidt et al., 1995), limbs (Bladt et al., 1995) and nervous system (Ebens et al., 1996). Low levels of HGF can lead to the appearance of congenital malformations in the nervous system (Trovato et al., 2007). Impact of this factor on fetus continues after delivery of the child, that is confirmed by high levels of HGF in the colostrum (Patki et al., 2012), the main initiator and first promoter of the neonatal development after birth.

HGF provides heart tissue regeneration after ischemia-reperfusion injury

(Ellison et al., 2011; Genead et al., 2012) and has been nominated as a pro-angiogenic cytokine (Atluri et al., 2008) due to improvements in myocardial and peripheral perfusion following therapy with HGF. In various disease models, HGF promotes cell survival and regeneration of cells in liver (Kosai et al., 1998; Huh et al., 2004; Borowiak et al., 2004; Kim et al., 2005; Giebler et al., 2009), kidneys (Ma et al., 2009; Dai et al., 2010) and skin (Chmielowiec et al., 2007).

Recognition of the faces of HGF present this growth factor as an interesting object of research studies in clinical perinatology as the expression of HGF definitely can have a significant impact on the course of pregnancy and its outcome.

1.1.3. Basic Fibroblast growth factor (bFGF) and receptor 1 (FGFR1)

Basic FGF or FGF2 is a member of the family of fibroblast growth factors, known to have impacts on vasculogenesis, wound healing and embryogenesis. It is also known as a mitogen for different cell types. Basic FGF is composed entirely of beta-sheet structure, comprising a three-fold repeat of a four-stranded antiparallel beta-meander (Figure 1.4.). The topology of bFGF is identical to that of Interleukin-1β (IL-1β); they share only 10% sequence identity and belong to a family of structurally related mitogenic factors.

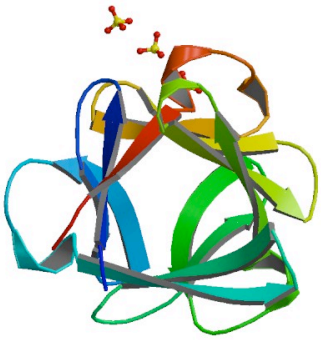

Figure 1.4. **Structure of the human basic FGF**
(Adapted from Zhang et al., 1991)

Role of bFGF in vasculogenesis has been researched widely. There was found an increased expression of bFGF in ischemic and infarcted myocardium (Scheinowitz et al., 1997; Scarborough et al., 2002); number of animal studies demonstrated significant improvement of angiogenesis in a damaged myocardium after systemic of local

application of bFGF (Lazarous et al., 1995; Lazarous et al., 1997; Shou et al., 1997; Rajanayagam et al., 2000; Laham et al., 2003: Biswas et al., 2004); intramyocardial administration seemed to be the most efficient route of administration. Efficiency of bFGF was found to be similar with Vascular endothelial growth factor (VEGF) (Hughes et al. 2004); the treated heart showed improvement of functionality (Liu et al., 2006). Application of bFGF has also been researched in animal studies with artificially ischemized limb; results demonstrated improved vascularization (Stark et al., 1998; Baffour et al., 2000; Lebherz et al., 2003). To acquire better result bFGF was applied together with HGF (Marui et al., 2005); for prolongation and safety of its activity it was used in combination with collagen matrix (Zhou et al., 2012) or biodegradable gelatin gel (Nakajima et al., 2004).

Members of FGF family FGF4 and FGF10 together with VEGF have been found to play a role on the establishment of pregnancy (Anteby et al., 2004; Natanson-Yaron et al., 2007), having significant impact on the branching morphogenesis of the placental villi, while bFGF has been found to regulate angiogenesis in the placental tissues (Riddell et al., 2012) through fibrocyte-like cells of villous stroma. The expression of bFGF in the placental tissues gradually decreased with the development of placenta (Wei et al., 2004), reflecting the decreasing need for angiogenesis with advancing of pregnancy.

Angiogenic factors, like bFGF, are investigated in pregnant patients with potential or evident problems of placental perfusion, like gestational diabetes, pre-eclampsia or fetal IUGR. There has been found increased level of bFGF in the cytotrophoblast and capillary endothelium of chorionic villi in patients with pre-eclampsia (Ozkan et al., 2008) and no differences of the levels of bFGF in the extravillous trophoblasts and decidual cells of IUGR cases (Ozkan et al., 2008). In the cases of gestational diabetes the increased level of bFGF was found in placental tissues (Hill et al., 1998; Arany et al., 1998) as well as the maternal and cord serum and amniotic fluid (Hill et al., 2005).

The members of FGF family 1-10, icluding bFGF, are binding to the Fibroblast growth factor receptors (FGFR). Fibroblast growth factor receptors consist of three immunoglobulin-like domains, a single transmembrane helix domain and an intracellular domain with tyrosine kinase activity (Figure 1.5.).

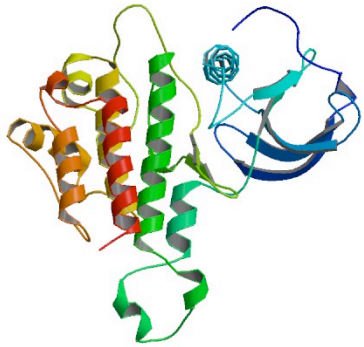

Figure 1.5. **Structure of FGFR1**
(Adapted from Bae et al., 2010)

To transphosphorylate FGFR1 into FGF-stimulated cell it is necessary to formate an asymmetric dimer between activated FGFR1 kinase domains (Bae et al., 2010).

FGFR1 has been found in different cells of placenta throughout the pregnancy: Höfbauer cells at the beginning of pregnancy and cytotrophoblast and syncitiotrophoblast later in pregnancy (Anteby et al., 2005) suggesting, that abundance of FGFR expression in Höfbauer cells implies that interactions between mesenchyme and trophoblast are important for the regulation of villous development.

All the described studies position research studies on growth factors as an auspicious resource for new knowledge and understanding of the processes in the maternal-fetal unit, having led to one or another outcome.

1.2. Cytokines

The course of pregnancy and the development of human fetus are accompanied by significant immune reactions of the maternal organism both protecting fetus from the potentially hazardous biological factors of own environment and providing fetal allograft against own immune response. Therefore tissues of the maternal-fetal unit could be expected to express both anti-inflammatory and pro-inflammatory cytokines and keep those processes in a balance, being disturbed in the cases of pathology and leading to the determined outcome.

1.2.1. Pro-inflammatory cytokines Tumor necrosis factor alpha (TNFα) and IL-1α

Time period before the onset of labor has the highest susceptibility to microbial invasion, even if there are no clinical signs of presence of inflammation processes. Therefore there are studies investigating presence and localization of pro-inflammatory cytokines in the maternal-fetal unit and their correlation with different perinatal processes. Increase of pro-inflammatory cytokines alters pre- and anti-inflammatory cytokine ratio, leading to pregnancy pathologies like pre-eclampsia (Xie et al., 2011; Chaparro et al., 2012; Lamarca et al., 2012; Taki et al., 2012); on the other hand it can be a protective measure in a case where pathogen reaches amnion and threaten the fetus (Mitchel et al., 2004).

One of the best known pro-inflammatory cytokines is that reflects a variety of processes in the placental tissues is **TNFα**. It has been investigated to indicate problems of pregnancy from the first trimester of pregnancy, indicating risk for pregnancy loss, congenital anomalies of fetus, pathologic course of pregnancy or its adverse outcome.

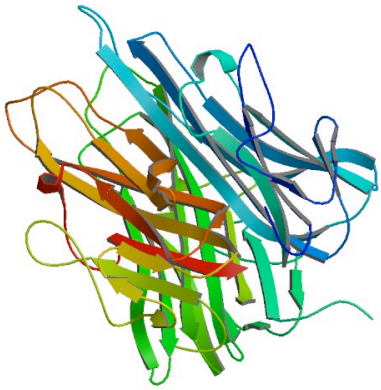

Figure 1.6. **The structure of the TNFα**
(Adapted from Eck et al., 1989)

TNFα is a protein hormone secreted by macrophages; three monomers associate intimately about a 3-fold axis of symmetry to form a compact bell-shaped trimer (Eck et al., 1989) (Figure 1.6.). The structure is homologous to several viral coat proteins, particularly satellite tobacco necrosis virus. TNFα is inducing apoptosis of the cells of extravillous trophoblast in the first trimester of pregnancy, leading to reduced utero-placental blood flow and possible loss of pregnancy (Wu et al., 2012); increase of the expression of TNFα leads to fetal rejection (Chabtini et al., 2012). Discontinuation of

anti-TNFα medications, applied for treatment of inflammatory bowel disease, till 30 weeks of pregnancy seems to be safe (Zelinkova et al., 2012). Placentas from miscarriages of the first trimester present increased levels of TNFα in the cases of abnormal karyotype (Calleja-Agius et al., 2012) comparing with the normal karyotype miscarriages, showing that abnormal karyotype exacerbates placental inflammatory response; levels of TNFα in the placental explants of euploid miscarriage pregnancies was still higher than of normal pregnancies (Calleja-Agius et al., 2012). In the cases of threatened miscarriages monocyte expression is lower, but free TNFα in plasma higher than in normal pregnancies (Calleja-Agius et al., 2011). Expression of TNFα as a pro-inflammatory cytokine has also been investigated in relations with premature rupture of membranes and chorionamnionitis. The expression of TNFα in cases of Premature rupture of membranes (PROM) is found not to be different (Roveran et al., 2009), although it shows increased expression in chorionamnionitis (Lockwood et al., 2006) weakening fetal membranes (Kobayashi et al., 2010) that possibly can lead to their premature rupture.

The above described studies propose TNFα as a complex indicator of well-being of the maternal-fetal environment, having impact and possibly anticipating the course and outcome of the pregnancy.

A clinically significant group of pro-inflammatory cytokines is family of **IL-1**.

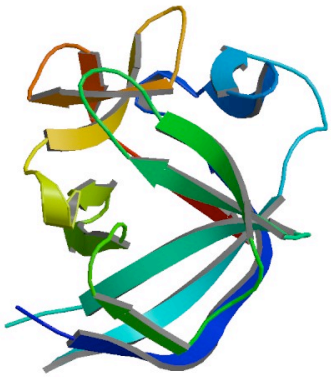

Figure 1.7. **Structure of Interleukin 1α**
(Adapted from Graves et al., 1990)

There are two types of IL-1: α and β, both produced by activated macrophages and other cell types, modulating immune and inflammatory responses. The core of the structure of IL-1α is a capped beta-barrell that possesses 3-fold symmetry and displays a

topology similar to that observed for IL-1β (Graves et al., 1990) (Figure 1.7.). There are authors suggesting that normal placenta produces very little IL-1 (Kauma et al., 1992); others show more noticeable expression of IL-1. Immune response by the production of IL-1α is found to be more expressed in pregnancy; IL-1α is higher in decidual than non-pregnant endometrial cells (Segerer et al., 2009) and it has been found in the cells of extravillous trophoblast and syncitiotrophoblast of placentas of various gestational ages (Paulesu et al., 1991). IL-1α shows an increased expression in the villous chorion in miscarriage cases in comparison with normal pregnancies (Pavlov et al., 2006); the level in miscarrige caese is increasing more if stimulated by lipopolysaccharide (LPS). Expression of IL-1α is more pronounced in pre-term than term placentas; stimulation with LPS causes different reactions in different compartments of placenta depending on the gestational age (Huleihel et al., 2004; Holcberg et al., 2008), indicating diverse types of response towards immune stimuli. The most active response towards LPS is seen in the decidua of the term placentas. Recent studies suggest therapeutic application of receptor antagonist of IL-1 after early non-invasive diagnosis of placental inflammation to prevent tissue damage and provide neuroprotection of fetus, improving the outcome of pregnancy (Girard et al., 2010; Girard et al., 2012).

1.2.2. Anti-inflammatory cytokine Interleukine 10 (IL-10)

IL-10 is a cytokine with a potency to down-regulate inflammatory processes and control immune processes (Wakkach et al., 2000: Groux et al., 2003; Yang et al., 2005); pharmacological activation of IL-10 is suggested for various autoimmune diseases (Zhou et al., 2005; Li et al., 2006). Structurally it is a homodimer, consisting of two identical protein molecules (Figure 1.8.).

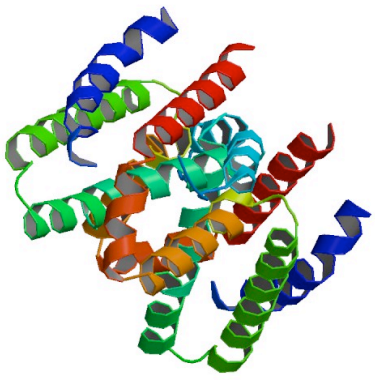

Figure 1.8. **Crystal structure of human IL-10**
(Adapted from Yoon et al., 2006)

IL-10 is involved in different stages of reproduction. Decreased level of IL-10 is found be related to recurrent pregnancy losses, including patients with thyroid autoimmunity (Kim et al., 2012; Parveen et al., 2012; Twig et al., 2012) although genetical predisposition is not found (Kaur and Kaur, 2011). Impact of IL-10 on the efficiency of in vitro fertilisation procedures is also not yet established (Persson et al., 2012), although the ratio Tumor necrosis factor α (TNFα): IL-10 seems to matter (Winger et al., 2012). In animal studies IL-10 is found to normalize endothelial function and regulate maternal blood pressure in early pregnancy (Orange et al., 2005; Tinsley et al., 2010), possibly having impact on the development of pre-eclampsia; in the term placentas of pre-eclamptic patients IL-10 has been found to be of lower expression of (Rein et al., 2003; Makris et al., 2006; Saito et al., 2007). In animal studies hypoxia induced defficiency of IL-10 has caused pathological conditions like pre-eclampsia (Kalkunte et al., 2010; Lai et al., 2011); suggesting roles of both hypoxia and defficiency of IL-10 in the pathogenesis of pre-eclampsia.

Establishment and prolongation of pregnancy is strongly associated with an immune tolerance of maternal immune system against the appearing allograft embryo, later developing into fetus, therefore high level of expression of anti-inflammatory cytokine IL-10 has been suggested as a precursor of successful pregnancy (Denison et al., 1998 Denney et al., 2011; Parveen et al., 2012; Brogin Moreli et al., 2012). The mechanism of the development of immune tolerance has been explained as a result of a reaction of maternal immune system towards trophoblast debris, shed during pregnancy into maternal circulation in large quantities (Abumaree et al., 2012), inducing secretion of anti-inflammatory cytokine IL-10 and reducing secretion of pro-inflammatory cytokines; other studies emphasize role of decidual macrophages, presenting enhancing their tolerance to semi-allogeneic fetus (Nagamatsu et al., 2010; Svensson et al., 2011).

Therefore the main role of IL-10 on the establishment and course of pregnancy could be undertaken as the management of ratio between anti-inflammatory and pro-inflammatory cytokines, ensuring maternal immune tolerance of fetal allograft, protecting from the pathological statuses like pre-eclampsia (Kalkunte et al., 2011) and guiding the pregnancy towards successful outcome.

1.3. Apoptosis of cells in the placental tissues

Apoptosis has been established as a programmed cell death different from the traumatic cell death (Kerr et al., 1965). A few years later term apoptosis has been proposed as a recognized mechanism of controlled cell deletion, which appears to play a complementary but opposite role to mitosis in the regulation of animal cell populations (Kerr et al., 1972).

Cellular disposal by the means of apoptosis does not cause inflammatory reaction in the surrounding tissues, as cellular constituents are not released in those tissues and are quickly phagocytosed by other cells, not producing anti-inflammatory cytokines (Savill and Fadok, 2000; Kurosaka et al., 2003).

There are two main apoptotic routes (Figure 1.9.): extrinsic pathway (death receptor) and intrinsic pathway (mitochondrial) (Danial and Korsmeyer, 2004; Meier and Vousden, 2007). The death receptor pathway is initited by extracellular stimuli which is detected by dead receptors, belonging to the family of tumor necrosis factor (TNF) receptors.

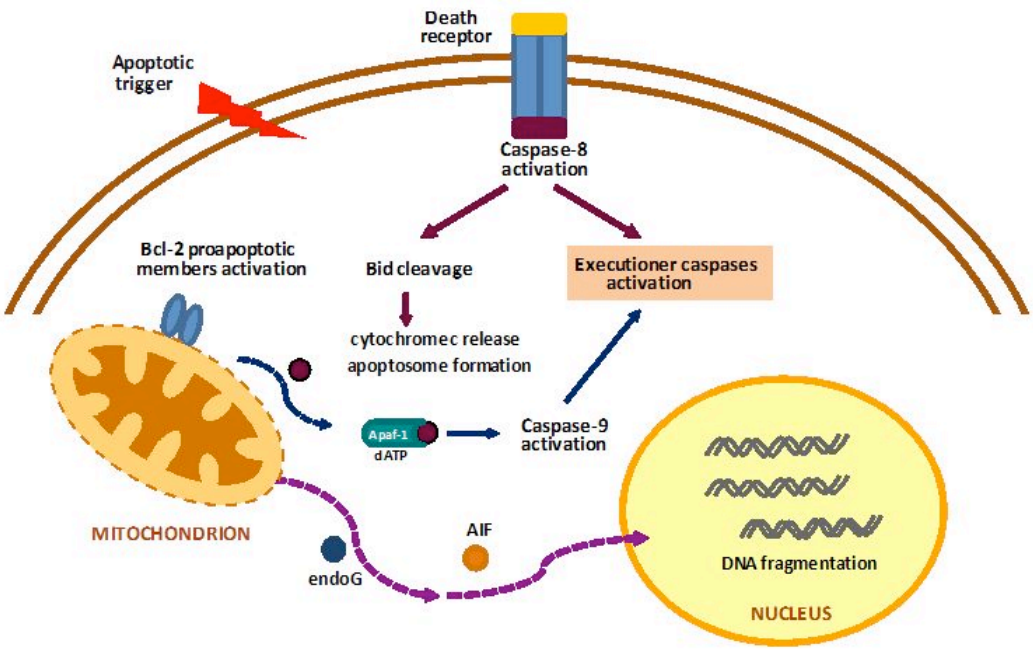

Figure 1.9. **Extrinsic and intrinsic apoptotic pathways**
(Adapted from Grasso et al., 2012)

Death receptor ligands become activated and interact by the means of their death domain with the protein motif Fas-associated death domain (FADD) in adapter proteins,

forming Death inducing signalling complex (DISC) which binds to the prodomain of the initiator caspase-8 (Peter et al., 2007), leading to activation of executioner caspases -3, -6, and -7 (Lavrik et al., 2005).

Initiation of mitochondrial pathway is characterized by the action of proapoptotic B-cell lymphoma (Bcl-2) proteins, promoting mitochondrial outer membrane permeabilization (MOMP); process is limited by antiapoptotic proteins of the same family (Kroemer et al., 2008). If the balance is in favour of the proapoptotic members of Bcl-2 family, the outer mitochondrial membrane is permeabilized through pore formation; cytochrome c and other proteins are released to cytosol. Cytochrome c binds to adaptor protein, formating apoptosome and activating caspase-9, leading to activation of executioner caspases -3, -6 and -7.

Morphologically cellular apoptosis includes several consecutive processes:

- It starts with blebbing, development of irregular bulges in the plasma membrane, and separation of cytoplasm containing cellular fragments (apoptotic bodies), leading to cell shrinkage; cell becomes smaller with dense cytoplasm and more tightly packed organelles;
- Nuclear fragmentation and pyknosis, the most characteristic feature of apoptosis (Elmore, 2007), which results from chromatin condensation in the nucleus;
- Chromosomal DNA fragmentation leads to karyorrhexis (destructive fragmentation of nucleus) and budding; apoptotic bodies at this point contain cytoplasm, organells and nuclear fragments;
- Theses bodies are subsequently phagocytosed by surrounding cells – eg., macrophages and parenchymal cells, and degraded within phagolysosomes.

Apoptosis normally is participating in the turnover of tissues; it has a special significance in the processes of reproduction and fetal development. At the beginning of pregnancy it is affecting maternal immune cells to enhance tolerance of fetal allograft (Kauma et al., 1999; Jerzak and Bischof, 2002). Further in the pregnancy it has a significant role in the morfogenesis and development of a placenta (Smith et al., 1997; Huppertz et al., 1998; Mayhew et al., 1999; Mayhew 2001; Hupperz et al., 2004), contributing to the growth and maturation of villi; apoptosis in placenta takes place through several pathways (De Falco et al., 2005). In the placentas of pathological pregnancies increased cell death could be found either by the means of apoptosis,

commonly associated with IUGR (Smith et al., 1997; Axt et al., 1999; Erel et al., 2001; Liu et al., 2002; Burton et al., 2009) or necrosis with either level of apoptosis (decreased, unchanged or increased), leading to abnormal maternal response (Huppertz et al., 2003; Kadyrov et al., 2006; Chen et al., 2010; Sharp et al., 2010; Chamley et al., 2011) and initiation of a pathway of pre-eclampsia. Recent studies suggest genetical predisposition of the development of IUGR in pregnancies with increased apoptosis in placental cells (Börzsönyi et al., 2012).

Number of apoptotic cells is found to be increased in the cases of PROM and Pre-term premature rupture of membranes (PPROM), related to high risk for infections (Tanir et al., 2005); various infectious agents have been found to have different impact on the trophoblast apoptosis: *Toxoplasma gondii* or *Plasmodium berghei* increase (Xu et al., 2012) and *Coxiella burnetii* decrease (Myers et al., 2012) the expression of programmed cell death.

It could be suggested that clearer understanding of processes leading or limiting apoptosis may provide new insights into placental pathologies; apoptosis appears to be an important target of placental research.

1.4. Protein of the basement membrane Collagen IV

Proper establishment of the maternal fetal interface placenta at the beginning of pregnacy, its efficient functioning throughout pregnancy as well as timely degradation of its structures at the time of delivery are extremely important for optimal fetal development and best pregnancy outcome. Basement membrane is one of the keystones of the tissue structure, ensuring its strength and having impact on its permeability; deviation of its strength in the placental tissues can either endanger its last till term or decrease functionality of placental barier. Unusual changes in the content of the components of the basement membrane in the placental tissues can indicate pathways of processes, leading to one or another outcome of pregnancy; their assesment can appear to be clinically significant.

First isolated from the basement membranes of canine glomeruli (Kefalides, 1966; Kefalides and Winzler, 1966) Collagen IV, the main constituens of *lamina basalis*, has proven itself to play a significant role in the physical strength of the basement membrane of various tissues (Kühn, 1995; Nerlich, 1995).

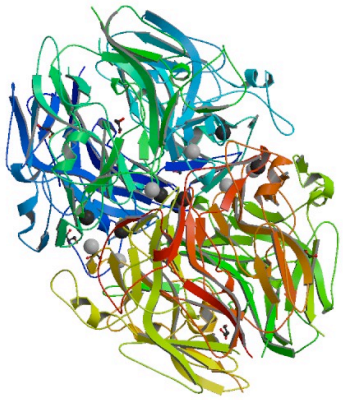

Figure 1.10. **Crystal structure of Collagen IV NC1 (non-collagenous) domain from the basement membrane of placenta**
(Adapted from Vanacore et al., 2004)

The chain of Collagen IV contains a long triple-helical collagenous domain flanked by a short 7S domain and a globular non-collagenous NC1 domain (Sundaramoorthy et al., 2002) (Figure 1.10.).

Six genes, encoding six different polypeptide chains of Collagen IV are differentially expressed during the embryonic development (Khoshnoodi et al., 2008), providing tissues with specific collagen IV networks. Alterations of Collagen IV content in the tissues are seen in the cases of in congenital Alport syndrome; detection of its expression in different basement membranes confirm the diagnosis (Kashtan, 1995; Lemmink et al., 1997; Garcia-Torres et al., 2000; Savige et al., 2003; Haas, 2009; Kashtan and Segal, 2011; Yao et al., 2012). Antibodies against NC1 domain of the Collagen IV with consecutive damage of glomerular basement membranes are found in patients with the autoimmune Goodpasture syndrome (Savige et al., 1991; Matsakura et al, 1993; MacDonald et al., 2006; Alenzi et al., 2012). External factors also can influence the expression of Collagen IV; its defective structure is seen in a diabetic kidney, leading to its damage (Raabe et al., 1998).

