Placental proteome in late-onset of fetal growth restriction

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Received June 10, 2022; Accepted September 16, 2022

DOI: 10.3892/mmr.2022.12872

Abstract. Fetal growth restriction (FGR) occurs when the fetus does not reach its genetically programmed intrauterine potential for growth and affects ~5-10% of pregnancies. This condition is one of the leading causes of perinatal mortality and morbidity associated with obstetric and neonatal complications. Placental dysfunction in FGR causes an impairment in the transfer of nutrients and oxygen from the mother to the developing fetus. Maternal adaptations to placental insufficiency may also play a role in the pathophysiology of FGR. The present study aimed to compare the proteome of the placentas of 18 women with the physiological course of pregnancy and eutrophic fetus [estimated fetal weight (EFW) >10th percentile; control group] and 18 women with late FGR (EFW <10th percentile) diagnosed after 32 weeks of pregnancy, according to the Delphi consensus (study group). The U. Mann-Whitney test was used to compare two independent groups. The R. Spearman correlation coefficient significance test was used to assess the existence of a relationship between the analyzed measurable parameters. P<0.05 was considered to indicate a statistically significant difference. The tests showed the presence of 356 different proteins which were responsible for the regulation of gene transcription control, inhibiting the activity of proteolytic enzymes, regulation of trophoblast proliferation and angiogenesis and inflammatory response. In the FGR placental proteome, other detected proteins were mostly involved in response to oxidative stress, cellular oxidation and detoxication, apoptosis, hemostatic and catabolic processes, energy transduction protein interactions, cell proliferation, differentiation and intracellular signaling. The present study used chromatographic mass-spectrometry to compare the placental proteome profiles in pregnancies complicated by late-onset FGR and normal pregnancy. Comparative analysis of proteomes from normal and FGR placentas showed significant differences. Further research is needed to clarify maternal and fetal adaptations to FGR.

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Key words: proteome, fetal growth restriction, placenta, lipid metabolism, peptides, oxidative stress

Introduction

Pregnancy is a condition that requires numerous adaptive changes to the mother, fetus and placenta. Adaptation processes at the level of cell physiology and individual systems occurring in the course of physiological and complicated pregnancy are extremely complicated. The physiological changes that occur in pregnancy are mainly caused by the placenta. However, the way in which the maternal tissues respond to the new demands of pregnancy development is hypothesized to influence the success of the pregnancy as well (1).

The proteome defines all the proteins within a cell or a tissue. The metabolome describes metabolites including small molecules, peptides, carbohydrates, lipids, nucleosides and catabolic product. They are connected by numerous aspects of cell signaling, protein degradation and generation and post-translational modification (2). In comparison to the non-pregnant state, the first trimester of pregnancy is characterized by systemic adaptation of the mother (2). How these adaptive processes are reflected in the maternal metabolome/proteome is not well characterized.

The principal findings of the study by Handelman *et al* (3) were that pregnant women and non-pregnant women differ in the abundance of 44% of the profiled plasma metabolites. This finding was explained by the inhibition of specific metabolic processes producing small molecules, the activation of catabolic processes consuming small molecules or expanding blood volume, leading to dilution. The metabolite differences and associated perturbed pathways reflected the physiological changes occurring in the first 16 weeks of normal pregnancy. Finally, metabolites like blood lysolipids and dipeptides were reported to change as a consequence of advancing gestation.

During the first two trimesters of pregnancy, there is a build-up of lipids in the mother's tissues. Circulating maternal metabolic products, such as triglycerides, cholesterol, free fatty acids and phospholipids, are intended to meet the energy needs of the fetus and ensure an adequate supply of mother's milk after delivery. This period is seen as an 'anabolic phase'

characterized by an increase in maternal fat accumulation and a progressive decrease in fasting glucose levels in as the gestational age increases, which is associated with a 10% reduction in insulin sensitivity compared to the pre-pregnancy period (4-6). Although fasting glucose is lowered, hepatic glucose production (via gluconeogenesis and glycogenolysis) is increased, leading to an increase in fasting insulin. As a consequence, decreased maternal liver sensitivity to insulin leads to increased hepatic glucose production (4). During the third trimester of pregnancy, the maternal metabolic state is characterized by a 'catabolic phase' in which peritoneal insulin sensitivity is further reduced and fat storage in the peritoneum and subcutaneous tissue is impaired, which is a source of calories for the mother and fetus (4,5). Insulin resistance in pregnant women increases significantly with gestational age in normal pregnancy to ensure adequate glucose transfer to the fetus and maternal inositols positively correlate with crown-rump length (CRL) (7). They are correlated with insulin sensitivity and may be mechanistically related to glucose homeostasis (8). These events demonstrate active changes in energy requirements during pregnancy. Other age-related carbohydrate metabolic changes include placental polyol pathways which are very active in the first trimester of pregnancy (9). One hypothesis regarding elevated levels of polyols in early pregnancy is that they are an early source of carbohydrates for the placenta and embryo. In addition, the polyol pathway may facilitate the re-oxidation of pyridine nucleotides under low oxygen conditions, helping to regulate intracellular pH during periods of high glycolysis (10).