Collagen IV containing basement membrane is underlying the cytotrophoblast of placental villi (Mori et al., 2007; Jones et al., 2008), ensuring their integrity and participating in the composition of placental barrier; abnormalities are associated with the pathologies of pregnancy and possibly adverse outcomes. Increased expression of Collagen IV or thickened basement membrane were found to be significantly higher in the placentas of patients with hypertension (Hu et al., 2004) or cigarette smoking (van der Velde et al., 1983; van der Velde et al., 1985; Asfhaq et al., 2003; Rath et al., 2011), leading to placental insufficiency (Khozhaĭ et al., 2010). Decreased expression of

Collagen IV and thinner basement membrane can be caused by certain infectious agents (Duaso et al., 2010).

1.5. Tissues degrading enzymes

Extracellular matrix (ECM) is a major component of the microenvironment of a cell and takes part in its proliferation, adhesion, migration, differentiation and death (Hynes, 2009); it is a constantly remodeling structure, normally well controlled (Rozario et al., 2010; Tsang et al., 2010) and in mismatch situations leading to pathological conditions (Muschler et al., 2010; Lu et al., 2011). Remodeling processes involve elective ECM degradation, provided by different enzymes, the most significant of whom are matrix metalloproteinases (MMP) (Reynolds, 1996; Shingleton et al., 1996; Xu et al., 2002; Cawston and Young, 2010), activated by different processes in the tissues and inhibited by their tissue inhibitors (Bischof, 2001). Collagen IV, ensuring the physical strength of basement membrane, is degraded by gelatinases MMP2 and MMP9, having impact on the integrity and strength of different tissues. Establishment, course and resolution of pregnancy is associated with persistent remodeling of placenta; coherence of degradation and developmental processes in its abundant ECM (Hopper et al., 2003) can determine success of pregnancy and its outcome. Pathological conditions of pregnancy, possibly leading to PPROM and pre-term delivery, are linked with imbalance between MMPs and their tissue inhibitors (Riley et al., 1999; Cocle et al., 2007; Weiss et al., 2007; Pasquier et al., 2008; Tency et al., 2012), positioning MMPs as a clinically interesting subject for perinatal research.

1.5.1. MMP9

MMP9 is another collagenase, involved in the breakdown of Collagen IV and possibly having impact on the integrity of tissues. MMP9 is seen in inflammatory atherosclerotic lesions and its over-expression is implicated in the vascular re-modelling events preceding plaque rupture and causing acute myocardial infarction (Rowsell et al., 2002). MMP9 activity may therefore represent a key mechanism in the pathogenesis of heart failure. The structure of MMP9 adopts typical fold of a MMP. The catalytic centre is composed of the active-site zinc ion, co-ordinated by three histidine residues and the

essential glutamic acid residue (Figure 1.11.). The presented structure designs specific inhibitors for MMP9.

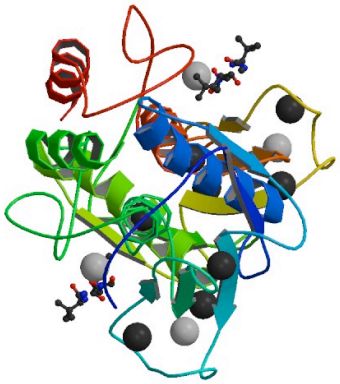

Figure 1.11. **MMP9 active site mutant-inhibitor complex**
(Adapted from Rowsel et al., 2002)

MMP9 is also a significant player of human reproduction. Its role in the trophoblast invasion is starting later than MMP2 from the 9th week of pregnancy (Staun-Ram et al., 2004) and increasing with advanced gestation. Increased level of MMP9 in the first trimester is associated with *missed abortion* (Nissi et al., 2013). Already in the first trimester pre-eclampsia related cytokines IL-1β and TNFα are associated with increased expression of MMP9, possibly promoting the development of pre-eclampsia (Lockwood et al., 2008). During pregnancy high activity of MMP9 ensures normal placental function; its decrease is associated with the development of fetal IUGR (Swierczewski et al., 2012); localization of MMP9 in placental tissues is highly specific (Xu et al., 2002). It has even been suggested that high activity of MMP9 can prevent development of pre-eclampsia (Coolman et al., 2007).

Preceeding and during parturition rupture of membranes, onset of labor and microbial invasion in the amniotic cavity are associated with the increased activity of MMP9 (Vadillo-Ortega et al., 1995; Maymon et al 2000; Fortunato and Menon, 2001; Goldman et al., 2003; Weiss et al., 2007), contributing to degradation of the ECM in the fetal membranes and placenta and facilitating rupture of membranes and detachment of placenta from the maternal uterus. Indomethacin decreases the expression of MMP9 ans MMP2 in the fetal membranes chorion and amnion, somehow arresting pre-term delivery (Ulug et al., 2001).

In term placentas increased expressions of MMP9 and MMP2 are found in patients with type 2 diabetes (Capobianco et al., 2012), probably activated by reactive nitrogen species.

Although MMP9 is already known to be a significant player in human pregnancy and parturition, there are still unanswered questions, for example: if labor process faces inappropriate activity of MMP9?

1.5.2. Other MMPs

MMP2 is an one of the enzymes, providing breakdown of Collagen IV, costituens of the basement membrane and is involved in various physiological and pathological processes. MMP2 like other MMPs is expressed as latent proenzyme that is activated by proteolytic cleavage (cysteine switch) (Figure 1.14.).

Increased serum levels of MMP2 and MMP9 have been found in virus hepatitis C (VHC) positive heroin addicts (Kovatsi et al., 2012) as well as breast carcinoma patients (Solai et al., 2010; Vasaturo et al., 2012), indicating impaired barrier function of the tissues. Topically increased expression of MMP2 in the endometrial tissues is seen in the patients with endometriosis (Chung et al., 2002; Uzan et al., 2004), suggesting therapeutical targets of this hardly curable disease (Jana et al., 2012).

MMP2 is playing a significant role in the cytotrophoblast invasion in the first trimester of pregnancy (Isaka et al., 2003; Staun-Ram et al., 2004) and in a normal pregnancy looses its role with advanced gestation although it has been found in certain types of cells in pre-term and term post-delivery placentas (Xu et al., 2002; Isaka et al., 2003). In the first trimester of pregnancy inflammatory cytokines do not have an impact on the expression of MMP2 (Lockwood et al., 2008) while in the third trimester initiation of pre-term labor, PPROM or microbial invasion in the uterine cavity have been found to be related with a decreased expression of MMP2 (Maymon et al., 2000). Incubated chorionic villi in the presence of *Trypanosoma cruzi* have shown increased expression of MMP2 (Castillo et al., 2012);

Media, conditioned by decidua, show increased expression of MMP2 with an onset of uterine contractions while increase of MMP9 are shown by amnion conditioned media; that emphasizes consecutive involvement of MMPs in the process of parturition (Golgman et al., 2003).

Involvement of the other MMPs on human pregnancy and parturition; their involvement seems to be quite specific and narrow. Decreased expression of MMP3 and MMP7 has been foud in the extravillous trophoblast of pre-eclamptic patients (Reister et al., 2006). Increased expression of MMP3 has been found in the myometrium at labor onset (O'Brien et al., 2007) and in the amnotic fluid at PPROM (Fortunato et al., 2001). Increased expression of MMP8 has been found in the amniotic fluid in cases of oligohydramnion, more susceptable to infections (Kim et al., 2011), proven cases of amniotic infection (Park et al., 2009) or microbial invasion in the amniotic cavity (Maymon et al., 2000). Detection of MMP8 in the amniotic fluid has even been suggested for clinical confirmation of intra-amniotic infection (Angus et al., 2001), important for cases of PPROM or threatening pre-term deliveries.

1.6. Homeobox gene products

Morphogenesis of the developing human embryo is determined by a large variety of homeobox genes, a large family of similar genes that direct the formation of many body structures during early embryonic development; presently the most researched part of homeobox containing genes are Hox genes. First found in *Drosophila melanogaster* as determinants of anterior-posterior axis, they have been proven to determine development of vertebrates, their nervous system (Mc Ginnis and Krumlauf, 1992; Krumlauf, 1994; Schneider-Maunoury et al., 1998; Carapuço et al., 2005; Tümpel et al., 2009) and blood, in altered cases leading to defective hemopoesis (Lawrence et al., 1997); Hox gene products or Hox proteins are working as transcription factors, activating or inhibiting the transcription process to RNA. A majority of the vertebrate Hox proteins bind DNA as heterodimers with the Pre-B-cell leukemia transcription factor 1 (Pbx1) homeodomain protein (Piper et al., 1999) (Figure 1.12.). The shown structure suggests a model for modulation of Hox DNA binding activity by Pbx1 and related proteins.

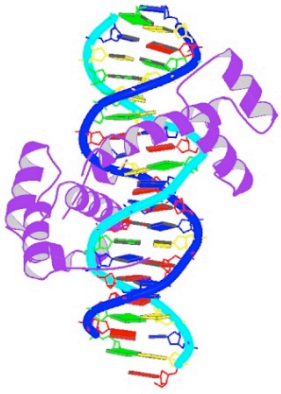

Figure 1.12. **Pbx1, homeobox protein HoxB1 and DNA ternary complex**
(Adapted from Piper et al., 1999)

Hox gene products have been researched in different fields of human medicine. The largest number of studies on Hox genes and their products are in oncology, as alteration in the transcription processes can contribute to the development of cancer (Cillo et al., 1992; Cillo, 1994-1995; Goodman and Scambler, 2001; Grier et al., 2005) and its activation (Chen et al., 2012). Large number of animal studies show impact of Hox genes on the development of leukemia (Lawrence et al., 1996; Magli et al., 1997; Thorsteinsdottir et al., 1997; Chiba, 1998; Shimamoto et al., 1998; Abramovich and Humphries, 2005; Argiropoulos and Humphries, 2007; Fröhling et al., 2007); research on humans confirm the importance of Hox genes on normal hemopoesis as well as development of malignancies (Celetti et al., 1993; Peschle et al., 1993; Giampaolo et al., 1994; Sauvageau et al., 1995; Thorsteinsdottir et al., 1997; Van Oostveen et al., 1999; Chung et al., 2009; Alharbi et al., 2012).

Hox gene products are highly associated with human reproduction. They are influencing development of female genital tract (Taylor, 2000); their altered expression lead to endometriosis and its caused infertility (Taylor et al., 1999; Kim et al., 2007; Cakmak et al., 2010; Zanatta et al., 2010). Hox gene products ensure implantation and decidualization (Taylor et al., 1999; Eun Kwon and Taylor, 1999; Daftary and Taylor, 2000; Vitiello et al., 2007; Lu et al., 2008), influence embryonic development and continue to act throughout lifetime (McGinnis and Krumlauf, 1992; Lappin et al.,2006). Hox gene products are involved in the maintenance of certain type of trophoblast cells (Amesse et al., 2003) and are needed for differentiation of cytotrophoblast into syncytiotrophoblast (Zhang et al., 2002).

Taking into account the above mentioned data we assumed that evaluation of Hox gene product positive cells in human placentas and correlation of the results with gestational time at delivery and pregnancy outcomes could be very interesting and clinically useful.

1.7. Correlations of the molecular factors in maternal-fetal unit with anthropometrical parameters of fetus

Impact of IGF1 on the human growth has been researched widely and is proved to have significant impact both on body size and proportions of children (Konen et al., 2009; Laron et al., 2012; Laron et al., 2012) as well as their ocular development (Bourla et al., 2006); IGF1 is used in the treatment of growth failure due to insufficiency of growth hormone, for example Laron syndrome. Level of IGF1 in the cord blood has been proposed for the confirmation of diagnosis of intrauterine growth restriction (IUGR) (Sifianou et al., 2011). IGF1 has also been found to participate in the development of fetal macrosomia not depending on maternal diabetc status (Roth et al., 1996). Receptor IGFR1, activated by IGF1, has also been found to be involved in the development of fetal macrosomia (Jiang et al., 2009).

HGF has more been described as a developmental agent of fetal nervous system (Trovato et al., 2007) and was found to be decreased in placentas malformed fetuses (Trovato et al., 2002); a few studies associate HGF with fetal growth as such, like animal studies that have shown decreased HGF in placentas of growth restricted (IUGR) mice fetuses (Somerset et al., 2000) and studies, having found less amount of HGF receptor in placentas of IUGR human fetuses (Baykal et al., 2005). Increased amount of HGF in the human colostrum also suggest its possible role in post-natal growth (Patki et al., 2012).

FGFR1 has not been descreibed to have a direct impact on the fetal growth, although its abundance in Höfbauer cells of placenta possibly confirm its role in the development of placental villi (Anteby et al., 2005), desirably leading to appropriate fetal growth.

Although interleukins are not usually associated with fetal growth as such, they have been found to have certain impact in patients with rheumatoid arthritis, in whom increased pro-inflammatory cytokines cause restriction of fetal growth while anti-inflammatory cytokines improve its growth (de Steenwinkel et al., 2012). Increased

level of pro-inflammatory cytokines in alcohol exposed mouse embryos (can also possibly suggest their impact on the fetal growth and development) (Roberson et al., 2012), as fetal alcohol syndrome is usually associated with growth restriction.

2. AIM AND TASKS 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.

The main tasks:

1. To provide statistical analysis of data of the women included in the study: age, some anthropometrical parameters, including placental weight, social characteristics, pregnancy data and risk factors; looking for correlations and differences between the study groups;
2. To provide statistical analysis of neonatal data: the most important anthropometrical parameters, some basic laboratory findings and clinical course; looking for correlations and differences between the study groups;
3. To detect distribution and appearance of growth factor and receptor positive cells in the samples of the acquired pre-term and term placentas of various gestational ages for evaluation of their impact on the placental and fetal growth and development;
4. To find out distribution and appearance of pro- and anti-inflammatory cytokine positive cells in the samples of the acquired pre-term and term placentas of various gestational ages for disclosure of their impact on the course of pregnancy and its outcome;
5. To reveal distribution and appearance of apoptotic cells in the samples of the acquired pre-term and term placentas of various gestational ages for assessment of the gestation and clinical course dependent non-destructive turnover of cells;
6. To determine distribution and appearance of tissue degrading enzyme positive cells in the samples of the acquired pre-term and term placentas of various gestational ages to assess their role on the onset of term and pre-term labor;
7. To ascertain presence of Hox gene products in the cells of the acquired post-delivery placentas of various gestational ages;

8. To look for the structures of the basement membrane in the tissues of the acquired pre-term and term placentas of various gestational ages for evaluation of gestation dependent status of the placental barrier;
9. To look for correlations between the factors of molecular processes in the placentas and pregnancy risk factors, gestational age of placenta, anthropometrical parameters and clinical indices of the mothers, placentas and newborns.

3. MATERIALS AND METHODS

3.1. Materials

On the 12th of March, 2009 the study was approved by the Ethics Committee of the Riga Stradins University. 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.

Exclusion criteria:

1. Maternal age less than 18 years (under-age);
2. Patients without antenatal care;
3. Patients with severe systemic disease;
4. Patients with positive status for human immunodeficiency virus (HIV);
5. Patients, having refused from the participation in the study.

Patient groups:

- Group 1 (healthy term): 14 patients of term deliveries from 37 gestational weeks with uncomplicated clinical courses and healthy children;
- Group 2 (pre-term): 25 patients of pre-term deliveries with premature children from 22 till 36 weeks of gestation;
- Group 3 (distress): 14 deliveries of various gestational ages (either pre-term or term) with documented fetal distress antenatally or during labor and delivery.

Immediately after delivery by a cut of a single use surgical knife there were taken two 1cm*1cm samples from symmetrically located places of the chosen placentas through all the layers of the placental tissues (Figure 3.1.) and placed into Picric Acid-Formaldehyde Fixation, having been originally described almost 50 years ago (Stefanini et al., 1967); samples were labeled with the study assigned number. After taking the samples patients were asked to complete the study survey. Samples were taken to the Institute of Anatomy and Anthropology of the Riga Stradins University for further processing. Patient data were **acquired from the medical records of the Riga Maternity hospital and the study survey.**

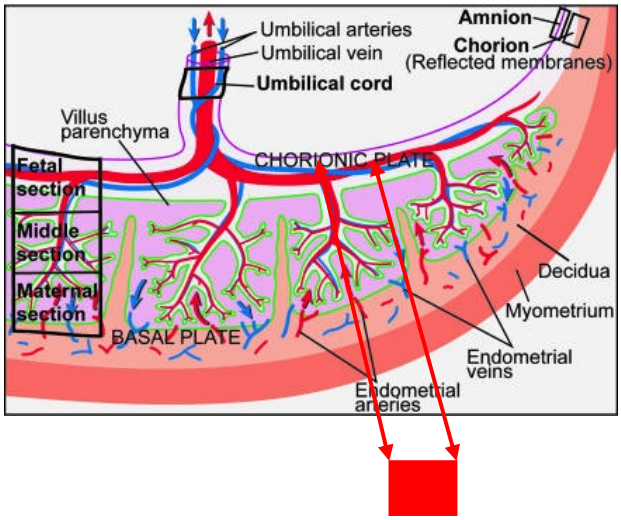

Figure 3.1. **Approximate cut-off of the placental samples**
Size ~ 1*1 cm (Adapted from Sood et al., 2006)

3.2. Methods

3.2.1. Patient data

Maternal and placental data were obtained from the medical record of Childbirth *(Dzemdību vēsture)* of the Riga Maternity hospital and the study survey:

- Maternal age, body weight before pregnancy and height, calculated body mass index (BMI=body mass(kg)/height(m)^2), weight gain during pregnancy;
- Pregnancy, number of previous childbirths;
- Course of pregnancy, risk factors (including harmful habits), pathologies;
- Gestational weeks at delivery, length of rupture of membranes (ROM), mode and course of delivery;
- Placental weight, macroscopic features.

Neonatal data were obtained from the medical record of Neonatal development *(Jaundzimušā attīstības vēsture)*:

- Gender, birthweight, body length, head and chest circumferences, calculated ponderal index (PI=100xbody mass(g)/height(cm)^3;
- Basic criteria of neonatal status after delivery: 1st and 5th minute Apgar scores (clinical evaluation), initial blood pH (homeostasis) and glucose level (nutrition).

3.2.2. Processing of the placental samples

Scheme of the processing of placental samples is shown in the Figure 3.2.

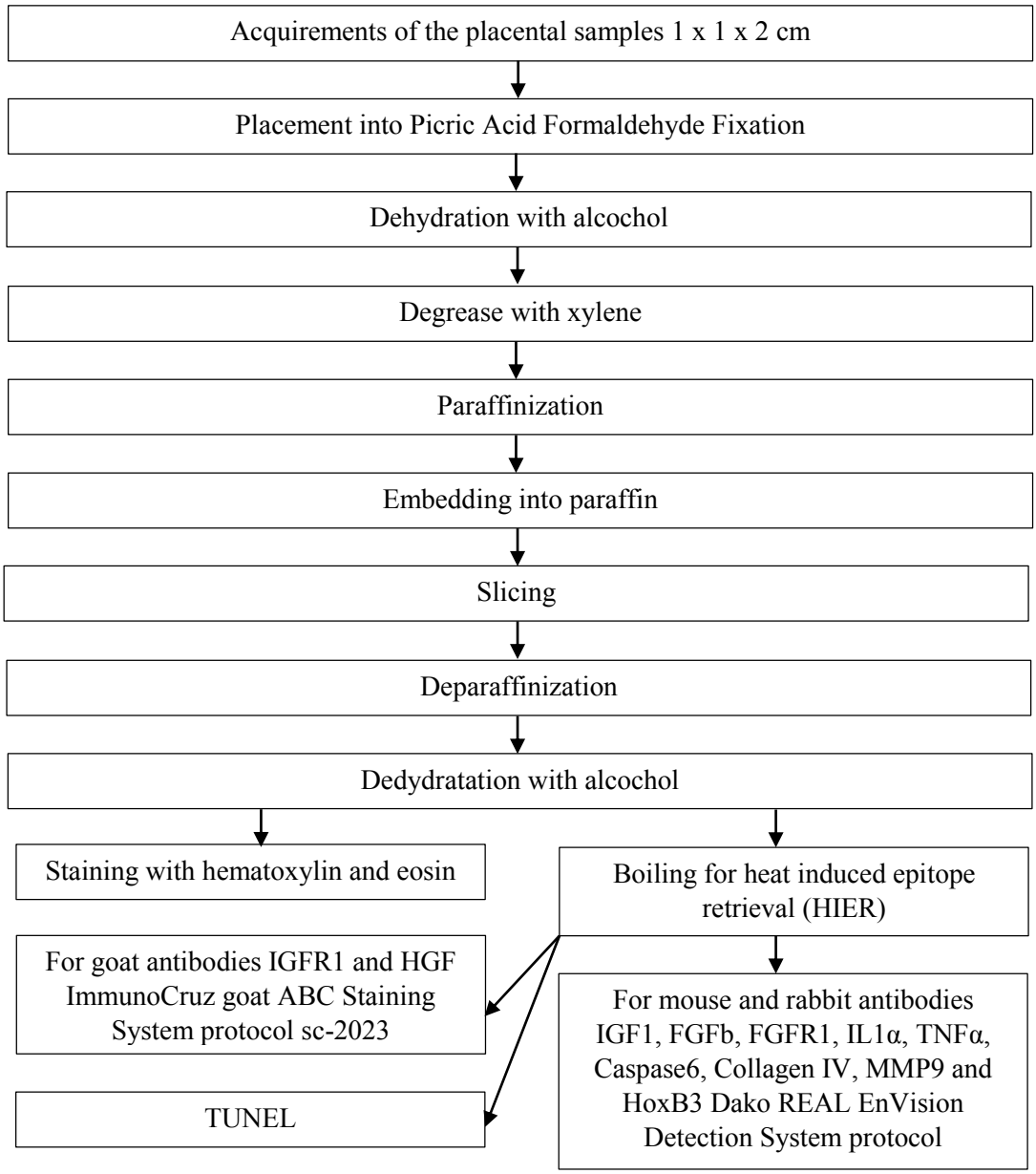

Figure 3.2. **Processing of the placental samples**

- **Routine staining with hematoxylin and eosin (H&E)**

Routine staining was provided in accordance with H&E Staining Method and Protocol (Avwioro, 2011; www.ihcworld.com) as follows:

1. Deparaffinization of sections, 2 changes of xylene, 10 minutes each;
2. Re-hydration in 2 changes of absolute alcohol, 5 minutes each;

3. 95% alcohol for 2 minutes and 70% alcohol for 2 minutes;
4. Washing briefly in distilled water;
5. Staining in Harris hematoxylin solution for 8 minutes;
6. Washing in running tap water for 5 minutes;
7. Differentiation in 1% acid alcohol for 30 seconds;
8. Washing in running tap water for 1 minute;
9. Bluing in 0.2% ammonia water or saturated lithium carbonate solution for 30 seconds to 1 minute;
10. Washing in running tap water for 5 minutes;
11. Rinsing in 95% alcohol, 10 dips;
12. Counterstaining in eosin-phloxine B solution (or eosin Y solution) for 30 seconds to 1 minute;
13. Dehydration through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each;
14. Clearing in 2 changes of xylene, 5 minutes each;
15. Mounting with xylene based mounting medium.

- **IHC staining with chosen antibodies**

Preparation of the samples for IHC processing with **mouse and rabbit antibodies** was provided in accordance with the Dako REAL^{TM} EnVision Detection System protocol (3rd edition, 2005) as follows:

1. Deparaffinization with xylene;
2. Dehydration with ethanol: $100^0 \rightarrow 96^0 \rightarrow 96^0 \rightarrow 70^0$;
3. Deparrafinized samples are placed into a slide holder;
4. The slide holder with slides are placed into a buffer container for HIER (heat induced epitope retrieval);
5. The container with the slide holder is placed into a microwave oven at least for 10' ($\geq$ 750V); buffer has to boil for 10 minutes;
6. The container with samples is taken out of the oven and remains cooling for 30 minutes;
7. The container is placed into a Tris-buffered saline (TBS) for washing;
8. Samples are blocked with the Peroxidase Block for 10 minutes;
9. Rinsing for 5 minutes, then rinsing with TBS for 5 minutes.

Staining with the DAKO EnVision^{TM} system:

1. Application of the primary antibody for 30 minutes;
2. Washing with TBS 2*5 minutes;
3. EnVision +/ bounding phase for 30 minutes;
4. Washing 2*5 minutes;
5. Application of Daco REAL™ EnVision™ Detection System (DAB) for 30 seconds to 1 minutes;
6. Washing 5 minutes;
7. Counterstaining with hematoxyllin and eosin (H&E) in correspondence with the Harris H&E staining protocol.

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) as follows:

1. After preparation of tissue sections, slides are incubated for 5-10 minutes in 0.1-1% hydrogen peroxide diluted in peroxidase blocking solution (PBS), deionized water (H_2O) or methanol to quench endogenus peroxidase activity;
2. Washing with PBS 2*5 minutes;
3. Incubation of the sections for one hour in 1.5% blocking serum in PBS and blotting of the excess blocking serum from the slides;
4. Incubation of the sections with primary antibody for 30 minutes at room temperature. Optimal antibody concentration is determined by titration; recommended range is 0.5-5.0 μg/ml, diluted in 1.5% blocking serum in PBS;
5. Washing with PBS 3*5 minutes;
6. Incubation of the sections for 30 minutes with biotinylated secondary antibody at approximately 1 μg/ml;
7. Washing with PBS 3*5 minutes;
8. Incubation of the sections for 30 minutes with AB enzyme reagent;
9. Washing with PBS 3*5 minutes;
10. Incubation of the sections in 1-3 drops peroxidase substrate for 10 minutes;
11. The section may be checked for staining by rinsing with H_2O and viewing under a microscope. If necessary, peroxidase substrate could be added and incubation continued. Sections are washed in deionized H_2O for 5 minutes;
12. Counterstain could be done with hematoxylin for 5-10 seconds. Samples are immediately washed with several changes of deionized H_2O.

13. For paraffin-embedded tissue sections, dehydratation is provided as follows: 2*95% ethanol for 10 seconds each, 2*100% ethanol for 10 seconds each, 3* xylenes for 10 seconds each. Excess of xylenes is wiped off;
14. Immediately 1-2 drops of permanent mounting medium is added; tissue section covered with a glass coverslip.

Table 3.1.

Antibodies, used in the study for IHC processing

	Antibody	Clone	Species reactivity	Manufacturer	Working 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 6	EP13254	rabbit	Abcam	1 : 200
10.	CollagenIV	CIV94	mouse	Invitrogen	1 : 30
11.	MMP9	polyclonal	rabbit	Santa Cruz	1 : 250
12.	HoxB3	polyclonal	rabbit	Santa Cruz	1 : 100

- **Staining of apoptotic cells by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)**

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):

1. Deparaffinization of the tissue slides:
   - Xylene 2*4 minutes;
   - Ethanol: 99^0 2*2 minutes → 95^0 2*2 minutes → 70^0 2*2 minutes;
   - Distilled water 7-10 minutes;
   - Washing in PBS (pH = 7.55) 10 minutes.
2. Slides were put into PBS+0.25% of TrixtonX-100 for 10 minutes at room temperature (on the vibrator);
3. Blocking of endogenous peroxidase activity was provided putting them for 30 minutes into 3% H_2O_2;
4. Washing 3*5 minutes;

5. Antigen retrieval: placement of slides into a plastic coplin jar in 0.2 ml of citrate buffer; boiling for 10 minutes in a microwave oven (700V). The slides should not run dry; jar is filled with distilled water when needed. Slides should cool down in the distilled water. Slides are washed in PBS;
6. Coverage of tissue cut with DNase, washing with PBS and blocking of slides in 0.1% bovine serum albumin (BSA);
7. Incubation of slides for 30 minutes with TUNEL mix (1:10) in 37^0C;
8. Washing in PBS;
9. Staining of slides in a converter – horse-radish peroxidase (POD) for 30 minutes in 37^0C;
10. Washing in PBS;
11. Developing with DAB substrate solution kit (Vector, Cat. number SK4100) for 7 minutes;
12. Washing in tap water for 5 minutes;
13. Counterstaining with hematoxyllin and eosin (H&E) in correspondence with Harris H&E staining protocol.

Apoptosis of the placental cells was detected by a TUNEL In situ cell death kit, manufactured by Roche, in a working dilution of 1:10.

- **Negative and positive controls**

Negative and positive controls were provided to avoid background staining and non-specific bounding of secondary antibodies. For negative control was used omitting of primary antibodies, obtaining preparations with no staining. For positive controls were used tissues, known to contain molecular factors either from the manufacturer's recommendations or from previous research studies in the Institute of Anatomy and Anthropology: tissues of human placenta for IGF1 and IGFR1, 6 weeks embryo for HGF, fetal liver for FGFb, umbilical cord for FGFR1, chicken intestines for IL-10, human small intestines for IL-1į, human psoriatic skin for TNFį, human breast cancer for Caspase, human skin for Collagen IV, human heart for MMP9 and human Fallopian tube for HoxB3 products. Most of the factors presented so convincing immunostaining in placental tissues that possibly can be used for positive controls in other research studies.

3.2.3. Evaluation of findings

Samples, routinely stained with H&E were evaluated qualitatively, looking for compliance of the visual maturation of placenta with gestational age at delivery as well as unusual findings with possible clinical significance.

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):

- 0 for none (0) indicator positive cells or ECM structures (in a visual field);
- 0,5 for occasional (0/+) indicator positive cells or ECM structures;
- 1 for few (+) indicator positive cells or ECM structures;
- 2 for moderate amount (++) of indicator positive cells or ECM structures;
- 3 for numerous (+++) indicator positive cells or ECM structures;
- 4 for abundance (++++) of indicator positive cells or ECM structures.

Number of apoptotic cells was detected in 10 visual fields; rounded average was stated as the stated finding of the sample.

3.2.4. Statistical processing of the results

For more detailed evaluation of the acquired data and more advanced interpretation of the results, epidemiological evaluation and correlation analysis were done in the following sections:

1. In the whole study, including 53 delivery cases;
2. In three study groups: 14 patients healthy 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 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.

Pearson product-moment correlation was used to inspect the linear correlation of mother and neonate specific data with their respective indicators, as well as between the indicators themselves. The correlation was used to infer associative relationships. Pearson correlation 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.

As the tested hypotheses do not stipulate the relationship direction, 2-tailed Pearson's correlation was used for non-directional analysis. Visual inspection for outliers by spotting wayward values in correlation analyses were performed to secure robustness checks. Data processing softwares Microsoft Excel 2013 Preview and IBM SPSS 19.0 were used; the reported statistical significance was set at $p<0.05$, as well as $p<0.01$ statistical significance is sometimes reported for more comprehensive research purposes.

4. RESULTS

4.1. Maternal data

The study included 53 delivery patients of different ages and numbers of pregnancies and childbirths; gestational time at the time of delivery varied from 22 till 40 weeks. 30 from the deliveries (57%) were vaginal and 23 (43%) were by Cesarean section (CS), 9 of them (20%) were emergency CS due to fetal distress.

Table 4.1.

Maternal data

	Minimum	Maximum	Mean ± SD
Maternal age	18	39	29.79 ± 5.6
Pregnancy	1	7	2.59 ± 1.64
Delivery	1	6	1.75 ± 0.98
Weeks	22	40	33.02 ± 5.18

Histogram of the maternal age showed distribution close to normal (Figure 4.1.).

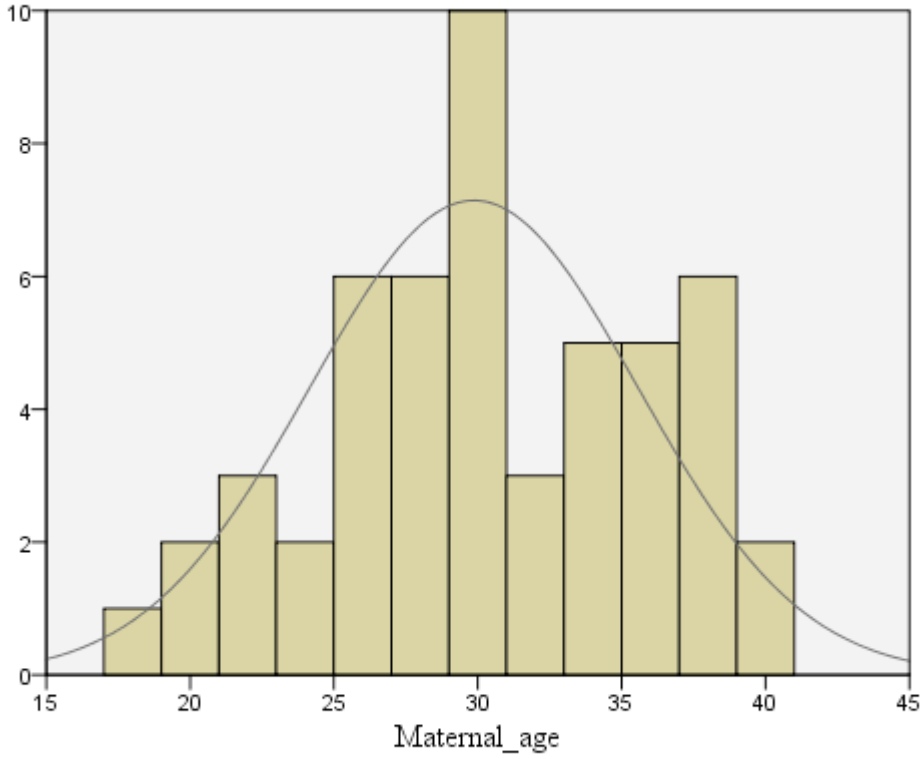

Figure 4.1. **Histogram of the distribution of the maternal age**

Slightly more than a half (57%) of the women were in legal marriage and two thirds (62%) resided in one of the 6 largest cities of Latvia. Almost half (47%) had an educational level of a university and all of them were committed to antenatal care.

Maternal BMI before the pregnancy varied from 17.2 till 36.6 (kg/m^2) with the mean of 23.74±4.72 kg/m^2. This one and the other maternal anthropometric measurements are shown in the Table 4.2.

Table 4.2.

Maternal anthropometric parameters

	Minimum	Maximum	Mean ± SD
Weight before the pregnancy (kg)	46	109	65.82 ± 12.75
Body height (cm)	150	182	166.33 ± 7.025
BMI before the pregnancy (kg/m^2)	17.20	42.60	23.79 ± 4.74
Weight gain during pregnancy (kg)	0	26	10.90 ± 5.81

Maternal age showed strong statistically significant correlation with the number of pregnancies and number of deliveries (Table 4.3.).

Table 4.3.

Correlations between the maternal data and risk factors

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

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

In the whole study maternal anthropometric parameters, including BMI, evaluated by Pearson 2-tail test, did not show statistically significant correlations with the maternal age, numbers of pregnancies or deliveries, as well as the scope of risk factors during actual pregnancy or gestational time at delivery ($p>0.05$).

The **study group G1 (term)** included 14 cases of normal term deliveries from 37 till 40 weeks of pregnancy, 9 of the deliveries were vaginal spontaneous and 5 by Cesarean section (Table 4.4.).

Table 4.4.

Maternal data of the study group G1 (term)

	Obstetrical findings	Age	BMI (kg/m^2)[4] before the actual pregnancy	Weight gain (kg)	ROM[5]
1.	GI PI w37[1], vaginal term	27	109/(1.60)^2 = 42.6	8	15
2.	GIII PIII w37, term CS[2]	37	55/(1.64)^2 = 20.4	8	0

Table 4.4. continued p.45

Table 4.4. (End)

	Obstetrical findings	Age	BMI (kg/m^2)[4] before the actual pregnancy	Weight gain (kg)	ROM[5]
3.	GII PII w37, term CS, breech presentation.	25	61/(1.60)^2 = 23.8	9	0
4.	GII PII w38, term CS	27	65/(1.56)^2 = 26.7	12	0
5.	GII PII w39, vaginal term	28	61/(1.52)^2 = 26.4	6	10
6.	GI (IVF[3]) PI w39, term CS, inborn glaucoma	39	61/(1.73)^2 = 20.4	13	0
7.	GI PI w39, vaginal term	23	81/(1.75)^2 = 25.5	14	
8.	GI PI w39, vaginal term	19	61/(1.67)^2 = 21.9	11	7
9.	GII PI w39, vaginal term	32	60/(1.62)^2 = 22.9	18	1
10.	GIII PIII w39, vaginal term	30	60/(1.72)^2 = 20.3	18	2
11.	GI PI, vaginal term, 39 weeks	25	70/(1.64)^2 = 26.0	19	9
12.	GI PI w40, term CS	21	59/(1.68)^2 = 20.9	15	0
13.	GI PI w40, vaginal term	21	61/(1.73)^2 = 20.4	19	4
14.	GIV PII, vaginal term, 40 weeks	37	83/(1.65)^2 = 30.5	12	5

1 – GI PI w37 – Gravidity I (1st pregnancy), Parity I (1st delivery), 38 weeks of pregnancy
2 – CS – Cesarean section
3 – IVF – *In vitro* fertilization
4 – BMI – Body mass index = body weight (kg)/ (height (m))^2
5 – ROM – Length of ruptured membranes in hours

The **study group G2 (pre-term)** included 25 cases of pre-term deliveries from 22 till 36 weeks of pregnancy, 17 vaginal and 8 by Cesarean section (Table 4.5.).

Table 4.5.

Maternal data of the study group G2 (pre-term)

	Obstetrical findings	Age	BMI[5] (kg/m^2) before the actual pregnancy	Weight gain (kg)	ROM[6]
1.	GI PI w22[1], pre-term vaginal	20	71/(1.70)^2 = 24.6	0	0
2.	GII PII w23, pre-term vaginal, PPROM[2]	36	58/(1.66)^2 = 21.0	10	74
3.	GIV PII w24, pre-term vaginal, chorionamnionitis	30	64/(1.76)^2 = 20.7	4	12
4.	GI PI w28, pre-term vaginal	18	79/(1.68)^2 = 28.0	0	0
5.	GVII PIII w28, pre-term vaginal, VHC[3] positive, syphilis in 2002, PPROM	34	88/(1.65)^2 = 32.3	7	144
6.	GI PI w28, pre-term vaginal	34	51/(1.56)^2 = 21.0	6	16
7.	GII PII w28, pre-term vaginal, PPROM	37	64/(1.58)^2 = 25.6	13	103
8.	GII PII w29, pre-term vaginal, PPROM	34	64/(1.64)^2 = 23.8	8	23
9.	GIV PIV w30, pre-term vaginal, PPROM, bartholinitis	32	55/(1.55)^2 = 22.9	4	359

Table 4.5. continued p.46

Table 4.5. (End)

	Obstetrical findings	Age	BMI[5] (kg/m^2) before the actual pregnancy	Weight gain (kg)	ROM[6]
10.	GII PII w30, pre-term vaginal, PPROM	36	64/(1.67)^2 = 22.9	15	54
11.	GII PII w30, pre-term vaginal	25	53/(1.64)^2 = 19.7	7	0
12.	GIV PI w31, pre-term CS[4]	28	59/(1.72)2 = 19.9	15	5
13.	GIV PI w31, pre-term CS	28	59/(1.72)2 = 19.9	15	5
14.	GII PII w31, pre-term CS, PPROM	34	60/(1.76)^2 = 19.4	13	185
15.	GI PI w31, pre-term vaginal, PPROM	35	89/(1.56)^2 = 36.6	4	40
16.	GI PI w32, pre-term CS, breech presentation	23	54/(1.66)^2 = 19.6	4	0
17.	GVII PVI w32, pre-term vaginal, breech presentation, VHC positive, chronic glomerulonephritis	30	80/(1.81)^2 = 24.4	4	0
18.	GIV PIII w33, pre-term CS	35	85/(1.78)^2 = 26.8	3	0
19.	GI PI w33, pre-term vaginal, PPROM	21	51/(1.65)^2 = 18.7	14	104
20.	GIV PII w33, pre-term vaginal, glucose intolerance, PPROM	37	81/(1.65)^2 = 29.8	8	48
21.	GII PI w34, pre-term CS, PPROM, transverse position of fetus	27	50/(1.64)^2 = 18.6	26	21
22.	GIII PII w34, pre-term vaginal	29	75/(1.68)^2 = 26.6	20	1
23.	GIII PII w34, pre-term vaginal	29	75/(1.68)^2 = 26.6	20	1
24.	GII PI w34, pre-term CS	27	59/(1.72)^2 = 19.9	26	0
25.	GII PI w34, pre-term CS, breech presentation	29	82/(1.64)^2 = 30.1	17	4

1 – GI PI w22– Gravidity I (1st pregnancy), Parity I (1st delivery), 22 weeks of pregnancy;
2 – PPROM – preterm premature rupture of membranes;
3 – VHC – Virus hepatitis C;
4 – CS – Cesarean section;
5 – BMI (kg/m^2) = weight kg/(height m)^2;
6 – ROM – Length of ruptured mebranes in hours.

The **study group G3 (distress)** included 14 delivery cases with significant antenatal or intranatal distress from 23 weeks of pregnancy till term (Table 4.6.); 5 of the deliveries were term and 9 pre-term. Group included 4 stillbirth cases and 1 case of early neonatal death due to consequences of perinatal asphyxia.

Table 4.6.

Maternal data of the study group G3 (distress)

	Obstetrical findings	Age	BMI (kg/m^2)[6] before the actual pregnancy	Weight gain (kg)	ROM[7]
1.	GI PI w23[1], induced vaginal pre-term, fetal renal failure (hydronephrosis), stillbirth	28	59/(1.72)^2 = 19.9	15	1

Table 4.6. continued p.47

Table 4.6. (End)

	Obstetrical findings	Age	BMI (kg/m^2)[6] before the actual pregnancy	Weight gain (kg)	ROM[7]
2.	GVII PIV w29, pre-term emergency CS[2] due to fetal distress, breech presentation, PPROM[3]	32	55/(1.55)^2 = 22.9	4	140
3.	GII PII w28, pre-term delivery emergency CS due to placental abruption and fetal distress, bicornate uterus	36	58/(1.66)^2 = 21.0	10	0
4.	GI PI w28, vaginal pre-term with fetal distress, chorionamnionitis	25	53/(1.64)^2 = 19.7	7	12
5.	GIV PI w30, pre-term emergency CS due to fetal distress, twin MH MA[4] pregnancy, demise of the other fetus	28	59/(1.72)^2 = 19.9	15	0
6.	GVI PII w31, pre-term emergency CS due to placental abruption and fetal distress	34	60/(1.76)^2 = 19.4	13	0
7.	GII PII w32, pre-term emergency CS due to fetal distress, mild preeclampsia, IUGR[5], oligohydramnion.	36	64/(1.67)^2 = 22.9	15	0
8.	GII PII w33, pre-term emergency CS due to severe pre-eclampsia and fetal distress	35	89/(1.56)^2 = 36.6	4	0
9.	GI PI w35, pre-term emergency CS due to fetal distress	34	64/(1.64)^2 = 23.8	8	0
10.	GIII PIII w37, term emergency CS due to placental abruption and fetal distress, stillbirth	37	64/(1.58)^2 = 25.6	13	21
11.	GV PII w38, vaginal term, stillbirth	23	54/(1.66)^2 = 19.6	4	1
12.	GIV PIII w38, term emergency CS due to fetal distress	34	51/(1.56)^2 = 21.0	6	4
13.	GI PI w40, vaginal term with fetal distress	21	51/(1.65)^2 = 18.7	14	10
14.	GIII PI w40, term emergency CS due to fetal distress, stillbirth	18	79/(1.68)^2 = 28.0	0	0

1 – GI PI w23 – Gravidity I (1st pregnancy), Parity I (1st delivery), 23 weeks of pregnancy;
2 – CS – Cesarean section;
3 – PPROM – Preterm premature rupture of membranes;
4 – MH MA – Monochorionic monoamniotic;
5 – IUGR – Intra-uterine growth restriction;
6 – BMI (kg/m^2) = weight kg/(height m)^2;
7 – ROM – Length of ruptured mebranes in hours.

The mean values of the maternal parameters in the study groups G1 (term) and G2 (pre-term) showed some statistically significant differences. T-test for the equality of means revealed statistically significantly higher numbers of pregnancies in G2 (pre-term) than in G1 (term) (Table 4.7.); length of rupture of membranes (ROM) was also higher in G2 (pre-term).

Table 4.7.

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

	Study groups	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

There were no differences of the mean values of those parameters between the study groups G1 (term) and G3 (distress) as well as G2 (pre-term) and G3 (distress) indicating, that the main reason of the difference is length of gestation. Similar findings were found between pre-term and term delivery patients of the whole study (Table 4.8.).

Table 4.8.

Comparison of the means of parameters between term and pre-term patients

	Groups	Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
Pregnancy	Term Pre-term	2.05 2.91	1.026 1.855	0.235 0.328	0.040*
Length of ROM	Term Pre-term	5.11 42.38	5.806 76.613	1.332 13.543	0.010**

* Correlation is significant at the 0.05 level.
** Correlation is significant at the 0.01 level.

4.2. Neonatal data

Totally were researched **53 neonatal cases**: 34 of patients were premature, born from 22 till 35 weeks of gestation with a weight from 540 till 2390 g; 19 term neonates were born from 37 till 40 weeks of gestation with a weight from 2740 till 4630 g. The median time of gestation was 33.06 weeks ± 5.09. Mean anthropometrical parameters of the whole study are shown in the Table 4.9.

Table 4.9.

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

Most of the babies (41 from 53) were appropriate for the gestational age (AGA); five were small for the gestational age (SGA) and nine – large for the gestational age (LGA) (Figure 4.2.). Appropriateness for the gestational age was evaluated in accordance with the Fetus Growth Charts Graphs and Calculators.

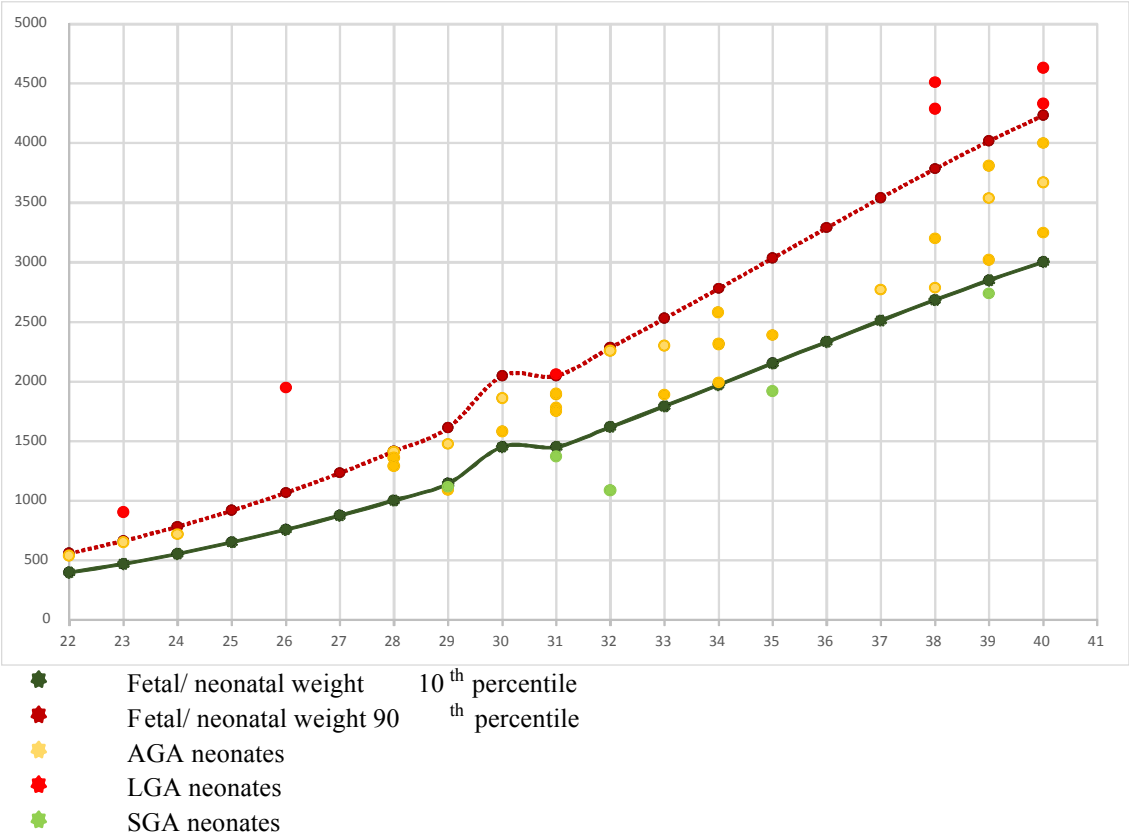

Figure 4.2. **Appropriateness for the gestational age**

Four of SGA babies were premature: 1114 g in 29 weeks, 1370 g in 31 week, 1088 g in 32 weeks and 1920 g in 35 weeks; one was a mature baby of 2740 g of body weight in 39 gestational weeks. From LGA babies three were premature: 905 g in 23 weeks, 1948 g in 26 weeks and 2060 g in 31 week; four babies were mature: two 38 weeks babies of 4510 g and 4290 g, one 39 gesatational weeks baby of 4410 g and two 40 weeks babies of 4330 g and 4630 g of body weight.