The placenta serves a key role in regulating the metabolic environment in pregnancy. The human placenta is adapted from the initial hypoxic environment in the first trimester to increased oxygenation in the second trimester of pregnancy when the spiral arteries remodel (11,12). The oxygen concentration in the interstitial junctions increases from 2-3% in the 8th week of pregnancy to 8.5% in the 12th week of pregnancy (13). These changes are accompanied by increased oxidative stress and, as a consequence, an increase in placental antioxidant factors maintaining redox homeostasis. These changes are particularly evident in the metabolism of hexadecanoic acid, erythritol and 2-deoxyribose (14). Placental cholesterol has also been found to be elevated in correlation with CRL. Higher cholesterol levels may be the result of increased levels of progestogen hormones. Maternal cholesterol is a precursor to both progesterone and estrogen (10).

Placental dysfunction is the main culprit in fetal growth restriction (FGR) causing an impairment in the transfer of nutrients and oxygen from the mother to the developing fetus. Maternal adaptations to placental insufficiency may also play a role in the pathophysiology of FGR (15).

FGR affects ~5-10% of pregnancies and is the leading cause of perinatal mortality and morbidity (16). This condition occurs due to placental dysfunction when the fetus does not reach its genetically programmed intrauterine potential for growth. FGR is associated with obstetric and neonatal complications and the development of cardiometabolic diseases at an older age (17,18). The etiology of FGR is complex and related to fetus (genetic and chromosomal abnormalities and congenital metabolic disorders), placenta (impaired placentation and implantation) and pregnant woman (placental dysfunction of vascular origin) (16,18). Risk factors of developing FGR include: Smoking, hypertension, severe chronic anemia, pregestational diabetes mellitus, autoimmune diseases, congenital malformations and infections, chromosomal abnormalities. unfortunately, the presence of these risk factors is only found in 30% of cases (19).

In clinical practice FGR must be distinguished from small for gestational age (SGA) fetuses, which represent constitutionally smaller fetuses. In contrast to SGA fetuses, FGR is pathological condition associated with insufficiency of placenta and less supply of oxygen and nutrients to the developing fetus. The current standard of recognition FGR is based on ultrasound examinations. FGR can be divided as early or late onset according to gestational age at onset/recognition (32 weeks). Early-onset FGR is associated with gestational hypertension and/or pre-eclampsia in ≤70% and represents ~20-30% of cases of FGR whereas late-onset FGR represents 70-80% of all FGR and to a lesser extent is associated with the development of hypertensive disorders of pregnancy (20). According to the DELPHI consensus early-onset FGR is diagnosed before 32 weeks of gestation when estimated fetal weight (EFW) or abdominal circumference(AC) is <3rd centile or there is absent end-diastolic flow in the umbilical artery (UA) or AC or EFW is <10th centile combined with a pulsatility index (PI) >95th centile in either the UA or uterine artery (21,22). For late FGR (≥32 weeks), the following criteria must be fulfilled: AC or EFW <3rd centile and four contributory parameters (EFW or AC <10th) centile, AC or EFW crossing centiles by > two quartiles on growth charts and cerebroplacental ratio <5th centile or UA-PI >95th centile (21,22).

There is no effective antenatal therapy for FGR. Hence, delivery of the newborn remains the only option to avoid stillbirth. When this occurs preterm, the further risk of morbidity and mortality is introduced (23,24). In current clinical practice for monitoring the growth restricted fetus cardiotocography and Doppler sonography are used. In the management of late-onset FGR the cerebroplacental ratio (CPR) is important because it not only allows a more precise diagnosis of late-onset perinatal outcomes but also helps to predict unfavorable perinatal outcomes (25-27). The two subtypes of FGR show different pathogenic and clinical features. Defective placentation, due to a poor trophoblastic invasion of the maternal spiral arteries, is hypothesized to play a central role in the pathogenesis of early-onset preeclampsia and FGR (28).

One of the promising diagnostic methods is comparative proteomics based on the analysis of the protein profile in normal and abnormal tissues (29). Reviews have highlighted the proteomic approaches that have been used to explore pre-eclampsia (PE), FGR and preterm birth (30-34).

The aim of the present study was to compare the proteome of the placentas in women with fetal growth restriction and with a physiological course of pregnancy.