In the whole sample **anthropometric parameters**: body weight, body length, head and chest circumference **were directly proportional to the gestational age**, as shown in the Table 4.10.

Table 4.10.

Correlation of the anthropometric parameters of the neonates

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

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

The **study group G1 (term)** included 14 liveborn term neonates (7 girls and 7 boys) with a birth weight from 2740 g (SGA) till 4410 g (LGA) (Table 4.11.) and good health status; all of them were discharged home in the first week of life.

Table 4.11.

Neonatal data of the study group G1 (term)

	Neonatal findings	GA[4]	A[5]	Gender	Birth weight (g)	Body length (cm)	Head[6] (cm)	Chest[7] (cm)
1.	VSD[1]	37	8/8	Female	3020	49	34	35
2.	VSD	37	7/9	Female	3200	50	34	35
3.	Healthy	37	7/8	Female	3270	50	35	32
4.	Healthy	38	8/9	Male	4290	57	37	37
5.	Healthy	39	7/8	Female	2740	50	35	32
6.	Healthy	39	8/9	Male	3230	53	36	34
7.	Healthy	39	7/8	Female	3540	52	36	35
8.	VSD	39	8/9	Male	3670	52	35	35
9.	Healthy	39	8/9	Female	3810	55	35	36
10.	Healthy	39	8/9	Male	4000	54	36	35
11.	Healthy	40	7/8	Male	3250	54	35	33
12.	Transitory RD[2]	40	6/8	Female	3670	54	35	35
13.	Healthy	40	8/9	Male	4180	55	37	36
14.	LGA[3] Cephalohematoma Hyperbilirubinemia	39	7/8	Male	4410	56	37	36

1 – VSD – Ventricular septal defect
2 – RD – Respiratory distress
3 – LGA – Large for the gestational age
4 – GA – Gestational age in weeks
5 – A – Apgar scores (1st minute/ 5th minute)
6 – Head – Head circumference
7 – Chest – Chest circumference

The **study group G2 (pre-term)** included 25 live-born pre-term neonates (16 girls and 9 boys) from 22 till 36 weeks of gestation with the birth weight from 540 g

till 2580 g and various clinical courses (Table 4.12.), mainly determined by prematurity related health issues. 4 of the pre-term neonates were discharged home in the first week of life, 20 transferred to the Children hospital for futher treatment and 1 died in the NICU of the Maternity hospital from extreme prematurity.

Table 4.12.

Neonatal data of the study group G2 (pre-term)

	Neonate	GA[10]	A[11]	Gender	Birth weight (g)	Body length (cm)	Head[12] (cm)	Chest[13] (cm)
1.	Prematurity RDS IVH II Hyperbilirubinemia	22	6/6	Female	540	30	22	20
2.	Prematurity RDS Pneumonia	23	1/2	Male	650	28	22	20
3.	Prematurity RDS PDA[7] IVH II	24	3/5	Female	720	32	22	20
4.	Prematurity RDS Positive RPR[8]	28	4/6	Female	1130	36	23	24
5.	Early neonatal sepsis Prematurity RDS NE[3] IVH[4] II-III	28	6/7	Female	1190	38	26	23
6.	Prematurity RDS[1] Hyperbilirubinemia	28	7/8	Female	1290	37	27	24
7.	Prematurity Transitory RD IVH I Hyperbilirubinemia	28	7/7	Female	1410	41	27	25
8.	Prematurity Transitory RD NE Hyperbilirubinemia	29	7/7	Male	1476	39	26	24
9.	Prematurity Transitory RD IVH II Hyperbilirubinemia	30	5/7	Female	1580	41	28	25
10.	Prematurity IVH II Transitory RD[5]	30	7/8	Male	1710	42	28	26
11.	Prematurity RD NE	30	6/7	Male	1750	44	30	29
12.	Prematurity. SGA[6] Transitory RD Hyperbilirubinemia	31	7/7	Female	1370	38	31	24
13.	Prematurity Transitory RD Hyperbilirubinemia	31	7/7	Female	1780	45	31	26
14.	Prematurity RDS IVH II Hyperbilirubinemia	31	7/7	Male	1888	40	31	29
15.	Prematurity Pneumonia IVH II Bilateral renal pielectasy Hyperbilirubinemia	31	7/8	Male	2060	46	30	29
16.	Prematurity Transitory RD SA[9]	32	7/7	Male	1940	46	30	28
17.	Prematurity Transitory RD Hyperbilirubinemia	32	7/7	Female	2258	49	30	29

Table 4.12. continued p.52

Table 4.12. (End)

	Neonate	GA[10]	A[11]	Gender	Birth weight (g)	Body length (cm)	Head[12] (cm)	Chest[13] (cm)
18.	Prematurity Early neonatal sepsis NE IVH II	33	7/7	Female	1948	46	32	28
19.	Prematurity VSD[2]	33	7/7	Female	1992	45	32	28
20.	Prematurity RD Hyperbilirubinemia	33	7/8	Male	2300	45	33	31
21.	Prematurity	34	7/8	Female	2310	48	32	29
22.	Prematurity	34	7/8	Female	2320	42	33	32
23.	Prematurity Transitory RD Hyperbilirubinemia	34	7/7	Female	2390	47	33	29
24.	Prematurity Transitory RD Meningitis Partial ileus	34	7/8	Male	2450	48	32	29
25.	Prematurity Transitory RD	34	7/7	Female	2580	44	33	32

1 – RDS – Respiratory distress syndrome, caused by the deficiency of surfactant
2 – VSD – Ventricular septal defect, the most common congenital heart defect
3 – NE – Neonatal encephalopathy, clinical assessment of neurologic disturbance
4 – IVH – Intraventricular haemorrhage, non-traumatic brain injury of premature neonates
5 – RD – Respiratory distress, clinical signs of respiratory failure
6 – SGA – Small for the gestational age
7 – PDA – Patent ductus arteriosus
8 – RPR – Rapid plasma reagin, possibly indicating syphilis
9 – SA – Non-traumatic subarachnoid haemorrhage
10 – GA – Gestational age
11 – A – Apgar scores (1st minute/ 5th minute)
12 – Head – Head circumference
13 – Chest – Chest circumference

The **study group G3 (distress)** included 14 live or stillborn neonates, born after significant ante- or intranatal distress, from 23 till 40 weeks of gestation (7 girls, 7 boys); 5 were term and 9 pre-term (Table 4.13.). Neonates presented different clinical courses: 2 were discharged home in the first week of life, 8 transferred to the Children hospital for further treatment; 4 of the children were stillborn.

Table 4.13.

Neonatal data of the study group G3 (distress)

	Neonate	GA[9]	A[10]	Gender	Birth weight (g)	Body length (cm)	Head[11] (cm)	Chest[12] (cm)
1.	Bilateral hydronephrosis Secondary pulmonary hypoplasia and anomalies of limbs	23	0/0	Male	905	34	24	22
2.	Prematurity NE[1] IVH II RDS[2]	28	6/7	Male	1090	35	27	23

Table 4.13. continued p.53

Table 4.13. (End)

	Neonate	GA[9]	A[10]	Gender	Birth weight (g)	Body length (cm)	Head[11] (cm)	Chest[12] (cm)
3.	Prematurity Transitory RD Bacteremia (E.coli).	28	6/7	Female	1360	41	27	24
4.	Prematurity RD Pneumonia IVH I Hyperbilirubinemia	29	6/7	Female	1114	40	27	25
5.	Prematurity Transitory RD Anaemia (TTT[3])	30	7/7	Male	1860	41	30	28
6.	Prematurity RD Pneumonia IVH II	31	7/8	Male	1896	44	30	28
7.	Prematurity, SGA[4] Transitory RD[5] Hyperbilirubinemia.	32	7/7	Female	1088	38	26	23
8.	Prematurity Transitory RD	33	7/7	Female	1888	43	31	28
9.	Prematurity SGA	35	7/7	Male	1920	45	31	28
10.	Perinatal asphyxia	37	0/0	Female	2770	49	33	32
11.	Perinatal asphyxia	38	0/0	Female	2786	54	32	29
12.	HIE[6] LGA[7]	38	0/2	Male	4510	55	39	36
13.	Early neonatal sepsis (clinically GBS[8])	40	1/2	Male	4330	56	36	35
14.	Perinatal asphyxia LGA	40	0/0	Female	4630	59	37	37

1 – NE – Neonatal encephalopathy
2 – RDS – Respiratory distress syndrome
3 – TTT – Twin-to-twin transfusion
4 – SGA – Small for the gestational age
5 – RD – Respiratory distress
6 – HIE – Hypoxic ischemic encephalopathy, meeting the criteria, defined by ACOG and AAP task force (Longo and Hankins, 2009)
7 – LGA – Large for the gestational age
8 – GBS – Group B streptococci
9 – GA – Gestational age in weeks
10 – A – Apgar scores (1st minute/ 5th minute)
11 – Head – Head circumference
12 – Chest – Chest circumference

Evaluating **anthropometrical parameters** of the neonates, appeared strong significant differences of the neonatal anthropometrical parameters between the study groups G1 (term) and G2 (pre-term) as well as between G1 (term) and G3 (distress) (Table 4.14.).

Table 4.14.

Comparison of neonatal anthropometrical parameters between the study groups

	Study groups	N	Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
Birthweight	G1 (term)	14	3591.43	503.019	134.438	0.000**
	G2 (pre-term)	25	1721.28	578.325	115.665	
	G1 (term)	14	3591.43	503.019	134.438	0.002**
	G3 (distress)	14	2296.21	1322.32	353.406	

Table 4.14. continued p.54

Table 4.14. (End)

	Study groups	N	Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
Body length	G1 (term)	14	52.93	2.495	0.667	0.000**
	G2 (pre-term)	25	41.48	5.665	1.133	
	G1 (term)	14	52.93	2.495	0.667	0.002**
	G3 (distress)	14	45.29	8.062	2.155	
Head circumference	G1 (term)	14	35.50	1.019	0.272	0.000**
	G2 (pre-term)	25	28.96	3.691	0.738	
	G1 (term)	14	35.50	1.019	0.272	0.001**
	G3 (distress)	14	30.71	4.410	1.179	
Chest circumference	G1 (term)	14	34.71	1.490	0.398	0.000**
	G2 (pre-term)	25	26.52	3.537	0.707	
	G1 (term)	14	34.71	1.490	0.398	0.000**
	G3 (distress)	14	28.43	4.957	1.325	

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

Evaluating other parameters, appeared **strong significant differences** between the 1st and 5th minute evaluations of the **Apgar scores** (Apgar, 1953) between G1 (term) and G2 (pre-term) neonates; differences of the Apgar scores of neonates in the study group G3 (distress) presented strong significant differences with neonates of both other groups G1 (term) and G2 (pre-term) (Table 4.15.).

Table 4.15.

Comparison of Apgar score evaluation of neonates in the study groups

	Study groups	N	Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
1st minute Apgar score	G1 (term)	14	7.43	0.646	0.173	0.002**
	G2 (pre-term)	25	6.28	1.514	0.303	
	G1 (term)	14	7.43	0.646	0.173	0.001**
	G3 (distress)	14	3.86	3.348	0.895	
1st minute Apgar score	G2 (pre-term)	25	6.28	1.514	0.303	0.004**
	G3 (distress)	14	3.86	3.348	0.895	
5th minute Apgar score	G1 (term)	14	8.50	0.519	0.139	0.000**
	G2 (pre-term)	25	6.92	1.256	0.251	
	G1 (term)	14	8.50	0.519	0.139	0.000**
	G3 (distress)	14	4.36	3.388	0.905	
	G2 (pre-term)	25	6.92	1.256	0.251	0.002**
	G3 (distress)	14	4.36	3.388	0.905	

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

4.3. Placental data

Samples were taken from 53 post-delivery placentas with no remarkable anomalies, with a weight from 220 g till 930 g, with a mean of of 448.95 ± 157.503 g.

4.3.1.The mean values of the placental weight

The **mean values of the placental weight** in the study groups varied (Table 4.16.).

Table 4.16.

Placental weight in the study groups

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

Comparison of the mean values of the placental weight in the study groups showed significantly lower mean placental weight in G2 (pre-term) in comparison with G1 (term) ones (Table 4.17.). Differences with the mean value of the placental weight in the study group G3 (distress) were not significant.

Table 4.17.

The mean values of placental weight in the study groups

		Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
Placental weight	G1 (term)	663.33	102.63	59.255	0.007**
	G2 (pre-term)	403.39	145.39	30.432	

** Correlation is significant at the 0.01 level

Evaluating differences between the values of the mean weight of **pre-term and term placentas** in the whole study, significance was even stronger (Table 4.18.).

Table 4.18.

The mean values of the placental weight in the whole study

		Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
Placental weight	Study term	637.50	91.924	32.50	0.000**
	Study pre-term	401.81	133.54	23.607	

4.3.2. Correlations between the placental weight and maternal parameters

Evaluating correlations of the placental weight with maternal parameters: age, pregnancy, number of childbirths, weight, length, body mass index (BMI) prior to the actual pregnancy and weeks of gestation **in the whole study** we found **statistically significant positive correlations** of the placental weight with maternal weight before and weight gain during actual pregnancy (Table 4.19.). **Strong correlations** appeared between the placental weight and weeks of gestation.

Table 4.19.

Placental weight and maternal parametrs: the whole study

		Weight of placenta
Weeks of gestation	Pearson corr.	0.537**
	Sig. (2-tailed)	0.001
Weight before pregnancy	Pearson corr.	0.342*
	Sig. (2-tailed)	0.036
Weight gain in pregnancy	Pearson corr.	0.342*
	Sig. (2-tailed)	0.036

* Correlation is significant at the 0.05 level (2-tailed);
** Correlation is significant at the 0.01 level (2-tailed).

Correlating placental weight with the maternal parameters **in the study groups** our findings differed: in the study group G1 (term) placental weight did not correlate with any of the maternal factors, in G2 (pre-term) it correlated with the gestation and maternal weight prior to pregnancy and in the study group G3 (distress) placental weight correlated with the maternal weight gain during pregnancy (Table 4.20.).

Table 4.20.

Placental weight and maternal parametrs in the study groups

Maternal parameter		Weight of placenta
Study group G2 (pre-term)		
Weeks of gestation	Pearson corr.	0.631**
	Sig. (2-tailed)	0.001
Weight before pregnancy	Pearson corr.	0.492*
	Sig. (2-tailed)	0.020
Study group G3 (distress)		
Weeks of gestation	Pearson corr.	0.544*
	Sig. (2-tailed)	0.044

* Correlation is significant at the 0.05 level;
** Correlation is significant at the 0.01 level.

Comparing mean weight of **term and pre-term placentas of the whole study** in term cases we found strong correlation of the placental weight with the maternal

weight gain during pregnancy; in pre-term cases correlation of the placental weight with the weeks of gestation (Table 4.21.).

Table 4.21.

Placental weight and maternal parametrs: term placentas of the whole study

Maternal parameter		Weight of placenta
Term placentas of the whole study		
Weight gain in pregnancy	Pearson corr. Sig. (2-tailed)	0.863** 0.006
Pre-term placentas of the whole study		
Weeks of gestation	Pearson corr. Sig. (2-tailed)	0.358* 0.044

* Correlation is significant at the 0.05 level;
** Correlation is significant at the 0.01 level.

4.3.3. Correlations between the placental weight and neonatal parameters

In the whole study placental weight directly correlated with all the assessed neonatal anthropometrical parameters: body weight and length, head and chest circumferences (Table 4.22.).

Table 4.22.

Placental weight and neonatal parametrs: the whole study

Parameter		Weight of placenta
Body weight	Pearson corr. Sig. (2-tailed)	0.749** 0.000
Body length	Pearson corr. Sig. (2-tailed)	0.692** 0.044
Head circumference	Pearson corr. Sig. (2-tailed)	0.725** 0.000
Chest circumference	Pearson corr. Sig. (2-tailed)	0.788* 0.000

* Correlation is significant at the 0.05 level;
** Correlation is significant at the 0.01 level.

In the study groups we found the following correlations (Table 4.23.): in the study group G1 (term) placental weight positively correlated with the chest circumference of the neonate and strong negative correlation was seen with neonate's ponderal index. Study group G2 (pre-term) presented positive correlations between the placental weight and all the assessed neonatal anthropometrical parameters; in this study group weight of placenta also positively correlated with both Apgar score grades. In the study group G3 (distress) placental weight had only positive correlation with the ponderal index of the neonate.

Table 4.23.

Placental weight and neonatal parametrs: the study groups

Neonatal parameter		Weight of placenta
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 G2 (pre-term)		
Body weight	Pearson corr. Sig. (2-tailed)	0.746** 0.000
Body length	Pearson corr. Sig. (2-tailed)	0.743** 0.000
Head circumference	Pearson corr. Sig. (2-tailed)	0.697** 0.000
Chest circumference	Pearson corr. Sig. (2-tailed)	0.781* 0.000
1st minute Apgar score	Pearson corr. Sig. (2-tailed)	0.551** 0.006
5th minute Apgar score	Pearson corr. Sig. (2-tailed)	0.603** 0.002
Study group G3 (distress)		
Ponderal index	Pearson corr. Sig. (2-tailed)	0.554* 0.040

* Correlation is significant at the 0.05 level;
** Correlation is significant at the 0.01 level.

Weight of **term placentas of the whole s**tudy did not show correlations with any of the neonatal parameters, while weight of **pre-term placentas of the whole study** presented strong positive correlations with all the assessed neonatal anthropometrical parameters (Table 4.24.).

Table 4.24.

Placental weight and neonatal parametrs: pre-term placentas of the whole study

Parameter		Weight of placenta
Body weight	Pearson corr. Sig. (2-tailed)	0.606** 0.000
Body length	Pearson corr. Sig. (2-tailed)	0.566** 0.000
Head circumference	Pearson corr. Sig. (2-tailed)	0.548** 0.001
Chest circumference	Pearson corr. Sig. (2-tailed)	0.629** 0.000

** Correlation is significant at the 0.01 level

4.3.4. Routine microscopy of the placental samples

Placentas were of various maturity: placentas of the late second trimester (22-27 weeks) appeared to be young (Appendix, Figures 1., 2. and 3.), while from the beginning of the third trimester they presented to be transititonal (Appendix, Figures 4. and 5.), acquiring certain signs of ageing approaching term (Appendix, Figures 6. and 7.).

4.4. Immunohistochemical (IHC) findings in the placental samples

4.4.1. Growth factors and receptors

Most of the researched growth factors were found in almost all the samples. Amount of **IGF1** containing cells did not correlate with the gestational age of placenta and appeared to be from 0 till abundant (++++) in placentas of different gestational ages (Appendix, Figures 8.-12.). IGF1 positive were different types of placental cells: cytotrophoblast, syncitiotrophoblast, proliferation buds, extravillous trophoblast, Höfbauer cells, cells of extreambryonic mesoderm and amnial epithelium.

Findings **in the study groups** (Table 4.25.):

- Placental samples of the study group G1 (term) presented occasional (0/+) till numerous (+++) IGF1 positive cells in a visual field;
- Placentas of the study group G2 (pre-term) contained none (0) till abundant (++++) IGF1 positive cells in a visual field (Appendix, Figures 8.-10.);
- In the placentas of the study group G3 (distress) were seen occasional (0/+) till numerous (+++) IGF1 positive cells in a visual field (Appendix, Figures 11. and 12.).

Table 4.25.

Results of semi-quantitative evaluation of IGF1 positive cells in placentas of the study groups

	G1 (term)	G2 (pre-term)	G3 (distress)
1.	++	+++	+
2.	0/+	+	++
3.	++	++	+
4.	++	+	+
5.	+	+	0/+
6.	+	+	+
7.	+	+	+

Table 4.25. continued p.60

Table 4.25. (End)

	G1 (term)	G2 (pre-term)	G3 (distress)
8.	+	0	++
9.	+	+++	+++
10.	0/+	++	++
11.	0/+	+++	++
12.	+++	++	++
13.	++	+++	++
14.	+	++	+
15.		++	
16.		++++	
17.		++	
18.		+	
19.		+	
20.		+	
21.		0/+	
22.		++	
23.		+++	
24.		+	
25.		+	

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. Found mean rank values of the amount of IGF1 positive cells are shown in the Table 4.26.

Table 4.26.

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

	Group	The mean ± SD
1.	The whole study	1.58 ± 0.86
2.	Study group G1 (term)	1.32 ± 0.75
3.	Study group G2 (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

Differences between the mean rank values of IGF1 positive cells in the placentas of the study groups as well as between term and pre-term placentas were not statistically significant.

Amount of **IGFR1** containing cells presented significant negative correlation with the gestational age, although amounts from none (0) to abundant (++++) they were seen in the placentas of all the gestational ages (Appendix, Figures 13.-18.). IGFR1 positive were different types of placental cells: cytotrophoblast, syncitiotrophoblast,

proliferation buds, extravillous trophoblast, Höfbauer cells and cells of extraembryonic mesoderm.

Findings **in the study groups** (Table 4.27.):

- Placental samples of the study group G1 (term) presented none (0) till numerous (+++) IGFR1 positive cells in a visual field;
- Placentas of the study group G2 (pre-term) contained none (0) till abundant (++++) IGFR1 positive cells in a visual field (Appendix, Figures 13.-17.);
- In the placentas of the study group G3 (distress) were seen occasional few (+) till abundant (++++) IGFR1 positive cells in a visual field (Appendix, Figure 18.).

Table 4.27.

Results of semi-quantitative evaluation of IGFR1 positive cells in placentas of the study groups

	G1 (term)	G2 (pre-term)	G3 (distress)
1.	+	+++	+++
2.	0/+	++	+++
3.	+++	+++	++
4.	++	+	++
5.	++	0/+	++
6.	+	+++	++
7.	++	++++	+++
8.	+	++++	++
9.	++	++++	++
10.	+	++	++
11.	0	+++	++
12.	0/+	+++	+
13.	+	++	++++
14.	0/+	++++	++
15.		++++	
16.		++++	
17.		++	
18.		+	
19.		0/+	
20.		+	
21.		0/+	
22.		+++	
23.		++	
24.		+	
25.		++	

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. Found mean rank values of the amount of IGFR1 positive cells are shown in the Table 4.28.

Table 4.28.

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

	Group	The mean ± SD
1.	The whole study	2.06 ± 1.12
2.	Study group G1 (term)	1.25 ± 0.83
3.	Study group G2 (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

Comparison of the mean rank values of IGFR1 positive cells in placental cells identified **statistically significant differences between the study groups** G2 (pre-term) and G3 (distress) with the study group G1 (normal term) as well as between term and pre-term placentas of the whole study.

Table 4.29

Differences between the mean rank values of IGFR1 positive cells

	Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
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**
Term Pre-term	1.553 2.338	0.9113 1.1396	0.2091 0.1954	0.009**

** Correlation is significant at the 0.01 level

Amount of **HGF** containing cells did not correlate with the gestational age of placenta and appeared from none (0) till abundant (++++) in a visual field in the placentas of all the gestational ages (Appendix, Figures 19.-24.). HGF positive were different types of placental cells: cytotrophoblast, syncitiotrophoblast, proliferation buds, extravillous trophoblast, Höfbauer cells and cells of extraembryonic mesoderm.

Findings **in the study groups** (Table 4.30.):

- Placental samples of the study group G1 (term) presented none (0) till numerous (+++) HGF positive cells in a visual field (Appendix, Figures 19.-20.);
- Placentas of the study group G2 (pre-term) contained occasional (0/+) till abundant (++++) HGF positive cells in a visual field (Appendix, Figures 21.- 23.);

- In the placentas of the study group G3 (distress) were seen occasional few (+) till abundant (++++) HGF positive cells in a visual field (Appendix, Figure 24.).

Table 4.30.