Materials and methods

Patients between 32-36 weeks' gestation with singleton pregnancy with late onset FGR who were hospitalized in the Department of Obstetrics and Pathology of Pregnancy at the Medical University of Lublin between 2019 and 2021 were recruited for the present study. The criteria for excluding patients from the analysis were: Multiple pregnancy, presence of

any antenatal infections, positive TORCH test result, treatment with antibiotics during pregnancy, any form of hypertension in pregnancy, pre-pregnancy and gestational diabetes, nephropathy, thyroid dysfunction and any other general diseases before pregnancy, using any drugs or stimulants, nicotinism and fetuses with birth defects and chromosomal abnormalities.

After obtaining informed written consent, 36 pregnant women who delivered by cesarean section were involved in the study, including 18 women with physiological pregnancy and eutrophic fetus (EFW >10th percentile; control group) and 18 women with late FGR (EFW <10th percentile; study group) diagnosed after 32 weeks of pregnancy, according to Delphi consensus (21). The age range of the control group was 18-38 years whereas the age range of the study group was 19-37 years old. To estimate the weight of the fetus during an ultrasound scan, a regression equation was used considering the biparietal diameter, the length of the femur and the head and abdominal circumferences, as proposed by Hadlock *et al* (35). Doppler measurements of the umbilical artery free loop were obtained within 1 week of delivery using a Voluson E9 with RA4B 3D 4-8 MHz curvilinear probe (Cytiva). The PI, RI and CPR were then calculated. PI=(*S*-*D*)/*A* and RI=(*S*-*D*)/*S*, where *S* is the systolic peak, *D* is the end diastolic flow and *A* is the temporal average frequency. Whereas cerebroplacental ratio (CPR) is the ratio between the PI of middle cerebral and umbilical artery (PI MCA/PI UA) and reflect the distribution of cardiac output in favor of cerebral blood flow. It is one of the parameters that has the best accuracy in predicting perinatal outcomes (36). In response to intrauterine hypoxia, redistribution of fetal blood flow to the brain occurs and the value of CPR decreases <1. In cases of late-onset FGR, the tolerance to hypoxia is lower than in early-onset FGR (37).

Clinical information on mothers was obtained from standardized medical records and patient interviews, including smoking, age, weight and body mass index (BMI) at the start of the first trimester, pregnancy weight gain and TORCH. BMI was calculated as body weight (kg)/height $(m)^2$. The following data on infants were also obtained from medical records: Gestational age at delivery, sex and birth weight of the newborn, placental weight, body length, head circumference and neonatal complications. Gestational age was determined based on the date of the last menstruation and the first-trimester ultrasound scan (based on CRL). Birth weight and placenta weight, body length and head circumference were measured immediately after birth using appropriate measuring tools.

The material for proteomic research was fragments of normal placentas constituting control and fragments of placentas collected from women diagnosed with FGR. All samples were collected by trained personnel as follows: In aseptic conditions, during the cesarean section, immediately after the delivery of the child, the placenta was placed in sterile containers containing ice. Specimen collection personnel were wearing a sterile protective apron, face masks and sterile gloves to ensure sterility throughout the sampling process. The placentae were weighed and collected. A total of four placenta biopsies of 1.0x1.0x1.5 cm from each placenta was obtained. The collection site was ~3-4 cm from the umbilical cord insertion site peripherally from four different placental quadrants. Only sections from the inner part of the placenta were collected for examination to eliminate possible infections during cesarean section (risk of contamination). Sections from 18 placentae from women with impaired fetal growth and 18 control placenta were qualified for the study. Each placenta sample was placed in a sterile, labeled cryovial, then frozen in liquid nitrogen and stored at -80˚C until DNA extraction and further analysis.

Written informed consent was obtained from all subjects included and the study was performed in accordance with the principles of the Helsinki Declaration The research was issued by the Bioethics Committee at the Medical University of Lublin (approval no. KE-0254/87/2020). Derived data supporting the findings of this study are available from the corresponding author on request.

Identification of proteins

Protein isolation. Each test was performed in duplicate. Proteins isolated from the tested material and control (18 samples FGR and 18 control) were analyzed in a polyacrylamide (PAA) gel, then pooled and analyzed using mass spectrometry (MS). The obtained results were searched using the Mascot algorithm (MASCOT 2.4.1; https://www.matrixscience.com/) against the Uniprot database 2019_02 (559228 sequences; 200905869 residues, https://www.uniprot.org/) with a filter searching for human proteins. In order to prepare samples for further analyses, protein isolation was performed. 100 mg of each tissue was crushed in a mortar with liquid nitrogen and subsequently 500 μl of isolation buffer (0.1% Tris-Cl, 10% glycerol) was added and gently mixed. The mixture was then transferred into 1.5 ml Eppendorf tubes and centrifuged at 10,000 x g for 10 min at 4˚C. Finally, the supernatant was transferred to new tubes and frozen at -20˚C for further study.