Results of semi-quantitative evaluation of HGF positive cells in placentas of the study groups

	G1 (term)	G2 (pre-term)	G3 (distress)
1.	+	++	++
2.	+	+	+++
3.	++	+++	+
4.	++	++	+
5.	+	+++	+
6.	+++	++	++
7.	++	+++	+
8.	+	+++	+++
9.	0/+	++++	++
10.	+	++	+++
11.	0	++	++
12.	+	+++	+
13.	0/+	+	+++
14.	0/+	+	0/+
15.		++	
16.		+	
17.		0/+	
18.		++	
19.		0/+	
20.		+	
21.		+	
22.		0/+	
23.		+	
24.		+	
25.		+	

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. Found mean rank values of the amount of HGF positive cells are shown in the Table 4.31.

Table 4.31

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

	Group	The mean ± SD
1.	The whole study	1.61 ± 0.94
2.	Study group G1 (term)	1.17 ± 0.80
3.	Study group G2 (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

Differences between the mean rank values of HGF positive cells in the placentas of the study groups as well as between term and pre-term placentas were not statistically significant.

Basic FGF containing cells with predominantly weak immunoreactivity were seen in just a few placentas with the best view in two term placentas shown in the Appendix, Figures 25. and 26.; due to very small number of visually identifiable samples with bFGF positive cells this indicator was excluded from further resarch in our study.

Amount of **FGFR1** containing cells did not correlate with the gestational age of placenta, from occasional (0/+) to abundant (++++) in a visual field they were found in all the placentas (Figures 27.-34.). FGFR1 contained different types of placental cells: cytotrophoblast, syncitiotrophoblast, proliferation buds, extravillous trophoblast, Höfbauer cells, cells of extraembryonic mesoderm and amnial epithelium.

Findings **in the study groups** (Table 4.32.):

- Placental samples of the study group G1 (term) presented few (+) till abundant (++++) FGFR1 positive cells in a visual field (Appendix, Figures 27.-28.);
- Placentas of the study group G2 (pre-term) contained occasional (0/+) till abundant (++++) FGFR1 positive cells in a visual field (Appendix, Figures 29.-34.);
- In the placentas of the study group G3 (distress) were seen occasional (0/+) till abundant (++++) FGFR1 positive cells.

Table 4.32.

Results of semi-quantitative evaluation of FGFR1 positive cells in placentas of the study groups

	G1 (term)	G2 (pre-term)	G3 (distress)
1.	++	+++	++
2.	+++	+++	++
3.	+++	++++	++++
4.	+++	+++	+++
5.	++	++	++
6.	+	+++	++
7.	+++	+++	++
8.	++	++	+++
9.	++	+++	++
10.	+	++	+++
11.	++	++++	0/+
12.	+++	++	+++
13.	+++	++	+
14.	++++	++	+
15.		++	
16.		+	
17.		+	
18.		0/+	
19.		+	
20.		0/+	
21.		++	
22.		+++	
23.		++	
24.		++++	
25.		+++	

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. Found mean rank values of the amount of FGFR1 positive cells are shown in the Table 4.33.

Table 4.33.

The rank values of FGFR1 positive cells in different divisions of the study

	Group	The mean ± SD
1.	The whole study	2.31 ± 0.95
2.	Study group G1 (term)	2.46 ± 0.88
3.	Study group G2 (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

Differences between the mean rank values of FGFR1 positive cells in the placentas of the study groups as well as between term and pre-term placentas were not statistically significant.

Correlations between the rank values of growth factors

Correlating the rank values of the growth factors **among themselves** in the placentas of **the whole study** we found, that correlations between all of them are positive; statistically strong correlations presented the rank value of IGFR1 with IGF1 and HGF (Table 4.34.).

Table 4.34.

Correlation between the rank values of the growth factors and receptors in the whole study

		IGF1	IGFR1	HGF
IGF1	Pearson corr. Sig. (2-tailed)	1	0.363** 0.007	0.173 0.215
IGFR1	Pearson corr. Sig. (2-tailed)	0.363** 0.007	1	0.469** 0.000
HGF	Pearson corr. Sig. (2-tailed)	0.173 0.215	0.469** 0.000	1

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

Correlations between findings **in the study groups**: in the study group G1 (term) there were no statistically significant correlations; the rank values of IGF1 positively correlated with the rank values of IGFR1 in the placentas of the study group G2 (pre-term) and with the rank values of HGF in the placentas of the study group G3 (distress) (Table 4.35.)

Table 4.35

Correlations between the rank values of the growth factors in the study groups

Parameter		IGFR1	HGF
Study group G2 (pre-term)			
IGF1	Pearson Correlation Sig. (2-tailed)	0.430* 0.032	-0.030 0.888
Study group G3 (distress)			
IGF1	Pearson Correlation Sig. (2-tailed)	-0.022 0.941	0.590* 0.026

* Correlation is significant at the 0.05 level

Correlations between findings in **term and pre-term** placentas: we found similar correlation between the rank values of IGFR1 and HGF in the term placentas of

the whole study and in the study group G3 (distress); in pre-term placentas the rank values of IGFR1 correlated with IGF1 and with HGF (Table 4.36.).

Table 4.36

Correlations between the rank values of the growth factors in term and pre-term placentas of the whole study

Parameter		IGF1	HGF
Term placentas of the whole study			
IGFR1	Pearson Correlation Sig. (2-tailed)	0.227 0.349	0.606** 0.006
Pre-term placentas of the whole study			
IGFR1	Pearson Correlation Sig. (2-tailed)	0.368* 0.032	0.498** 0.003

* Correlation is significant at the 0.05 level;
** Correlation is significant at the 0.01 level (2-tailed).

Correlations of the rank values of growth factors with maternal parameters

Correlating the rank values of the growth factor and receptor positive cells in placentas with maternal parameters in **the whole study** we found statistically significant negative correlations between the rank values of IGFR1 and HGF with the maternal weight gain during pregnancy and a negative correlation of the rank value of IGFR1 with the gestational time at delivery (Table 4.37.).

Table 4.37.

Correlations of the rank values of the growth factors and receptors with maternal parameters in the whole study

Parameter		IGFR1	HGF
Weight gain	Pearson Correlation Sig. (2-tailed)	-0.283* 0.044	-0.301* 0.032
Gestation	Pearson Correlation Sig. (2-tailed)	-0.427** 0.001	-0.263 0.057

** Correlation is significant at the 0.01 level (2-tailed);
* Correlation is significant at the 0.05 level (2-tailed).

Correlations of the rank values of the growth factor and receptor positive cells in placentas of the **study groups** (Table 4.38.): in the study group G1 (term) we found statistically significant positive correlations of the rank values of HGF with the maternal BMI before the actual pregnancy; rank values of FGFR1 showed negative correlations with the maternal weight before pregnancy. In the study group G2 (pre-term) was found a statistically significant negative correlation between the rank values of IGF1 and placental weight and in the study group G3 (distress) rank values of HGF were lower in patients with a larger number of deliveries.

Table 4.38.

Correlations of the rank values of the growth factors with maternal parameters in the study groups

Parameter		IGF1	HGF	FGFR1
Study group G1 (term)				
Maternal BMI before pregnancy	Pearson Correlation	-0.168	0.601*	-0.517
	Sig. (2-tailed)	0.565	0.023	0.071
Maternal weight before pregnancy	Pearson Correlation	-0.289	0.524	-0.644*
	Sig. (2-tailed)	0.317	0.054	0.017
Study group G2 (pre-term)				
Weight of placenta	Pearson Correlation	-0.472*	-0.009	0.038
	Sig. (2-tailed)	0.023	0.969	0.863
Study group G3 (distress)				
Pregnancy	Pearson Correlation	-0.229	-0.538*	0.215
	Sig. (2-tailed)	0.431	0.047	0.460

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the rank values of the growth factor and receptor positive cells in **term and pre-term** placentas of the whole study with maternal parameters (Table 4.39.) we found negative correlation of the rank value of HGF in term placentas with maternal weight gain during pregnancy; the rank value of HGF in pre-term placentas had negative correlation with the number of pregnancies and the rank value of IGFR1 in the same placentas had negative correlation with weight gain during pregnancy.

Table 4.39.

Correlations between the rank values of the growth factors and maternal parameters in term and pre-term placentas of the whole study

Parameter		IGFR1	HGF
Term placentas of the whole study			
Weight gain in pregnancy	Pearson Correlation	0.041	-0.470*
	Sig. (2-tailed)	0.867	0.042
Pre-term placentas of the whole study			
Pregnancy	Pearson Correlation	-0.238	-0.364*
	Sig. (2-tailed)	0.176	0.034
Weight gain in pregnancy	Pearson Correlation	-0.430*	-0.243
	Sig. (2-tailed)	0.011	0.166

* Correlation is significant at the 0.05 level

Correlations of the rank values of growth factors with neonatal parameters

Evaluating correlations of the rank values of the growth factor and receptor positive cells with **neonatal parameters** in **the whole study** we found a number of correlations (Table 4.40.): the rank value of HGF showed negative correlation with both evaluations of the Apgar score of the baby and his initial glucose level ($p<0.05$); the rank values of IGF1, IGFR1 and HGF all had negative correlations with the

neonatal head circumference; the rank values of IGF1 and IGFR1 also with the chest circumference and the rank values of IGFR1 also with the birthweight.

Table 4.40.

Correlations of the rank values of growth factors and receptors with neonatal parameters in the whole study

Parameter		IGF1	IGFR1	HGF	FGFR1
1st minute Apgar score	Pearson Correlation Sig. (2-tailed)	-0.067 0.631	-0.159 0.255	-0.312* 0.023	0.067 0.634
5th minute Apgar score	Pearson Correlation Sig. (2-tailed)	-0.106 0.452	-0.230 0.097	-0.346* 0.011	0.100 0.476
Birthweight	Pearson Correlation Sig. (2-tailed)	-0.229 0.099	-0.298* 0.030	-0.217 0.119	0.043 0.760
Head circumference	Pearson Correlation Sig. (2-tailed)	-0.273* 0.048	-0.306* 0.026	-0.280* 0.042	-0.002 0.987
Chest circumference	Pearson Correlation Sig. (2-tailed)	-0.282* 0.041	-0.347* 0.011	-0.263 0.058	0.028 0.840
Blood glucose	Pearson Correlation Sig. (2-tailed)	0.093 0.524	0.113 0.439	0.058 0.692	-0.300* 0.036

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the rank values of the growth factor and receptor positive cells in placentas with neonatal parameters **in the study groups**, we found statistically significant correlations, shown in the Table 4.41.: in the study group G1 (term) the rank value of IGF1 showed positive correlation with the initial blood glucose level of the neonate and negative correlation with his chest circumference, the rank value of IGFR1 had positive correlation with baby's initial blood pH and negative correlation with his ponderal index, the rank value of FGFR1 had negative correlation with the initial blood glucose level of the baby.

Table 4.41.

Correlations of the rank values of growth factors with neonatal parameters in the study groups

Parameter		IGF1	IGFR1	FGFR1
Study group G1 (term)				
Blood glucose	Pearson Correlation Sig. (2-tailed)	-0.223 0.464	-0.024 0.938	-0.638* 0.026
Study group G2 (pre-term)				
Ponderal index	Pearson Correlation Sig. (2-tailed)	-0.133 0.546	-0.430* 0.041	-0.300 0.164
Chest circumference	Pearson Correlation Sig. (2-tailed)	-0.412* 0.041	-0.194 0.354	-0.198 0.342
Blood pH	Pearson Correlation Sig. (2-tailed)	0.294 0.153	0.428* 0.033	0.011 0.957
Blood glucose	Pearson Correlation Sig. (2-tailed)	0.444* 0.026	0.169 0.419	-0.320 0.119

* Correlation is significant at the 0.05 level (2-tailed)

Evaluation of the correlations of the rank values of the growth factor and receptor positive cells in **term and pre-term** placentas of the whole study with the maternal paramaters we found significant negative correlations between the rank values of IGFR1 in term placentas with the Apgar scores and initial blood pH (Table 4.42.); in pre-term placentas correlation between the rank value of IGFR1 with the initial blood pH of the neonate was positive.

Table 4.42.

Correlations of the rank values of growth factors with the neonatal parameters in term and pre-term placentas of the whole study

Parameter		IGFR1
Term placentas of the whole study		
1st minute Apgar score	Pearson Correlation Sig. (2-tailed)	-0.569* 0.011
5th minute Apgar score	Pearson Correlation Sig. (2-tailed)	-0.506* 0.027
Blood pH	Pearson Correlation Sig. (2-tailed)	-0.667* 0.050
Pre-term placentas of the whole study		
Blood pH	Pearson Correlation Sig. (2-tailed)	0.394* 0.023

* Correlation is significant at the 0.05 level (2-tailed)

We compared also means of the rank values of the growth factors among appropriate for the gestational age (AGA), small for the gestational age (SGA) and large for the gestational age (LGA) neonates and got significantly higher rank values of IGFR1 in the placentas of LGA neonates in camparison with SGA ones (Table 4.43.).

Table 4.43.

Correlations between the means of the graded expression of IGFR1 in the placentas of AGA, SGA and LGA babies

Parameter		Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
IGFR1	SGA LGA	16.00 26.67	5.477 10.00	2.449 3.333	0.024*

* Correlation is significant at the 0.05 level (2-tailed)

Differences between AGA neonates and SGA as well as AGA and LGA neonates were not statistically significant.

4.4.2. Cytokines

Amount of **IL-1α** positive cells did not correlate with the gestational age of placenta and was found in the placentas of various gestational ages from none (0) to

numerous (+++) in a visual field (Appendix, Figures 35.-41.). Different types of placental cells were IL-1α positive: cytotrophoblast, extravillous trophoblast, Höfbauer cells and cells of extraembryonic mesoderm.

Findings **in the study groups** (Table 4.44.):

- Placental samples of the study group G1 (term) presented occasional (0/+) to numerous (+++) IL-1α positive cells in a visual field (Appendix, Figures 35.-37.);
- Placentas of the study group G2 (pre-term) contained none (0) till numerous (+++) IL-1α positive cells in a visual field (Appendix, Figures 38.-41.);
- In the placentas of the study group G3 (distress) were seen none (0) till numerous (+++) IL-1α positive cells in a visual field.

Table 4.44.

Results of semi-quantitative evaluation of IL-1α positive cells in placentas of the study groups

	G1 (term)	G2 (pre-term)	G3 (distress)
1.	++	++	+
2.	+	++	+
3.	++	0/+	0/+
4.	+++	+	++
5.	+++	+++	++
6.	++	++	+++
7.	++	+++	++
8.	+	++	++
9.	0/+	0	+
10.	+	+	+
11.	+	0/+	0
12.	++	++	+
13.	+	+	++
14.	+++	0/+	+
15.		0/+	
16.		0/+	
17.		+	
18.		+	
19.		0/+	
20.		+	
21.		++	
22.		++	
23.		++	
24.		+++	
25.		+	

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. Found mean rank values of the amount of IL-1α positive cells are shown in the Table 4.45.

Table 4.45.

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

	Group	The mean ± SD
1.	The whole study	0.811 ± 0.7353
2.	Study group G1 (term)	1.75 ± 0.85
3.	Study group G2 (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

Differences between the mean rank values of IL-1α positive cells in the placentas of the study groups as well as between term and pre-term placentas were not statistically significant.

Amount of **TNFα** positive cells did not correlate with the gestational age of placenta and appeared from none (0) till numerous (+++) in a visual field in the placentas of various gestational ages (Appendix, Figures 42.-45.). TNFα positive were Höfbauer cells and cells of extravillous trophoblast.

Findings **in the study groups** (Table 4.46.):

- Placental samples of the study group G1 (term) presented none (0) till numerous (+++) TNFα positive cells in a visual field (Appendix, Figures 35.-37.);
- Placentas of the study group G2 (pre-term) contained none (0) till numerous (+++) TNFα positive cells in a visual field (Appendix, Figures 38.-40.);
- In the placentas of the study group G3 (distress) were seen none (0) till moderate (+++) TNFα positive cells in a visual field (Appendix, Figure 41.).

Table 4.46.

Results of semi-quantitative evaluation of TNFα positive cells in placentas of the study groups

	G1 (term)	G2 (pre-term)	G3 (distress)
1.	++	+++	++
2.	+	0/+	+
3.	++	+	0/+
4.	+++	0/+	0/+
5.	++	0	0/+

Table 4.46. continued p.73

Table 4.46. (End)

	G1 (term)	G2 (pre-term)	G3 (distress)
6.	0/+	+	0
7.	0	+	0/+
8.	+	++	+
9.	0	+++	0/+
10.	+	+	0/+
11.	0/+	+	0
12.	+	++	+
13.	++	+	+
14.	0/+	0/+	+
15.		0/+	
16.		+	
17.		+	
18.		+	
19.		0/+	
20.		+	
21.		0/+	
22.		+	
23.		0/+	
24.		+	
25.		+	

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. Found mean rank values of the amount of IL-1α positive cells are shown in the Table 4.47.

Table 4.47.

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

	Group	The mean ± SD
1.	The whole study	2.311 ± 0.9468
2.	Study group G1 (term)	1.18 ± 0.96
3.	Study group G2 (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

Differences between the mean rank values of TNFα positive cells in the placentas of the study groups as well as between term and pre-term placentas were not statistically significant.

Amount of **IL-10** containing cells **significantly decreased with advancing gestational age** of placenta, althought they were seen from few (+) to abundant (++++) in a visual field in all the placental samples of different gestational ages (Appendix,

Figures 46.-51.). There were different types of IL-10 positive placental cells: cytotrophoblast, syncitiotrophoblast, extravillous trophoblast, Höfbauer cells, cells of extraembryonic mesoderm, amnial epithelium.

Findings **in the study groups** (Table 4.48.):

- Placental samples of the study group G1 (term) presented none (0) till numerous (+++) IL-10 positive cells in a visual field (Appendix, Figure 46.);
- Placentas of the study group G2 (pre-term) contained none (0) till numerous (+++) IL-10 positive cells in a visual field (Appendix, Figures 47.-49.);
- In the placentas of the study group G3 (distress) were seen none (0) till moderate (+++) IL-10 positive cells in a visual field (Appendix, Figures 50.-51.).

Table 4.48.

Results of semi-quantitative evaluation of IL-10 positive cells in placentas of the study groups

	G1 (term)	G2 (pre-term)	G3 (distress)
1.	+++	+++	+++
2.	+++	++	+++
3.	+++	++++	++++
4.	+++	+++	+++
5.	+++	+++	++
6.	++	++++	+++
7.	+	++++	+++
8.	++++	+++	++++
9.	++	++++	++
10.	+++	++++	+++
11.	+	++++	+
12.	++++	+++	++++
13.	+++	++++	++++
14.	+++	++++	+++
15.		++++	
16.		++++	
17.		++	
18.		+	
19.		+	
20.		++	
21.		++	
22.		++++	
23.		+++	
24.		++++	
25.		++++	

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. Found mean rank values of the amount of IL-10 positive cells are shown in the Table 4.49.

Table 4.49.

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

	Group	The mean ± SD
1.	The whole study	3.019 ± 0.9505
2.	Study group G1 (term)	2.71 ± 0.91
3.	Study group G2 (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

Correlating the mean rank values of cytokines in the placentas of AGA, SGA and LGA neonates we found a statistically significantly higher rank value of IL-10 in the placentas of LGA neonates comparing with the AGA ones (Table 4.50.).

Table 4.50.

Correlations between the mean rank values of IL-10 in AGA and LGA groups

Parameter		Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
IL-10	AGA LGA	28.72 34.44	10.047 5.270	1.609 1.757	0.024*

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the rank values of the **cytokine positive cells** in placentas of **the whole study** we found positive correlations between the rank values of IL-10 and TNFα (Table 4.51.).

Table 4.51.

Correlations between the rank values of IL-10 and TNF in the whole study

Parameter		IL-10
TNFα	Pearson Correlation Sig. (2-tailed)	0.291* 0.045

* Correlation is significant at the 0.05 level (2-tailed)

Looking for correlations between rank values of cytokine positive cells in placentas of **the study groups** appeared statistically significant ones in the study group G1 (term) between the rank value of a pro-inflammatory cytokine TNFα and rank values of both a pro-inflammatory cytokine IL-1α and anti-inflammatory cytokine IL-10 (Table 4.52.).

Table 4.52.

Correlation between the rank values of cytokines in the study groups

		IL-10	IL-1α
Study group G1 (term)			
TNFα	Pearson corr. Sig. (2-tailed)	0.606* 0.048	0.698* 0.017

* Correlation is significant at the 0.05 level (2-tailed)

Assessment of the correlations between the rank values of cytokine positive cells **in term and pre-term** placentas of the whole study appeared a strong positive correlation in the term placentas between anti-inflammatory cytokines IL-1α and TNFα (Table 4.53.).

Table 4.53.

Correlation between the rank values of cytokines in term and pre-term placentas of the whole study

		IL-1α
Term placentas		
TNFα	Pearson corr. Sig. (2-tailed)	0.659** 0.005

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

Looking for correlations of the rank values of cytokine positive cells in placentas with maternal parameters in **the whole study** we found negative correlations of the anti-inflammatory cytokine IL-10 with maternal weight gain during pregnancy and gestational age of the placenta (Table 4.54.).

Table 4.54.

Correlations of the rank value of cytokines in placentas of the whole study with maternal parameters

Parameter		IL10
Weight gain	Pearson Correlation Sig. (2-tailed)	-0.424** 0.002
Gestation	Pearson Correlation Sig. (2-tailed)	-0.346* 0.011

* Correlation is significant at the 0.05 level (2-tailed);
** Correlation is significant at the 0.01 level (2-tailed).

Correlating the rank values of cytokine positive cells in placentas of **the study groups** with maternal parameters we found a number of statistically significant correlations (Table 4.55.): in the study group G1 (term) the rank value of TNFα positive cells in placentas positively correlated with maternal age, number of pregnancies and childbirths; in the study group G2 (pre-term) were seen positive correlations of the rank

value IL-10 with gestational age and maternal weight gain and strong positive correlation of IL-1α with length of rupture of membranes (ROM); in the study group G3 (distress) appeared negative correlations of the rank values of IL-1α with maternal weight gain during pregnancy.

Table 4.55.

Correlations of the rank values of growth factors with maternal parameters in the study groups

Parameter		IL-10	IL-1α	TNFα
Study group G1 (term)				
Maternal age	Pearson Correlation	0.300	0.388	0.614*
	Sig. (2-tailed)	0.297	0.171	0.044
Pregnancy	Pearson Correlation	0.393	0.095	0.628*
	Sig. (2-tailed)	0.165	0.746	0.039
Parity	Pearson Correlation	0.223	0.350	0.622*
	Sig. (2-tailed)	0.443	0.219	0.041
Study group G2 (pre-term)				
Weight gain	Pearson Correlation	-0.512*	0.126	-0.142
	Sig. (2-tailed)	0.012	0.567	0.540
Gestation	Pearson Correlation	-0.440*	-0.133	-0.033
	Sig. (2-tailed)	0.028	0.526	0.880
ROM	Pearson Correlation	-0.114	0.536**	0.191
	Sig. (2-tailed)	0.605	0.008	0.406
Placental weight	Pearson Correlation	-0.468*	-0.144	-0.165
	Sig. (2-tailed)	0.033	0.533	0.487
Study group G3 (distress)				
Weight gain	Pearson Correlation	-0.169	-0.623*	0.185
	Sig. (2-tailed)	0.564	0.017	0.526
Gestation	Pearson Correlation	-0.170	-0.613*	0.132
	Sig. (2-tailed)	0.562	0.020	0.654

* Correlation is significant at the 0.05 level (2-tailed);
** Correlation is significant at the 0.01 level (2-tailed).

Correlating the rank values of cytokine positive cells in **term and pre-term** placentas of the whole study with maternal parameters we found positive correlations of the rank values of TNFα positive cells in term placentas with numbers of pregnancies, negative correlations of the rank values of TNFα positive cells in pre-term placentas with maternal body height and negative correlations of the rank values of IL-10 positive cells in pre-term placentas with maternal weight gain during pregnancy and gestational age of placenta Table 4.56.).

Table 4.56.

Correlations between the rank values of the cytokines with maternal parameters in term and pre-term placentas of the whole study

Parameter		IL-10	IL-1α	TNFα
Term placentas of the whole study				
Pregnancy	Pearson Correlation Sig. (2-tailed)	0.400 0.090	-0.032 0.896	0.523* 0.038
Pre-term placentas of the whole study				
Body height	Pearson Correlation Sig. (2-tailed)	0.085 0.633	-0.193 0.274	-0.439* 0.012
Weight gain in pregnancy	Pearson Correlation Sig. (2-tailed)	-0.542** 0.001	-0.025 0.888	-0.061 0.742
Gestation	Pearson Correlation Sig. (2-tailed)	-0.456** 0.007	-0.203 0.249	-0.061 0.742

* Correlation is significant at the 0.05 level;
** Correlation is significant at the 0.01 level.

Correlating the rank values of cytokine positive cells in the placentas of **the whole study** with neonatal parameters we found just one statistically significant negative correlation of the rank values of IL-10 positive cells with neonatal head circumferences (Table 4.57.).

Table 4.57.

Correlations of the rank value of researched cytokines with the neonatal parameters in the whole study

Parameter		IL10
Head circumference	Pearson Correlation Sig. (2-tailed)	-0.280* 0.042

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the rank values of cytokine positive cells in placentas of **the study groups** with neonatal parameters we found statistically significant negative correlations in the study groups G2 (pre-term) and G3 (distress) (Table 4.58.): in the study group G2 (pre-term) rank values of IL-10 showed negative correlations with neonatal birthweight and rank values of IL-1α presented negative correlations with initial blood pH and 1st minute Apgar score; in the study group G3 (distress) the rank value of IL-1α showed negative correlations with all the measured anthropometric parameters, the rank values of TNFα presented positive correlation with the ponderal index and negative correlations with initial blood pH.

Table 4.58.

Correlations of the rank values of the cytokine with neonatal parameters in the study groups

Parameter		IL-10	IL-1α	TNFα
Study group G2 (pre-term)				
Birthweight	Pearson Correlation	-0.421*	-0.271	-0.124
	Sig. (2-tailed)	0.036	0.190	0.573
Blood pH	Pearson Correlation	0.104	-0.559**	0.299
	Sig. (2-tailed)	0.621	0.004	0.166
1st minute Apgar score	Pearson Correlation	-0.066	-0.526**	0.280
	Sig. (2-tailed)	0.754	0.007	0.196
Study group G3 (distress)				
Birthweight	Pearson Correlation	0.056	-0.535*	0.377
	Sig. (2-tailed)	0.850	0.048	0.184
Body length	Pearson Correlation	-0.065	-0.612*	0.172
	Sig. (2-tailed)	0.825	0.020	0.557
Ponderal index	Pearson Correlation	0.128	0.023	0.603*
	Sig. (2-tailed)	0.663	0.937	0.023
Head circumference	Pearson Correlation	-0.020	-0.562*	0.407
	Sig. (2-tailed)	0.946	0.036	0.149
Chest circumference	Pearson Correlation	0.124	-0.548*	0.404
	Sig. (2-tailed)	0.673	0.042	0.152
Blood pH	Pearson Correlation	-0.059	0.388	-0.855**
	Sig. (2-tailed)	0.870	0.268	0.002
Blood glucose	Pearson Correlation	-0.085	-0.352	0.768**
	Sig. (2-tailed)	0.815	0.319	0.009

* Correlation is significant at the 0.05 level (2-tailed);
** Correlation is significant at the 0.01 level (2-tailed).

Looking for correlations between the rank values of cytokines in **term and pre-term** placentas of the whole study with neonatal parameters appeared several statistically significant ones (Table 4.59.): the rank values of IL-1α positive cells in term placentas showed positive correlations with both Apgar score evaluations of neonates and the rank values of TNFα positive cells in term placentas revealed positive correlations with ponderal index of neonates; rank values of IL-10 positive cells in pre-term placentas presented negative correlations with neonatal birthweight and chest circumference and rank values of IL-1α positive cells in pre-term placentas had negative correlations with initial blood pH of neonate and his 1st minute Apgar score evaluation.

Table 4.59.

Correlations between the rank values of cytokines with neonatal parameters in term and pre-term placentas of the whole study

Parameter		IL-10	IL-1α	TNFα
Term placentas of the whole study				
Ponderal index	Pearson Correlation Sig. (2-tailed)	0.297 0.263	0.444 0.085	0.592* 0.016
1st minute Apgar score	Pearson Correlation Sig. (2-tailed)	-0.084 0.733	0.484* 0.036	0.192 0.477
5th minute Apgar score	Pearson Correlation Sig. (2-tailed)	-0.082 0.739	0.480* 0.038	0.262 0.327
Pre-term placentas of the whole study				
Birthweight	Pearson Correlation Sig. (2-tailed)	-0.387* 0.024	-0.319 0.066	-0.050 0.786
Head circumference	Pearson Correlation Sig. (2-tailed)	-0.363* 0.035	-0.308 0.077	-0.115 0.531
Blood pH	Pearson Correlation Sig. (2-tailed)	0.099 0.583	-0.463** 0.007	0.237 0.199
1st minute Apgar score	Pearson Correlation Sig. (2-tailed)	-0.170 0.337	-0.441** 0.009	0.161 0.379

* Correlation is significant at the 0.05 level;
** Correlation is significant at the 0.01 level.

4.4.3. Apoptosis of cells in the placental tissues

Apoptotic cells were identified in all the placental samples of various gestational ages (Appendix, Figures 52.-59.); their amount decreased with advancing gestational age of placenta. Apoptosis affected various cell types: cytotrophoblast, syncitiotrophoblast, extravillous trophoblast and cells of extraembryonic mesoderm.

Findings **in the study groups** (Table 4.60.):

- Placental samples of the study group G1 (term) presented 1 till 35 apoptotic cells in a visual field (Appendix, Figure 52.);
- Placentas of the study group G2 (pre-term) contained 2 till 75 apoptotic cells in a visual field (Appendix, Figures 53.-57.);
- In the placentas of the study group G3 (distress) were seen 1 till 72 apoptotic cells in a visual field (Appendix, Figures 58.-59.).

Table 4.60.

Number of apoptotic cells in a visual field in placentas of the study groups

	G1 (term)	G2 (pre-term)	G3 (distress)
1.	4.82 ± 2.09	9.09 ± 4.93	2.64 ± 2.01
2.	1.18 ± 1.40	2.73 ± 1.90	43.55 ± 10.77
3.	1.64 ± 1.57	25.73 ± 4.31	13.64 ± 5.08

Table 4.60. continued p.81

Table 4.60. (End)

	G1 (term)	G2 (pre-term)	G3 (distress)
4.	0.91 ± 0.83	57.64 ± 7.26	20.09 ± 5.65
5.	1.45 ± 1.69	52.91 ± 8.24	17.18 ± 4.62
6.	13.73 ± 5.52	26.91 ± 3.39	2.64 ± 2.46
7.	2.82 ± 1.99	75.91 ± 14.42	35.36 ± 7.02
8.	24.09 ± 3.91	21.91 ± 4.87	17.82 ± 4.87
9.	35.36 ± 5.10	44.73 ± 7.88	71.91 ± 10.32
10.	6.36 ± 2.69	43.91 ± 6.76	40.09 ± 7.74
11.	14.27 ± 4.52	31.36 ± 6.25	1.27 ± 1.62
12.	19.18 ± 2.75	16.91 ± 4.37	14.55 ± 4.63
13.	28.91 ± 4.11	14.18 ± 3.74	22.27 ± 3.35
14.	23.55 ± 5.37	34.73 ± 4.41	14.91 ± 4.37
15.		36.00 ± 3.52	
16.		28.09 ± 7.88	
17.		6.64 ± 3.01	
18.		33.27 ± 5.59	
19.		8.00 ± 3.32	
20.		12.09 ± 4.59	
21.		24.18 ± 6.27	
22.		32.18 ± 5.04	
23.		11.27 ± 5.18	
24.		17.91 ± 3.27	
25.		10.18 ± 5.23	

The mean numbers of the apoptotic cells in a visual field in the study groups was different (Table 4.61.).

Table 4.61.

The rank values apoptotic cells in different divisions of the study

	Group	The mean ± SD
1.	The whole study	22.15 ± 17.82
2.	Study group G1 (term)	11.85 ± 11.718
3.	Study group G2 (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

Differences are evident and between the study groups G1 (term) and G2 (pre-term) as well as term and pre-term placentas of the whole study statistically significant (Table 4.62.).

Table 4.62.

Correlations between the mean values of apoptotic cells

	Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
G1 (term) G2 (pre-term)	11.85 27.16	11.718 17.899	3.250 3.580	0.003**
All term placentas All pre-term placentas	13.00 27.00	13.052 18.246	3.076 3.129	0.003**

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

Correlating the numbers of apoptotic cells in a visual field with the maternal parameters in **the whole study**, appeared a statistically negative significant correlation with the gestational age of placenta (Table 4.63.)

Table 4.63.

Correlation of the number of apoptotic cells in a visual field with maternal parameters in the whole study

Parameter		Apoptosis
Gestation	Pearson Correlation Sig. (2-tailed)	-0.278* 0.046

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the numbers of apoptotic cells in placentas of **the study groups** with maternal parameters, we found a statistically significant correlation of the number of apoptotic cells in placentas of the study group G1 (term) with the number of pregnancies of the mother (Table 4.64.).

Table 4.64.

Correlations of the number of apoptotic cells in placentas with the maternal parameters in the study groups

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

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the number of apoptotic cells in **term and pre-term** placentas of the whole study with **maternal parameters** we found a statistically significant correlation of the number of apoptotic cells in term placentas with the number of pregnancies of the mother (Table 4.65.).

Table 4.65.

Correlations of the numbers of apoptotic cells in term and pre-term placentas with the maternal parameters of the whole study

Parameter		Apoptosis
Term placentas of the whole study		
Pregnancy	Pearson Correlation Sig. (2-tailed)	-0.529* 0.024

* Correlation is significant at the 0.05 level

Correlating the number of apoptotic cells in placentas **of the whole study** with **neonatal parameters** we found statistically significant negative correlations with neonatal birthweight, head and chest circumferences (Table 4.66.).

Table 4.66.

Correlation of the apoptotic cells in a visual field with neonatal parameters in the whole study

Parameter		Apoptosis
Birthweight	Pearson Correlation Sig. (2-tailed)	-0.319* 0.021
Head circumference	Pearson Correlation Sig. (2-tailed)	-0.277* 0.047
Chest circumference	Pearson Correlation Sig. (2-tailed)	-0.310* 0.025

* Correlation is significant at the 0.05 level (2-tailed)

4.4.4. Protein of the basement membrane Collagen IV

For evaluation of the structural integrity of the placenta we wanted to research two proteins of the basement membrane – Collagen IV and Laminin. Collagen IV, the main protein of a layer of basement membrane *lamina basalis*, has been described as a scaffold of the whole formation (Paulsson, 1992), ensuring its integrity; we were looking for its amount in different post-delivery placentas and found it in our preparations. Laminin, another important protein of the basement membrane, was not visually detected in the preparations of the post-delivery placentas of our study.

Amounts of Collagen IV were more in term placentas, althought it was seen in most of the placentas of the study (Appendix, Figures 60.-64.); amounts varied from none (0) till abundant (++++) in a visual field. Collagen IV was found in *lamina basalis*, constituent of the basement membrane and stroma of placental villi.

Findings **in the study groups** (Table 4.67.):

- Placental samples of the study group G1 (term) presented from none (0) till abundance (++++) of Collagen IV in a visual field (Appendix, Figure 60.);

- Placentas of the study group G2 (pre-term) contained from none (0) till abundance (++++) of Collagen IV in a visual field (Appendix, Figures 61.-63.);
- In the placentas of the study group G3 (distress) was seen from none (0) till abundance (++++) of Collagen IV in a visual field (Appendix, Figure 64.).

Table 4.67.

Results of semi-quantitative evaluation of Collagen IV in the placentas of the study groups

	G1 (term)	G2 (pre-term)	G3 (distress)
1.	++	+++	+++
2.	+++	+++	+++
3.	++	++++	++++
4.	++++	+++	++
5.	++	++	0/+
6.	+++	+	++
7.	+++	+	++
8.	++	0	+++
9.	++	0/+	0
10.	++	+	+
11.	0	++	0
12.	0/+	++	+++
13.	++	++	+
14.	0	0	+
15.		++	
16.		+	
17.		+++	
18.		0	
19.		0	
20.		0	
21.		0/+	
22.		+++	
23.		+	
24.		0/+	
25.		0	

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. Found mean rank values of the amount Collagen IV are shown in the Table 4.68.

Table 4.68.

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

	Group	The mean ± SD
1.	The whole study	1.50 ± 1.24
2.	Study group G1 (term)	1.96 ± 1.20
3.	Study group G2 (pre-term)	1.42 ± 1.23
4.	Study group G3 (distress)	1.81 ± 1.32
5.	Term placentas of the whole study	2.14 ± 1.28
6.	Pre-term placentas of the whole study	1.39 ± 1.16

Correlating the mean rank values of Collagen IV in the study groups we did not find statistically significant differences while comparison of the main rank values between term and pre-term placentas of the whole study revealed statistically more structures of Collagen IV in the term placentas (Table 4.69.).

Table 4.69.

Correlations between the means of the rank values of Collagen IV between term and pre-term placentas of the whole study

	Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
All term placentas	2.14	1.28	3.020	0.040*
All pre-term placentas	1.39	1.16	2.015	

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the rank values of Collagen IV in the the whole study, study groups and in term and pre-term placentas of the whole study with the maternal parameters we did not find any correlations in the whole study or in any of the study groups; in term placentas of the whole study the rank values of Collagen IV showed positive correlations with the numbers of pregnancies of the mothers and in pre-term placentas negative correlations with her weight before the actual pregnancy (4.70.).

Table 4.70.

Correlation of the rank values of Collagen IV in term and pre-term placentas of the whole study with maternal parameters

Parameter		Collagen IV
Term placentas		
Pregnancy	Pearson Correlation	0.494*
	Sig. (2-tailed)	0.037
Pre-term placentas		
Maternal weight before pregnancy	Pearson Correlation	-0.363*
	Sig. (2-tailed)	0.038

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the rank values of Collagen IV in the whole study, study groups and in term and pre-term placentas of the whole study with neonatal parameters statistically significant correlations appeared in the study group G3 (distress), where the rank value of Collagen IV presented positive correlation with the neonatal birthweight and chest circumference (Table 4.71.).

Table 4.71.

Correlation of the rank values of Collagen IV in placentas of the study groups with neonatal parameters

Parameter		Collagen IV
G3 (distress)		
Birthweight	Pearson Correlation Sig. (2-tailed)	0.585* 0.036
Chest circumference	Pearson Correlation Sig. (2-tailed)	0.616* 0.025

* Correlation is significant at the 0.05 level (2-tailed)

4.4.5. Tissue degrading enzyme MMP9

Our choice for the reasearch of the degrading enzymes of the ECM, actively participating in the tissue remodeling processes and actual for placenta, were matrix metalloproteinases. We could not visually identify MMP2 positive cells in our preparations of post-delivery placentas due to weak immunoreactivity and we excluded it from further evaluation.

MMP9 positive cells appeared to be more in pre-term placentas although the amount did not correlate with the gestational age. Amount of MMP9 positive cells was from none (0) to numerous (++++) in a visual field (Appendix, Figures 65.-70.). Various cell types were MMP9 positive: cytotrophoblast, syncitiotrophoblast, extravillous trophoblast, extraembryonic mesoderm and Höfbauer cells.

Findings **in the study groups** (Table 4.72.):

- Placental samples of the study group G1 (term) presented from none (0) till moderate (++) amount of MMP9 positive cells in a visual field (Appendix, Figure 65.);
- Placentas of the study group G2 (pre-term) contained from none (0) till abundance (++++) of MMP9 positive cells in a visual field (Appendix, Figures 66. and 67.);
- In the placentas of the study group G3 (distress) was seen from none (0) till

abundance (++++) of MMP9 positive cells in a visual field (Appendix, Figure 68.-70.).

Table 4.72

Results of semi-quantitative evaluation of MMP9 in placentas of the study groups

	G1 (term)	G2 (pre-term)	G3 (distress)
1.	++	0	0
2.	++	0	0
3.	+	+++	+++
4.	+	0	++
5.	0	0	0
6.	0	+	+
7.	0	+	++
8.	0	0/+	++++
9.	+	+++	++
10.	+	++	++
11.	0/+	+++	++
12.	++	++++	++++
13.	0/+	++	++++
14.	+	+	+++
15.		+	
16.		+++	
17.		+	
18.		++	
19.		+	
20.		0/+	
21.		++	
22.		+	
23.		0/+	
24.		++	
25.		+	

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. Found mean rank values of the amount of MMP9 positive cells are shown in the Table 4.73.

Table 4.73.

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

	Group	The mean ± SD
1.	The whole study	1.44 ± 1.20
2.	Study group G1 (term)	0.86 ± 0.74
3.	Study group G2 (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

Evaluating differences between the mean rank values of MMP9 we found a significantly higher mean rank value of MMP9 in the study group G3 (distress) (Table 4.74.); other differences between the groups as well as between term and pre-term placentas of the whole study were not significant.

Table 4.74.

Differences of the rank values of MMP9 positive cells in placentas of the study groups

	Mean	SD	Std. Error Mean	Sig. (t-test for Equality of Means)
G1 (term)	0.857	0.745	0.199	0.009**
G3 (distress)	2.071	1.439	0.384	

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

We did not get any statistically significant correlations of the rank values of MMP9 in the placentas of the whole study, study groups or term and pre-term placentas of the whole study with **maternal parameters**.

Correlating the rank values of MMP9 in the placentas of the **whole study** with **neonatal parameters**, we found statistically significant positive correlation with the inial blood pH and negative correlations with head circumference and 5th minute Apgar score (Table 4.75.).

Table 4.75.

Correlations between the rank values of MMP9 in the placentas of the study groups with neonatal parameters

Parameter		MMP9
Head circumference	Pearson Correlation	-0.271*
	Sig. (2-tailed)	0.049
5th minute Apgar score	Pearson Correlation	-0.271*
	Sig. (2-tailed)	0.050
Initial blood pH	Pearson Correlation	0.399**
	Sig. (2-tailed)	0.009

* Correlation is significant at the 0.05 level (2-tailed);
** Correlation is significant at the 0.01 level (2-tailed).

Correlating the rank values of MMP9 in the placentas of the **study groups** with neonatal parameters, we found several statistically significant correlations (Table 4.76.). In the study group G1 (term) the rank values of MMP9 showed positive correlation with birth weights of neonates and their chest circumferences; in the study group G2 (pre-term) was seen a significant positive correlation with the initial blood pH; in the study group G3 (distress) appeared a negative correlation with neonatal blood glucose.

Table 4.76.

Correlations between the rank values of MMP9 in the placentas of the study groups with neonatal parameters

Parameter		MMP9
G1 (term)		
Birthweight	Pearson Correlation Sig. (2-tailed)	0.622* 0.018
Chest circumference	Pearson Correlation Sig. (2-tailed)	0.550* 0.042
G2 (pre-term)		
Blood pH	Pearson Correlation Sig. (2-tailed)	0.464* 0.020
G3 (distress)		
Blood glucose	Pearson Correlation Sig. (2-tailed)	-0.713* 0.021

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the rank values of MMP9 **in term and pre-term** placentas of **the whole study** with the neonatal parameters we found a statistically significant positive correlation between the rank value of MMP9 positive cells in pre-term placentas with the initial blood pH (Table 4.77.).

Table 4.77.

Correlations between the rank values of MMP9 in term and pre-term placentas of the study with neonatal parameters

Parameter		MMP9
Pre-term		
Blood pH	Pearson Correlation Sig. (2-tailed)	0.388* 0.026

* Correlation is significant at the 0.05 level (2-tailed)

4.4.6. Products of the homeobox gene HoxB3

Amount of **HoxB3** product positive cells did not correlate with the gestational age of placenta and from occasional (0/+) to numerous (+++) in a visual field were seen in all the placentas of the study (Appendix, Figures 71.-74.). Different types of placental cells were HoxB3 positive: cytotrophoblast, syncitiotrophoblast, extravillous trophoblast and Höfbauer cells.

Findings **in the study groups** (Table 4.78.):

- Placental samples of the study group G1 (term) presented from occasional (0/+) till numerous (+++) HoxB3 positive cells in a visual field;
- Placentas of the study group G2 (pre-term) contained from occasional (0/+) till numerous (+++) HoxB3 positive cells in a visual field (Appendix, Figure

71.);

- In the placentas of the study group G3 (distress) were seen occasional (0/+) till numerous (+++) HoxB3 positive cells in a visual field (Appendix, Figures 72.-74.).

Table 4.78.

Results of semi-quantitative evaluation of HoxB3 in placentas of the study groups

	G1 (term)	G2 (pre-term)	G3 (distress)
1.	+++	+	+
2.	+	+	++
3.	+++	+++	0/+
4.	+	++	+++
5.	+	0/+	+
6.	+	++	+
7.	+++	++	++
8.	0/+	++	0/+
9.	+++	0/+	++
10.	+	+	+
11.	0/+	++	0/+
12.	0/+	++	++
13.	+	++	++
14.	+	0/+	+
15.		0/+	
16.		++	
17.		+	
18.		+	
19.		+	
20.		++	
21.		+	
22.		+	
23.		0/+	
24.		++	
25.		0/+	

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. Found mean rank values of the amount of HoxB3 positive cells are shown in the Table 4.79.

Table 4.79.

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

	Group	The mean ± SD
1.	The whole study	1.44 ± 1.20
2.	Study group G1 (term)	1.25 ± 0.27
3.	Study group G2 (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

Differences between the mean rank values of HoxB3 positive cells in the placentas of the study groups as well as between term and pre-term placentas were not statistically significant.

Correlating the rank values of HoxB3 products with **maternal parameters** in placentas of **the whole study** and the study groups as well as in term and pre-term placentas of the whole study, the only positive correlation that appeared was with the maternal body height in the study group G3 (distress) (Table 4.80.).

Table 4.80.

Correlations between the rank values of HoxB3 products in placentas of the study groups with neonatal parameters

Parameter		HoxB3 products
G3 (distress)		
Maternal body height	Pearson Correlation Sig. (2-tailed)	0.573* 0.032

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the rank values of HoxB3 products in the placentas of **the whole study** with **neonatal parameters** we got a statistically significant negative correlation with the ponderal index of the neonate (Table 4.81.).

Table 4.81.

Correlations between the rank values of HoxB3 products in placentas of the whole study with neonatal parameters

Parameter		HoxB3 products
Ponderal index	Pearson Correlation Sig. (2-tailed)	-0.323* 0.018

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the rank values of HoxB3 products in the placentas of **the study groups** we got a statistically significant positive correlation with the body length of neonate in the study group G1 (term) (Table 4.82.).

Table 4.82.

Correlations between the rank values of HoxB3 products in placentas of the study groups and neonatal parameters

Parameter		HoxB3 products
G1 (term)		
Body length	Pearson Correlation Sig. (2-tailed)	0.541* 0.046

* Correlation is significant at the 0.05 level (2-tailed)

Correlating the rank values of HoxB3 products in the placentas of **term and pre-term placentas** of **the whole study** we got a strong negative correlation with the ponderal index of neonates in pre-term cases of the whole study (Table 4.83).

Table 4.83.

Correlations between the rank values of HoxB3 products in term and pre-term placentas of the whole study and neonatal parameters

Parameter		HoxB3 products
Pre-term		
Ponderal index	Pearson Correlation Sig. (2-tailed)	-0.461** 0.008

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

4.5. Correlations between the researched molecular factors

Correlations between the rank values of the researched molecular factors were also provided in three sections of the study: in the whole study, in the study groups and in the term and pre-term placentas of the whole study.

4.5.1. Correlations between the growth factors and cytokines

Those were the largest groups of molecular factors in the study and showed the highest number of statistically significant correlations. In the whole study the growth factors IGF1 and two receptors IGFR1 and FGFR1 presented positive correlations with the anti-inflammatory cytokine IL-10 (Table 4.84.), one of significant players in the maternal immune tolerance against fetal allograft, ensuring ongoing pregnancy.

Table 4.84.

Correlation of the rank values of the growth factors and cytokines in the placentas of the whole study

		IL-10	TNFα
IGF1	Pearson corr. Sig. (2-tailed)	0.362** 0.008	0.277 0.057

Table 4.84. continued p.93

Table 4.84. (End)

		IL-10	TNFα
IGFR1	Pearson corr. Sig. (2-tailed)	0.432** 0.001	0.320* 0.026
FGFR1	Pearson corr. Sig. (2-tailed)	0.474** 0.000	0.174 0.238

* Correlation is significant at the 0.05 level (2-tailed);
** Correlation is significant at the 0.01 level (2-tailed).

In the study group G1 (term) the rank value of IGF1 showed positive correlation with the rank value of TNFα (Table 4.85.); in the study group G2 (pre-term) rank values of the anti-inflammatory cytokine IL-10 positively correlated with the rank values of IGF1, IGFR1 and FGFR1 and the rank value of TNFα positively correlated with the rank value of IGFR1 as in the whole study, with new correlations appearing. The rank value of TNFα showed a positive correlation with the rank value of HGF and the rank value of the pro-inflammatory cytokine IL-1α had a negative correlation with the rank value of IGF1 suggesting restricted growth of a pre-term fetus in an inflammatory environment. In the study group G3 (distress) the rank value of IL-10 positively correlated with the rank value of FGFR1 probably suggesting undiagnosed situations of the growth of placental and fetal tissues with restricted functionality of placenta.

Table 4.85.

Correlation of the rank values of the growth factors and cytokines in placentas of the study groups

		IL-10	IL-1α	TNFα
G1 (term)				
IGF1	Pearson corr. Sig. (2-tailed)	0.481 0.081	0.317 0.269	0.699* 0.017
G2 (pre-term)				
IGF1	Pearson corr. Sig. (2-tailed)	0.0412* 0.041	-0.426* 0.034	0.300 0.165
IGFR1	Pearson corr. Sig. (2-tailed)	0.623** 0.001	-0.212 0.310	0.467* 0.025
HGF	Pearson corr. Sig. (2-tailed)	0.268 0.196	0.065 0.756	0.484* 0.019
FGFR1	Pearson corr. Sig. (2-tailed)	0.609** 0.001	0.223 0.283	0.193 0.378
G3 (distress)				
FGFR1	Pearson corr. Sig. (2-tailed)	0.552* 0.041	0.053 0.857	0.034 0.908

* Correlation is significant at the 0.05 level (2-tailed);
** Correlation is significant at the 0.01 level (2-tailed).

Similar correlations are seen also in the term and pre-term placentas of the whole study (Table 4.86.).

Table 4.86.

Correlation of the rank values of the growth factors and cytokines in the term and pre-term placentas of the whole study

		IL-10	IL-1α	TNFα
Term				
FGFR1	Pearson corr. Sig. (2-tailed) N	0.509* 0.031 19	0.356 0.147 19	0.221 0.429 19
Pre-term				
IGF1	Pearson corr. Sig. (2-tailed)	0.371* 0.031	-0.459** 0.006	0.316 0.078
IGFR1	Pearson corr. Sig. (2-tailed)	0.569** 0.000	-0.133 0.455	0.437* 0.012
HGF	Pearson corr. Sig. (2-tailed)	0.267 0.127	-0.080 0.653	0.498** 0.004
FGFR1	Pearson corr. Sig. (2-tailed)	0.513** 0.002	0.120 0.499	0.089 0.627

* Correlation is significant at the 0.05 level (2-tailed);
** Correlation is significant at the 0.01 level (2-tailed).

4.5.2. Correlations between the growth factors and apoptotic cells

The most noticable correlation, appearing between the graded values of the growth factors and the number of apoptotic cells in a visual field in different sections of the study, was positive correlation of the number of apoptotic cells with the graded value of HGF in the placentas of the whole study (Table 4.87.), pre-term placentas both in the study group G2 (pre-term) (Table 4.88.) and in all the pre-term placentas of the study (Table 4.89.). Similar correlation in the whole study is largely determined by the findings in pre-term placentas, as the significance appeared to be strong and pre-term placentas constitute the majority of the study placentas.

Table 4.87.

Correlation of the rank values of growth factors and the number of apoptotic cells in placentas of the whole study

Molecular factors		Apoptosis
HGF	Pearson corr. Sig. (2-tailed)	0.365** 0.008

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

Correlation in the study group G3 (distress) between the rank value of IGF1 and apoptosis (Table 4.88.) support the suggestion regarding undiagnosed prolongation with increasing mass and restricted functionality.

Table 4.88.

Correlation of the rank values of the growth factors and the number of apoptotic cells in the placentas of the study groups

Molecular factors		Apoptosis
G2 (pre-term)		
HGF	Pearson corr. Sig. (2-tailed)	0.558** 0.004
G3 (distress)		
IGF1	Pearson corr. Sig. (2-tailed)	0.602* 0.023

* Correlation is significant at the 0.05 level (2-tailed);
** Correlation is significant at the 0.01 level (2-tailed).

In pre-term placentas of the whole study also appeared a statistically significant positive correlation between the rank values of HGF positive cells in placental tissues and the amount of apoptotic cells (Table 4.89.).

Table 4.89.

Correlation of the rank values of the growth factors and the number of apoptotic cells in pre-term placentas of the whole study

Molecular factors		Apoptosis
HGF	Pearson corr. Sig. (2-tailed)	0.420* 0.013

* Correlation is significant at the 0.05 level (2-tailed)

4.5.3. Correlations between the growth factors and other molecular factors

In placentas of **the whole study** the rank value of Collagen IV presented positive correlation with the rank value of FGFR1 (Table 4.90.); similar correlation was found in the placentas of the study group G2 (pre-term) (Table 4.91.) and pre-term placentas of the whole study (Table 4.92.); the same finding in the study group G3 (distress) is coinciding, as this study group includes more pre-term than term placentas.

Table 4.90.

Correlation of the rank values of the growth factors and other factors in the placentas of whole study

Factor		Collagen IV
FGFR1	Pearson corr. Sig. (2-tailed)	0.341* 0.014

* Correlation is significant at the 0.05 level (2-tailed)

In term placentas of the study group G1 (term) (Table 4.91.) and in the whole study were found positive correlations of the rank values of Collagen IV with the rank values of HGF.

Table 4.91.

Correlation of the rank values of the growth factors and other factors in the placentas of the study groups

Factors		Collagen IV
G1 (term)		
HGF	Pearson corr. Sig. (2-tailed)	0.678* 0.011
G2 (pre-term)		
FGFR1	Pearson corr. Sig. (2-tailed)	0.403* 0.046
G3 (distress)		
FGFR1	Pearson corr. Sig. (2-tailed)	0.678* 0.011

* Correlation is significant at the 0.05 level (2-tailed)

In term placentas of the whole study appeared also positive correlations with the rank values of IGFR1 (Table 4.92.). In the study groups G2 (pre-term), G3 (distress) as well as pre-term placentas of the whole study the rank values of Collagen IV positively correlated with the rank values of FGFR1.

Table 4.92.

Correlation of the rank values of the growth factors and other factors in the term and pre-term placentas of the whole study

Factors		Collagen IV
Term placentas of the whole study		
IGFR1	Pearson corr. Sig. (2-tailed)	0.485* 0.041
HGF	Pearson corr. Sig. (2-tailed)	0.484* 0.042
Pre-term placentas of the whole study		
FGFR1	Pearson corr. Sig. (2-tailed)	0.377* 0.031

* Correlation is significant at the 0.05 level (2-tailed).

If the protein of the basement membrane Collagen IV basically correlated with the growth factor receptor FGFR1, the tissue degrading enzyme MMP9 showed positive correlations with IGF1 in the whole study, in placentas of the study group G2 (pre-term) and in pre-term placentas of the whole study (Table 4.93.) meaning, that those correlations in the pre-term placentas were not influenced by the clinical courses of the pre-term patients.

Table 4.93.

Correlation of the rank values of the growth factors and other factors in various sections of the study

Factors		MMP9
The whole study		
IGF1	Pearson corr. Sig. (2-tailed)	0.376** 0.006
Study group G2 (pre-term)		
IGF1	Pearson corr. Sig. (2-tailed)	0.420* 0.037
Pre-term placentas of the whole study		
IGF1	Pearson corr. Sig. (2-tailed)	0.374* 0.029

* Correlation is significant at the 0.05 level (2-tailed)

The rank values of homeobox gene product HoxB3 positive cells presented significant positive correlation in placentas of the study group G1 (term) with the rank value of IGFR1 (Table 4.94.).

Table 4.94.

Correlation of the rank values of the growth factors and other factors in the placentas of the study groups

Factors		HoxB3
Study group G1 (term)		
IGFR1	Pearson corr. Sig. (2-tailed)	0.624* 0.017

* Correlation is significant at the 0.05 level (2-tailed)

4.5.4. Correlations between other researched cellular factors

Looking for correlations of the rank values of cytokines in the placentas of the whole study we found positive correlations of the rank values of IL-10 with the rank values of MMP9 (Table 4.97.); in the study group G3 (distress) was found similar correlation and also a positive correlation with the rank values of Collagen IV (Table 4.95.), suggesting possible disbalance between strength of the basement membrane and the tissues degrading enzymes.

Table 4.95.

Correlation of the rank values of the cytokines and other factors in the placentas of whole study and the study groups

Factors		Collagen IV	MMP9
IL-10	Pearson corr. Sig. (2-tailed)		0.372** 0.006

Table 4.95. continued p.98

Table 4.95. (End)

Factors		Collagen IV	MMP9
G3 (distress)			
IL-10	Pearson corr. Sig. (2-tailed)	0.729** 0.005	0.548* 0.042

* Correlation is significant at the 0.05 level (2-tailed);
** Correlation is significant at the 0.01 level (2-tailed).

In placentas of the study group G3 (distress) we found another positive correlation of the rank value of MMP9 – with the rank value of HoxB3 gene product positive cells (Table 4.96.).

Table 4.96.

Correlation of the rank values of MMP9 and HoxB3 products in term and pre-term placentas of the whole study

Factors		HoxB3 products
Study group G3 (distress)		
MMP9	Pearson corr. Sig. (2-tailed)	0.395* 0.021

* Correlation is significant at the 0.05 level (2-tailed)

Other correlations were not statistically significant.

5. DISCUSSION

5.1. Anthropological issues of the study

Statistically strong differences between the values of the mean weight of **pre-term and term placentas** in the whole studys confirm gestation to be the main determinant of the placental weight. Interestingly addition of the placental weights from G3 (distress) group decreased the mean values of both term and pre-term placentas, probably indicating lower placental weight for the gestation to be a risk factor for the development of life threatening fetal distress. Other authors have correlated placental weight/ birthweight ratio and have found worse perinatal outcome in the cases of higher ratio (Shehata et al., 2011), somehow opposing our findings.

All the researched molecular factors in the post-delivery placentas we correlated with their **gestational age**. Amount of the researched growth factor and receptor positive cells varied depending on the gestational age of placenta: IGF1, IGFR1 and HGF positive cells seemed to be less in the placentas of more advanced gestational age, while FGFR1 positive cells appeared to be more prominent in the term placentas. Our study convincingly showed decrease of **IGFR1** positive cells in the placentas during the third trimester of pregnancy. We found negative correlation of the rank values of IGFR1 with the gestational weeks of pregnancy and higher amount of cells in pre-term placentas of various clinical courses and outcomes, suggesting that both in pathological and normal pregnancies in earlier gestational time IGFR1 has more significant role than at term, ensuring its growth and functional capabilities with increasing demands of fetus. The rank values of **IGF1** positively correlated with the rank values of IGFR1 both in the whole study and pre-term placentas, therefore the presence and role of IGF1 are also more important before the term. Several studies have researched the expression of IGFs at different terms of gestation. There are studies showing similar decrease of IGF1 in the placental tissues with the gestational time (Kumar et al., 2006; Kumar et al., 2012); others showed no significant changes throughout the course of pregnancy (Han et al., 1996) or increase in the maternal blood serum with the advanced gestation (Tennekoon et al., 2007); in the neonatal blood serum levels of IGF1 have also been found to increase with advanced gestation (Smith et al., 1997).

Our study suggests not only higher amount of IGF1 and IGFR1 positive cells in pre-term placentas, but also their impact on neonatal health in cases of pre-term deliveries. 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. As the blood pH is one of the most objective signs of neonatal systemic well-being and its initial values properly reflect the status of maternal-fetal environment we can assert that pre-term neonates with a larger amount of IGFR1 positive cells in their placentas are born in a potentially better health status. We have not find in the literature suggestions on this issue; coinciding findings are our positive correlations of the rank values of IGF1 in the placentas of the study group G2 (pre-term) and other studies, having found 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). The rank values of IGF1 did not show positive correlations with the blood glucose level in all the pre-term cases of our study, because it was not valid for distress cases, included in the study group G3 (distress). Therefore we suggest that high amount of IGFR1 positive cells in the placental tissues can improve survival of VLBW infants; increased amount of IGF1 encourage his growth potential and probably can be concidered for therapeutic application in those patients.

Our finding showed decrease of the rank value of **HGF** with advancing gestational age, although the direct calculations of the correlation showed only weak significance ($0.05 \leq p \leq 0.1$). In the whole study as well as in the term and pre-term placentas of the whole study the rank value of HGF showed positive correlation with the rank value of IGFR1, decreasing with the advancing gestational age; it also presented negative correlation with the head circumference of the neonate and maternal weight gain in pregnancy, having the same relevance. In the literature there are some studies, describing increase of the expression of HGF in placentas with the advanced gestation in the 1st and 2nd trimester of pregnancy (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; in the placentas of the study group G3 (distress) and pre-term placentas of the whole study the rank values of HGF presented negative correlation with the numbers of pregnancies of the mothers, suggesting decreased capabilities of the maternal organism like it could be suggested with increased maternal age.

In the study group G1 (term), including healthy patients with a normal course of pregnancy and a term delivery with a healthy child, the rank value of HGF positive cells in the placentas showed positive correlation with the maternal BMI before actual pregnancy. The maternal BMI in this study group 24.88 ± 5.99 kg/m^2 was quite close to

the upper limit of normal values 25 kg/m^2 and higher than in the whole study 23.79 ± 4.74 kg/m^2, therefore we believe this finding to match the 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; the higher rank value among the study groups had the study group G1 (term) and the term placentas of the whole study in comparison with the pre-term ones; differences were not statistically significant. Although it has been stated that FGF and FGFR strongly contribute to the growth and development of the placenta and fetus (Marzioni et al., 2005), not too much research is available on the expressions of FGFb and FGFR1 in the maternal-fetal unit, one of the found has described unchanging expression of FGFR1 throughout the second and third trimesters (Anteby et al., 2005).

We found significant negative correlation of the rank value of a anti-inflammatory cytokine **IL-10** with the gestational age in the study group G2 (pre-term) and all the pre-term placentas of the study, meaning that there were more IL-10 positive cells in pre-term placentas than in term ones regardless other clinical findings. This finding was strengthened by negative correlations of the rank value of IL-10 with anthropometrical parameters of the neonates: with head circumference in the whole study and the pre-term cases of the whole study and birthweight in the study group G2 (pre-term) and all the pre-term cases of the study. Such a finding suggest decrease of actuality of maternal immunological tolerance against fetus with advanced gestation and emphasizes importance of the tolerance against fetal allograft before the achievement of the term of pregnancy. In other studies IL-10 is decribed to be present in the placental tissues throughout the pregnancy (Dembinski et al., 2003); we did not find any correlations with the term of gestation.

Most of the studies researching pro-inflammatory cytokines in the maternal-fetal unit are focusing on the pre-term parturition and the role of inflammation in its triggering as well as identification of hidden chorionamnionitis, possibly having impact on the pregnancy course and outcome. As majority of our patients had delivered pre-

term, we expected to find more pro-inflammatory cytokine positive cells in the placentas of smaller gestational age. Unexprectedly we did not find any correlations of the rank values of pro-inflammatory cytokines **IL-1α** and **TNFα** with any of the gestation related parameters, suggesting that the hidden reasons of pre-term deliveries in our cases were not infections. We did not find correlations of the expression of pro-inflammatory cytokines with the gestational ages in the other studies as well. One of our findings with the pro-inflammatory cytokines in association with the gestation was in the study group G3 (distress), in which the rank values of IL-1α presented negative correlation with the gestational age meaning, that in earlier gestational age inflammatory signs have more significant impact on the clinical course of pregnancy.

Apoptosis is a process that is present in placenta throughout the pregnancy like all the other live tissues, providing its development. 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 (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 only from the third trimester. In the placentas of the whole study we also found a significant negative correlation between the gestational age and the average number of apoptotic cells in a visual field suggesting, that in the third trimester of pregnancy cellular apoptosis is seen more in placentas with earlier termination; we suggest that cellular death in full-term placentas is possibly changing the balance between apoptotic and necrotic paths of cellular death (Huppertz et al., 2003; Huppertz et al., 2004).

Interestingly in the pre-term placentas of our study, both in the study group G2 (healthy pre-term) and pre-term placentas of the whole study, number of apoptotic cells in a visual field positively correlated with the rank value of HGF, suggesting regenerative efforts of the tissues of pre-term placentas. In the study group G2 (healthy pre-term) correlation was stronger ($p=0.004$) than in the pre-term placentas of the whole study ($p=0.013$), confirming negative impact of distress circumstances on the compensatory abilities of placenta.

In our study the amount of **Collagen IV** positive structures in placental tissues increased with advanced gestation; in the whole study the rank values of Collagen IV was significantly higher in term placentas in comparison with the pre-term ones,

coinciding with the studies having suggested Collagen IV to be an indicator of fetal maturation (Papadopuolos et al., 2001); they did not find it in the placentas of the Ist and IInd trimester, but found in the IIIrd trimester. In the study group G3 (distress) we also found a significant positive correlation of the rank values of Collagen IV with the birth weight and chest circumference of the neonate, suggesting possibly undiagnosed cases of prolongation of pregnancy, playing a role in the development of a significant fetal distress.

Correlating the rank values of Collagen IV with the maternal and neonatal parameters we found a few statistically significant ones. In the term placentas of the whole study appeared a positive correlation of the rank values of Collagen IV with the maternal number of pregnancies, suggesting accelerated maturity of the maternal-fetal unit in consecutive pregnancies, possibly due to exhausted resources. This finding is coherent with the negative correlations of the rank value of HGF in placentas of the study group G3 (distress) and the pre-term placentas of the whole study with the number of maternal pregnancies, suggesting decrease of regenerative capacities as well as other studies, having found increased Collagen IV in unfavorable circumstances due to inflammation (Kumar et al., 2006) or hypoxia (Chen and Aplin, 2003; Chen et al., 2005), including due to maternal smoking (Jalali et al., 2010). Another correlation of our study, congruent with the mentioned suggestions, is negative correlation of the rank values of Collagen IV in all the pre-term placentas of the study with the maternal weight before pregnancy, allowing to reason her health and social status.

The expression of **MMP9** in placentas and fetal membranes has mainly been evaluated as a factor of parturition and rupture of membranes, both pre-term and term; expression is found to be higher in the cases of spontaneous vaginal deliveries (Xu et al., 2002; Sundrani et al., 2012); more pronounced in the pre-term group. We did not find significant correlations with the gestational age, the mean rank values of MMP9 in pre-term and term placentas did not show statistically significant differences, although it was higher in pre-term placentas. We suggest, that MMP9 has specific areas of the exposure in the maternal-fetal unit and gestation related impact should be evaluated further, taking into account types of cells and tissues; it corresponds with some other studies, having found MMP9 in specific locations of placenta (Demir-Weusten et al., 2007).

The significant role of initiation of pre-term labor is somehow confirmed by the correlations in our study of the rank values of MMP9 with the neonatal clinical

parameters. In the pre-term placentas of the study group G2 (pre-term) the rank value of MMP9 showed statistically significant positive correlation with the initial neonatal blood pH, that means that MMP9 positive cells were more in those pre-term placentas, whose pre-mature neonates objectively were in a better health status; this correlation was not seen in the placentas, having undergone circumstances of significant distress.

In the study group G1 (term) we found a significant positive correlations between the rank values of MMP9 positive cells in placentas and birthweight of the neonate; interestingly in the study group G3 (distress) positive correlation was seen between the rank values of Collagen IV positive structures in placenta and birthweight of the neonate; suggesting importance of initiation of parturition in the well-being of the neonate, ensured by the means of MMP9.

We found **HoxB3 products** in all the placentas of the study in various amounts, meaning that they are playing a certain role in the development of the maternal-fetal unit. Evaluating the rank values of HoxB3 product positive cells in the placentas of our study groups, we did not find ane significant differences between any of them as well as between the pre-term and term placentas of the whole study; in pre-term placentas were slightly more HoxB3 positive cells.

We were also looking how the amount of researched molecular factor positive cells correlate with the **appropriateness for the gestational age** of the neonate. In our study 39 neonates were appropriate for the gestational age (AGA), 5 were small for the gestational age (SGA) and 9 of them were large for the gestational age (LGA); they were of various gestational ages. We correlated the mean rank values of the growth factors in the placentas of AGA, SGA and LGA neonates and found a significantly higher mean rank value of **IGFR1** in LGA placentas in comparison with the SGA ones. This 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). This finding coincides 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.

Controversial to our findings are studies having found negative correlations of IGF1 in the cord blood with the birthweight (Pathmaperuma et al., 2007) or 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).

Our suggestion is that the expressions of IGF1 and IGFR1 in the placentas of SGA depend on the causes of growth restriction, placental metabolism and other molecular events and need furher research studies.

We did not find significant correlations of the rank values of **FGFR1** with the anthropometrical parameters of the neonates; in the placentas of the whole study they showed significant negative correlation with the maternal weight before the actual pregnancy, meaning higher rank value in the placentas of patients, possibly associated with compromised health or social status. Number of studies have associated increased expression of FGF and FGFR in the cases of pathological pregnancies like pre-eclampsia (Ozkan et al., 2008) or IUGR (Barut et al., 2010), somehow matching with our findings, although we did not find significant differences in the mean rank values of FGFR1 in the placentas of AGA, SGA and LGA neonates.

Interestingly we found negative correlation of the rank value of FGFR1 in the placentas of the whole study with the initial blood glucose levels of the neonates; similar correlation was found also in the study group G1 (term). Taking into account that the rank values of FGFR1 were higher in the term placentas and the study group G1(term) included healthy patients with no clinical reasons for hypoglycemia, it could be related with hyperinsulinemia of the neonates, matching the studies, that have found increased FGFR in macrosomic neonates of diabetic patients (Grissa et al., 2010) and suggesting undiagnosed cases of not so severe gestational diabetes.

Comparing the mean rank values of **IL-10** in the placentas of AGA, SGA and LGA neonates we found significantly higher rank values of IL-10 in the placentas of LGA neonates. As in our study appropriatenes for the gestational age was evaluated in all the gestations, 4 of 9 LGA and 4 of 5 of SGA neonates of our study were pre-term; we suggest that more IL-10 positive cells in the placental tissues provide favorable circumstances for the fetal growth. Interestingly the mean rank value of IL-10 in SGA placentas was also higher than in AGA ones although the difference was not statistically significant. Such a finding to our mind was a compensatory reaction of the placenta to restricted circumstances, as all the pre-term SGA pregnancies were discontinued due to compromised fetal blood supply with no threats of spontaneous termination of pregnancy; all the neonates were born in good condition for the gestational age. Other studies have more researced IUGR/ SGA cases and found lower expression of IL-10 in the placentas of SGA patients (Hahn-Zoric et al., 2002) and the mononuclear cells of the maternal blood in the cases of IUGR with placental insufficiency (Raghupathy et al.,

2012) suggesting reduction if IL-10 to be among the causes of the development of IUGR. The difference between our findings could be the difference of causes and mechanisms of the development of IUGR/ SGA.

We did not find any differences of the numbers of **apoptotic cells** in the placentas of SGA, LGA and AGA neonates; we found significant negative correlation of the average number of apoptotic cells with the birth weight, head and chest circumferences of the child in the whole study possibly in the line with negative correlations with the gestational ages. As our SGA and LGA neonates were of various gestational ages, we suggest that the molecular processes of the damages of placental barriers, leading to growth restriction, differ depending on the gestational age.

Some other studies have not found increased number of apoptotic cells in the placentas of SGA neonates (Roje et al., 2011), others describe significantly increased number of apoptotic cells in the placentas of pregnancies complicated by IUGR alone (Athapathu et al., 2003), as a consequence of pre-eclampsia (Longtine et al., 2012) or cigarette smoking (Vogt Isaksen et al., 2004). They suggested that the elevated apoptosis in cytotrophoblasts contributes to the placental dysfunction causing this disorder due to damage of the maternal-fetal interface; we suggest that this could be valid for a certain gestational age and processes, not being so damaging to initiate necrotic type of cellular death.

We did not find any differences of the rank values of **MMP9** in the placentas, depending on the appropriateness for the gestational age like studies, having found decreased expression of MMP9 in SGA cases (Swierczewski et al., 2012); we did not find differences of the rank values of MMP9 between the groups of patients with and without positive cervical or fetal membrane cultures like studies, having found activation of MMP9 by infectious agents and increased pro-inflammatory cytokines (Zaga-Clavellina et al., 2006; Zaga-Clavellina et al., 2011; Flores-Herrera et al., 2012).

Evaluating the rank values of **HoxB3 structure** positive cells in the placentas of SGA, AGA and LGA neonates, more were in SGA placentas, but the differences were also not significant.

5.2. Morphological issues of the study

We did not recognize any remarkable changes in the variety of **IGF1** or **IGFR1** positive cell types like described in other studies, having found changes of localization

of IGFR1 during the course of pregnancy: from the proliferating cytotrophoblasts in the first trimester to macrophages and endothelial cells at term (Hills et al., 2004; Hiden et al., 2009). In our study in the term placentas with signs of ageing cytotrophoblast did not cover the whole area of the placental villi, therefore they seemed to have less amount of positive cytotrophoblast cells; at the same time transitional term placentas with a high coverage of placental villi by cytotrophoblast showed high number of IGF1 or IGFR1 positive cells. As our data relate to the tissue changes, they are suggested to be original.

We found several statistically significant correlations of the rank values of IGF1 and IGFR1 with the other researched factors in different patient groups of our research. Placentas of the whole study showed positive correlations with the anti-inflammatory cytokine IL-10; similar correlations were found also in the pre-term placentas both in the study group G2 (pre-term), including healthy pre-term cases, and all the pre-term placentas of the study. We did not find in the literature correlations between those factors, as they all are considered to be players of the same team, ensuring placental and fetal growth and sustaining maternal immune tolerance towards fetal allograft. Interestingly we did not find such correlations in term placentas although not all of them were 40 gestational weeks old, meaning that at some point this mechanism becomes surplus.

In the pre-term placentas of the study group G2 (pre-term) as well as all the pre-term placentas of the study we found a statistically significant negative correlation of IGF1 with the pre-inflammatory cytokine IL-1α, matching with the results research studies on the negative impact of a pro-inflammatory cytokine IL-6 on IGF axis (Hsiao et al., 2011). Significantly in our study this correlation appeared in the pre-term placentas, accentuating molecular impact of IL-1α related inflammatory processes on the growth of pre-term fetus. Interestingly in the pre-term placentas the rank value of IGFR1 positively correlated with the pro-inflammatory cytokine TNFα, suggesting importance of protective inflammatory processes in those placentas; it is supported by our findings in term placentas, both in the study group G1 (term) and all the term placentas of the study, showing statistically significant positive correlations between the rank values of IGF1 and TNFα. We did not find data in the literature on similar (or controversial) findings in the placental tissues, but in children with growth hormone deficiency there was found higher TNFα level and no correlations of the level of TNFα with the levels of IGF1 (Andiran and Yordam, 2007).

Our data suggest that identification and assessment of the growth factor IGF1 and its receptor IGFR1 in the maternal-fetal environment can provide useful clinical information regarding growth and maturation of placenta and fetus and suggest the optimal way of the management of pregnacy for the achievement of better outcome. Therapeutic application of IGF1 in the cases of extreme pre-maturity is worthy of reflection.

In the pre-term placentas of our study both in the study group G2 (pre-term) and all the pre-term placentas of the study appeared two significant positive correlations of the rank values of **HGF** with the rank values of a pro-inflammatory cytokine TNFį and the average numbers of apoptotic cells. There are studies describing HGF to inhibit apoptosis in the placentas of pathological pregnancies like pre-eclampsia (Dash et al., 2005); our study suggests it to be valid in wider ranke placentas of pre-term deliveries. Our suggestion is also matching with the authors, declaring HGF to promote regeneration of tissues (Matsumoto et al., 2001; Nayeri et al., 2002; Urbanek et al., 2005; Mizuno et al., 2008; Dai et al., 2010) and have impact on inflammation processes (Nakamura et al., 2011).

Taking into account the above mentioned, it would be logical to expect to find the largest amount of HGF positive cells in the placentas of our study group G3 (distress), having faced life threatening circumstances. Our data really showed the highest mean rank value of HGF in this study group: 1.82 ± 0.91 against 1.17 ± 0.80 and 1.74 ± 0.98 (differences are not statistically significant); no correlations with the number of apoptotic cells or pro-inflammatory cytokines indicate other pathways of cellular damage and death in those placentas. The rank values of FGFR1 in the placentas of the whole study presented negative correlations with the 1st and 5th minute Apgar score evaluation of neonates, witnessing participation of HGF in problematic situations, leading to births of neonates with lower Apgar scors.

In the term placentas of our study – both in the study group G1 (term) and all the term placentas of the study the rank value of HGF positively correlated with the rank value of Collagen IV, supporting the previously mentioned suggestions on the regenerative features of HGF, having promoted intactness of the basement membranes of term placentas.

We suggest HGF to be a potent regeneration agent of the tissues; its absence in threatening situations can be undertaken as an indicator of placental decompensation.

IL-10 is known as the most potent anti-inflammatory cytokine, providing

maternal tolerance towards fetal allograft; the highest rank value of IL-10 in comparison with the other cytokines in all the placentas of our study seemed natural. Concordance between the anti-inflammatory and pro-inflammatory cytokines are known to be a keystone of a successful pregnancy (Bowen et al., 2002; Szukiewich, 2012), therefore their correlations in different clinical scenarios could be of a practical value. We did not find significant correlations of the rank values of **IL-10** with the rank values of IL-1α or TNFα in the whole study; in the placentas of the study group G1 (term) the rank value of IL-10 positively correlated with the rank value of a pro-inflammatory cytokine TNFα, but not with IL-1α; finding is in line with above described positive correlations of the rank values of the growth factors and receptors with the rank value of TNFα. Our findings suggests that IL-10 and TNFα are working in the same direction towards sustaining and protection of the pregnancy, while IL-1α has the opposite role. Other authors also have described the protective role of IL-10 (Rivera et al., 1998; Robertson et al., 2006), placing TNFα in a role of an antagonist. Our data coincide with studies, that describe simultaneous increase of IL-10 and TNFα as a reaction on infectious stimuli (Barrera et al., 2012; Mitchell et al., 2012), having seen simultaneous increase of both IL-10 and TNFα on the stimulation by LPS. As in our study positive correlations between IL-10 and TNFα appeared in the sudy group G1 (term), including healthy patients with term deliveries and good outcomes, our suggestion would be that they can react not only to infectious, but also antigen stimuli, not causing inflammation and happening as a microbial colonization prior to term delivery.

We also found significantly higher mean rank value of IL-10 in the patients with positive cervical or fetal membrane cultures both in the whole study and in the study group G2 (pre-term), supporting suggestion on the microbial stimuli on the expression of IL-10.

In all the placentas of our study and in the placentas of the study group G3 (distress) the rank values of IL-10 showed statistically significant positive correlation with the rank values of MMP9 while in a study, provided *in vitro*, is described decrease of MMP2 and MMP9 by introduction of IL-10 (Fortunato et al., 2001), suggesting role of IL-10 in the delay of the initiation of pre-term labor. Our study examined placental tissues, while the latter one examined amniochorion, therefore the reactions could be different. In our study group G3 (distress) termination of pregnancy was evidently the best choice of management, especially as the rank value of IL-10 in this study group presented also a positive correlation with the rank value of Collagen IV. In the study

group G2 (pre-term) as well as pre-term placentas of the whole study we did not see any significant correlations between the rank values of IL-10 and MMP9, indicating efforts of placenta by the means of IL-10 to avoid initiation of pre-term labor; absence of such a placental reaction was possibly seen due to the way of development of pre-term labor in our cases, as only 9 from the 25 pre-term deliveries of the study group G2 (pre-term) faced spontaneous onset of labor with uterine contractions and increase of this group of patients would provide significant correlations.

We did not find significant differences in the rank values of **IL-1α** and **TNFα** between the placentas of SGA, AGA and LGA neonates while other authors have described significantly higher expression of TNFα in placentas of growth retarded fetuses (IUGR) and suggested trophoblast giant cells to be the source of TNFα in those cases (Almasry et al., 2012). They described findings of idiopathic fetal growth retardation, while in our patients retardation was mainly of circulatory origin; we suggest that the unknown (idiopathic) cause could have been of inflammatory origin. Their findings somehow match with our findings in pre-term placentas both of the study group G2 (pre-term) and all the pre-term placentas of the study, showing statistically significant negative correlation between the rank values of a pro-inflammatory cytokine IL-1α and the most potent growth factor IGF1, meaning that IL-1α is related to growth restriction of pre-term fetuses. Interestingly that was not valid for TNFα, as TNFα did not show negative, but positive correlation with IGF1 in the study group G1 (term) and with IGFR1 and HGF in the study group G2 (healthy pre-term), confirming its non-involvement in growth restriction processes in healthy pre-term and term placentas.

Collagen IV is a major costituens of the basement membrane, playing a significant role in the tissue stability (Kalluri, 2003; Poschl et al., 2004); we expected and found this protein in all the post-delivery placentas of various gestational ages both beyond the cytotrophoblast and stroma of the villi, like described by other authors (Sati et al., 2008).

Looking for correlations between Collagen IV and other molecular factors of our study from the point of different sections we found positive correlations of the rank values of Collagen IV with the rank values of three growth factors and receptors: HGF, FGFR1 and IGFR1. In the term placentas of the study group G1 (term) as well as in term placentas of the whole study the rank values of Collagen IV showed positive correlations with HGF; in the latter ones also with the rank values of IGFR1, while in

the pre-term placentas of the study group G2 (healthy pre-term), study group G3 (distress) and pre-term placentas of the whole study the rank value of Collagen IV showed positive correlation with the rank values of FGFR1. Positive correlation with FGFR1 was so strong, that appeared in evaluation of the whole study as well suggesting the pathway of decreased functionality of placental tissues contrary to the way of maintenance of placental function. In the literature we did not find analogue correlations; some authors mentioned increased fibrosis in the placentals with increased expression of Collagen IV (Chen and Aplin, 2003; Chen et al., 2005).

Comparing the mean rank values of **MMP9** between the study groups we found a statistically significant difference between the study groups G1 (term) and G3 (distress); the amount of MMP9 positive cells in the placentas of the latter study group was higher that in term ones; other studies have not found changes in the expression of MMP9 in circumstances of oxygen deprivation (Merchant et al., 2004). We suggest increased MMP9 in a placenta of a significant distress being an emergency reaction on a life threatening situation, when termination of pregnancy is the only way of survival; it confirms remaining functional abilities of the placenta.

We found positive correlations of the rank values of MMP9 with the rank values of anti-inflammatory cytokine IL-10 in the placentas of the whole study and in the placentas of the study group G3 (distress), suggesting role of MMP9 in the development of parturition processes in the other cases than loss of immunological tolerance of fetal allograft; as IL-10 has shown a significant negative correlation with the gestational age of placenta, it confirms the impact of MMP9 on the initiation of pre-term labor, caused by different circumstances; it corresponds with the other studies looking for causative factors of pre-term deliveries and finding increased expression of MMP9 (Romero et al., 2002; Weiss et al., 2007; Karthikeyan et al., 2012).

We suggest MMP9 to be an important molecular factor of the cells of the maternal-fetal environment, resposible for timely initiation of labor, either pre-term or term, for the achievement of better possible outcome.

In the placentas of the study group G1 (term) the rank values of **HoxB3** products showed significant positive correlations with the rank values of the growth factor receptor IGFR1, impact of which in the term placentas was not so remarkable, but showed positive correlation with Collagen IV; in the placentas of the study group G3 (distress) the rank values of HoxB3 products presented significant correlations with the

rank values of MMP9. We suggest participation of the HoxB3 products in the process of determination of integrity of tissues; their particular impact requires further research.

We did not find any correlations of HoxB3 products with cytokines like described negative correlations with a pro-inflammatory cytokine (Sarno et al., 2006; Sarno et al., 2009) in the decidual cells, suggesting development of pathway for a pre-term delivery and influencing the outcome of pregnancy.

5.3. Clinical issues of the study

Evaluating correlations of the rank values of **IL-10** with the clinical findings we did not find any significant correlations either between the rank values of the study groups or between the rank values of IL-10 with clinically significant maternal or neonatal parameters, despite in another study there was described decreased level of IL-10 in hypoxic conditions (Peltier et al., 2011) suggesting also reduction of the placental responsiveness to anti-inflammatory action of IL-10 in low pO_2 circumstances; those results were achieved in the cultured humen placental explants and possibly do not reflect all the processes *in vivo*. We did not find correlations between the rank values of IL-0 and neonatal blood pH after delivery either in the whole study or in any of the groups separately ($p>0.05$), including placentas of the study group G3 (distress) with significant fetal distress prior or during labor and definitely associated with a significant oxygen deficiency. Our study included 4 stillbirth cases: 1 antenal and 3 intranatal deaths; in the placenta of antenatally demised fetus of 38 gestational weeks we saw few (+) IL-10 positive cells while in all the three intra-natal death cases we saw abundance (++++) of IL-10 positive cells, that could be explained by the compensatory reaction of the placenta to the reduction of anti-inflammatory action of IL-10 in hypoxic circumstances.

Taking into account the findings of other studies, we evaluated correlations between the rank values of IL-10 with clinical findings, indicating the lung development. In the study group G2 (pre-term) we found higher rank value of IL-10 in the placentas of the patients, having received exogenous surfactant and therefore having more severe respiratory problems. As in this study group the rank value of IL-10 presented negative correlation with the gestational age, higher probability of surfactant application appears to be natural and is not related to lung development as such. To compare data with other studies, having found less broncho-pulmonary dysplasia in

patients with higher expression of IL-10 (McGowan et al., 2009), further prospective research studies are needed, as development of lung complications in pre-mature children depend on much more circumstances than lung organogenesis.

We did not find any differences in the mean rank values of IL-10 in the placentas between the patients with vaginal delivery or elective cesarean section (CS) as well as between the patients having been in labor (vaginal and emergency CS) and having undergone elective CS; the findings are similar to the findings between vaginal delivery and elective CS patients in the cord blood (Tutdibi et al., 2012).

We did not find significant differences of the rank values of the pro-inflammatory cytokines **IL-1α** and **TNFα** in the patients with positive cervical or fetal membrane culture with pathogenic or oportunistic microorganisms, like described *in vitro* models, having found activation of pro-inflammatory cytokines IL-1β and TNFα in the human fetal membranes after infection (Flores-Herrera et al., 2012); they suggested E.coli and GBS to be the main caustive agents. We exclused from our study cases with clinically diagnosed chorionamnionitis and in our study positive cultures mostly did not match with inflammation signs in placenta or the membranes, therefore the rank values of pro-inflammatory cytokines between those patient groups did not differ.

In the study group G2 (healthy pre-term) we found a positive correlation between the rank values of a pro-inflammatory cytokine IL-1α and the time of membrane rupture; clinical explanation is clear as in PPROM emergence of inflammation signs in the maternal-fetal unit is an indication for the termination of pregnancy and such post-delivery placentas could be expected to contain larger number of pro-inflammatory cytokine positive cells; interestingly this was found to be valid for IL-1α and not for TNFα. We concidered this finding to coincide with the studies, suggesting higher expression pro-inflammatory cytokines in the amniotic fluid indicative for an onset of pre-term delivery in 7 days (Hillier et al., 1993; Figueroa et al., 2005).

Our study identified several clinically important compliances between the amount of pro-inflammatory cytokine positive cells in the placental tissues and clinical signs of the neonate. In the pre-term placentas of our study group G2 (healthy pre-term) and in pre-term placentas of the whole study the rank values of IL-1α presented statistically significant negative correlations with the initial neonatal blood pH and the 1st minute Apgar score, suggesting more IL-1α positive cells in the placental tissues to

be indicative for a worse status of a pre-mature neonate. In the study group G3 (distress) similar correlations showed the rank values of TNFα, suggesting involvement of different pro-inflammatory cytokines. Our data match the findings of studies, having found increased expression of IL-1 and IL-6 in maternal and fetal blood in acidotic circumstances (Prout et al., 2010) and increased TNFα in cases of neuro-developmental disorders of children (Carpentier et al., 2011); we suggest gestation dependent indicators and pathways of pathological processes, leading to impaired health of fetus and neonate.

In the study group G3 (distress) we unexpectedly found a strong positive correlation of the rank values of TNFα with the initial blood glucose of the neonates; blood glucose levels were taken prior to any, including glucose, infusions and therefore were indicative for maternal blood glucose and neonatal insulin status and correspond with a study, having found increased placental TNFα production in mothers with higher glycemic means (Moreli et al., 2012). As this correlation was valid in the distress group with a clinically significant fetal distress prior or during labor, we suggest that predisposition to the distress situation was probably provided by an undiagnosed gestational diabetes or a milder type of a glucose intolerance in pregnancy.

In accordance with literature data we evaluated different clinical factors, that could possibly correlate with the number of pro-inflammatory cytokine positive cells in the placental tissues. Patients of our study, having antenatally received Dexamethasone, did not show decreased number of TNFα positive cells unlike the study, having seen stepwise reduction of TNFα in the placental tissues with an increase of doses of Betamethasone (Xu et al., 2005). We also did not see any differences of the rank values of pro-inflammatory cytokines between patients of vaginal deliveries, emergency CS and elective CS, although other studies have found higher level of TNFα in laboring patients in comparison with not laboring ones (Jiang et al., 2012). As we evaluated appearance of cytokine positive cells in the placental tissues *in situ*, we possibly did not cover all the maternal-fetal unit, researched in the studies of tissue cultures.

Looking for the correlations between the rank values of **HoxB3** positive cells in the placentas of the study groups with maternal and neonatal parameters we found some statistically significant correlations. In the study group G3 (distress) the rank values of HoxB3 products showed positive correlation with the maternal body height meaning more HoxB3 product positive cells in taller women. We could undertake that as a coincidence if we would not have seen other correlations. In the whole study as well as

in the pre-term cases of the whole study the rank values of HoxB3 products in placentas presented a significant negative correlation with the ponderal indices of neonates, suggesting its impact on the longitudinal growth of a fetus; this was strengthened by a finding in the study group G1 (term) where the rank values of HoxB3 products showed positive correlations with the neonatal body length. Those findings presently could be perceived as unique and suggesting more significant impact of Hox genes than already described impact on the growth and development of placental tissues *per se* (Zhang et al., 2002; Amesse et al., 2003).

6. CONCLUSIONS

1. Mothers of pre-term deliveries present significantly higher number of pregnancies than mothers of normal term deliveries, but not deliveries suggesting, that termination of pregnancy increases risk for pre-term delivery. Pre-term delivery cases in general have longer period of ruptured membranes (ROM) than term ones. None of the maternal anthropometrical or social data differ between the study groups.
2. Weight of a pre-term delivery placenta directly depends on the gestational age, maternal weight gain in pregnancy and correlates with the main fetal anthropometrical parameters (weight, length, head and chest circumferences) suggesting impact of placental weight on the growth and development of fetus.
3. Neonatal anthropometrical data agree with their gestational age. Clinically important is a statistically significantly lower initial blood glucose level in pre-mature neonates, supporting importance of blood glucose monitoring of pre-mature neonates.
4. IGF1, IGFR1, HGF and FGFR1 characterize all the placental samples, suggesting permanent growth ability of the organ. Tendency of the decrease of numbers of IGF1, IGFR1 and HGF positive cells seems to depend on adaptation/ageing of placenta. Basic FGF does not play a significant role in the development of placenta beyond 22nd week of pregnancy while FGFR1 immunoreactive cells increase with the advanced gestation, indicating increasingly evolving maturation (growth, proliferation) of placenta, especially in the third trimester.
5. Presence of more IL-1α positive cells in pre-term and more TNFα positive cells in term placentas demonstrate the ability of the organ to produce inflammatory factors selectively with development of placental tissues, including proliferation of Höfbauer cells. Significant decrease of IL-10 positive cells with advanced gestation proves importance of maternal tolerance to fetal allograft in late second and early third trimester of pregnancy.
6. Apoptotic cells are features of all post-delivery placentas of various gestational ages; their number decrease with advanced gestation, suggesting a change to other ways of cellular disposal. In term placentas of different clinical scenarios number of apoptotic cells is smaller in patients with a history of a larger number of

pregnancies, suggesting restricted abilities of tissues for non-destructive turnover of cells.

7. From the components of the basement membrane, the most remarkable one – Collagen IV is seen in post-delivery placentas of various gestational ages with significant increase towards the term, suggesting maturation of placental barrier with ongoing pregnancy.
8. From the tissue degrading enzymes, MMP9 positive cells were seen in all the post-delivery placentas from 22 weeks of pregnancy till term; significant increase in distress placentas suggests possible role of MMP9 of this organ in the natural termination of pregnancy in its various stages.
9. Amount of HoxB3 positive cells, present in all the post-delivery placentas from 22 weeks of gestation, does not correlate with its gestational age, but with some neonatal anthropometrical indices, indicating HoxB3 role in the placental development itself and also longitudinal growth of fetus.
10. Term placentas after normal deliveries with a healthy child show less pronounced IGF1, IGFR1 and HGF and more pronounced FGFR1 amount as a characteristic feature for standard development. Advanced maternal age, number of pregnancies and deliveries in their medical history are significantly associated with increased amount of TNFα positive cells in placenta at term, suggesting higher risk of this group for ascending infections. Apoptosis is less pronounced with increasing number of pregnancies suggesting decrease of programming capabilities of placentas of consecutive pregnancies. Increase of MMP9 in term placentas of neonates with higher body mass and chest circumference suggests tissue strength of those placentas.
11. Pre-term placentas are characterized by larger amount of IGF1 and IGFR1 positive cells, suggesting stimulation of growth of this organ; increased amount of IL-1α and MMP9 positive cells prove enhanced placental remodeling in this stage of pregnancy as well as higher susceptibility for the development of inflammation.
12. Post-delivery placentas after event of significant fetal distress in various gestational ages are characterized by mismatch of pro- and anti-inflammatory cytokine TNFα, IL-1α and IL-10 positive cells, suggesting presence of disorders, possibly causing restriction of growth and development of placenta itself and the fetus, as well as instability of the placental local defense. Positive correlation of HGF positive cells with FGFR1 positive cells suggest enhanced fibrosis in regenerative efforts of

placenta, possibly leading to decrease of its functional capacity. Significantly higher amount of MMP9 positive cells in distress placentas possibly indicate its impact on natural termination of pregnancy in harmful circumstances possibly affecting fetal well-being.

7. PUBLICATIONS AND PRESENTATIONS

Articles

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1. Makroskopiskas, mikroskopiskas un imūnhistoķīmiskas izmaiņas placentā normāli noritošas grūtniecības gadījumā, to saistība ar augļa veselības stāvokli. Poster presentation in the scientific conference of Riga Stradins University, Riga, Latvia, 2009.
2. Pēcdzemdību placentas ekstracellulārās vides indikatoru saistība ar klīnisko atradni. Oral presentation in the scientific conference of Riga Stradins University, 2010.
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4. Imūnās atbildes citokīni dažāda gestācijas vecuma pēcdzemdību placentas. Oral presentation in the scientific conference of Riga Stradins University, Riga, Latvia, 2012.
5. Matricas metālproteināze MMP9 dažāda gestācijas vecuma pēcdzemdību placentas. Oral presentation in the scientific conference of Riga Stradins University, 2013.

Poster and oral presentations in international congresses and conferences

1. Apoptozi veicinošo citoķīmisko marķieru noteikšana placenta. Poster presentation in the 5th Congress of Latvian Obstetricians and Gynecologists, Baltic International conference in Obstetrics and Gynecology, Riga, Latvia, October 10-11, 2008.
2. Cyto-chemical factors in placenta at the time of delivery, indicating risk factors for increased apoptotic cell death. Poster presentation, 9th World Congress of Perinatal Medicine, Berlin, Germany, October 24-28, 2009.
3. Correlation of cytokines in placentas with the clinical findings. Poster presentation in the XXII European Congress of Perinatal Medicine, Granada, Spain, May 26-29, 2010.
4. Immunohistochemical (IHC) detection of HoxB3 genes in post-delivery placentas of various gestational ages. Oral presentation in the 2nd International congress of UENPS, Istanbul, Turkey, November 15-17, 2010.
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7. Cilvēka morfoģenēzi noteicoša gēna HoxB3 imūnhistoķīmiska identifikācijas dažādu gestācijas laiku pēcdzemdību placentas. Oral presentation in the 6th Congress of Latvian Gynecologists and Obstetricians, 4th Joint Royal College of Obstetricians and Gynecologists (RCOG)/ Latvian Association of Gynecologists and Obstetricians Eurovision Conference, Riga, Latvia, October 13-15, 2011.
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