SDS-PAGE analysis. In order to verify the protein composition of protein samples, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in a slab mini-gel apparatus according to Laemmli (38) using 10% polyacrylamide as the separating gel and 5% polyacrylamide as the stacking gel. The proteins were denatured by heating them to 100˚C in the presence of 2-mercaptoethanol for 5 min. Then, 50 μg of each sample was put into the gel (two technical replicates). After electrophoresis the resulting gels were fixed and stained using sensitive Coomassie Blue Staining (0.02% Coomassie Brilliant Blue G-250, 12 h staining at room temperature) (39). Separating gel at 10% was chosen in order to separate the whole protein spectrum of a sample, including proteins with high as well as low molecular weighs.

MS analysis. Stained protein bands were excised from the gel and analyzed by liquid chromatography (LC) coupled to mass spectrometer in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. Tryptic peptide mixtures were analyzed by LC-ESI-MS/MS using nanoflow HPLC and the LTQ-Orbitrap XL (Thermo Fisher Scientific, Inc.) as mass analyzer with two technical replicates. Spectrometer parameters were as follows: capillary voltage: 2.5 kV, cone: 40 V, N2 gas flow: 0 and m/z range 300-2,000, with positive ionization mode. Excised gel fragments were placed in 1.5 ml Eppendorf tubes filled with 10% methanol and 2% acetic acid. The proteins were digested using trypsin. The

Table I. Clinical characteristics and anthropometric measurements of mothers and newborns.

Variables	Control group n=	Study group (FGR) n=	P-value
Baseline characteristics			
Age (years)	30.2±6.5	28.2±5.6	0.466
Height (m)	1.7±0.06	1.67±0.08	0.373
Actual Weight (kg)	80.3±11.3	79.5±7	0.854
Weight before the pregnancy (kg)	63.6±11.3	66.7±6.3	0.458
BMI before the pregnancy (kg/m^2)	22±3.5	23.9±2.1	0.154
Weight gain (kg)	14 (12-28)	12.5 (11-15)	0.032
Weight of the placenta (kg)	515±46	328±53	<0.001
Parity	2 (1-4)	1 (1-3)	0.504
Gestation	2 (1-4)	1.5 (1-4)	0.699
Perinatal outcomes			
Gestational age at the delivery (weeks)	39 (38-41)	37 (35.4-40)	0.002
Fetal weight at birth (g)	3,540 (2,910-3,890)	2,300 (1,385-2,570)	<0.001
Neonatal length (cm)	54 (47-57)	48 (35-51)	0.001
APGAR 1 min (points)	9 (8-10)	8 (6-9)	0.002
APGAR 5 min (points)	10 (9-10)	10 (6-10)	0.597
Feto-placental Doppler before delivery			
UA PI	0.77 (0.72-0.91)	1.11 (0.98-1.9)	<0.001
MCA PI	1.44±0.21	1.31±0.22	0.191
UtA PI	0.79±0.05	0.93±0.17	0.025
CPR	1.703 (1.48-2.444)	0.995 (0.737-1.687)	<0.001

Values are shown as median [interquartile range]; statistical analysis was performed using a Mann-Whitney U test. Variables following a normal distribution are reported as mean ± standard deviation, statistical test for them was the standard t-test. BMI, body mass index; PI, pulsatility index; UA, umbilical artery; MCA, middle cerebral artery; UtA, uterine artery; CPR, cerebro-placental ratio; APGAR, Appearance, Pulse, Grimace, Activity, Respirations.

generated peptides were concentrated, desalted on an RP-C18 precolumn (LC Packings) and further separated by UltiMate nano-HPLC (LC Packings) using water containing 0.1% TFA as a mobile phase with a linear acetonitrile gradient (10-30%) over 50 min with the flow rate of 150 nl/min. The column was directly coupled to a nanospray ion source operating in a data-dependent MS to MS/MS switch mode. Proteins were identified by tandem mass spectrometry (MS/MS) via information-dependent acquisition of fragmentation spectra of multiple-charged peptides.

Protein identification algorithm. The spectral data were analyzed by MASCOT 2.4.1 (Matrix Science; www.matrixscience.com) and searched against the Uniprot 2019_02 (559228 sequences; 200905869 residues) database with Homo sapiens (human; 20492 sequences) filter. Mascot search criteria were as follows: protein scores >31 indicated identity or extensive homology (P<0.05); with carbamidomethyl (C) and oxidation (M) variable modifications; peptide mass tolerance ±50 ppm and fragment mass tolerance ±0.8 Da. Protein identifications were accepted when at least two peptide fragments per protein were identified.

Quantitative analysis. To achieve a non-label quantitative comparison of proteins between analyzed samples, the Exponentially Modified Protein Abundance Index (emPAI) was employed (40). The number of peptides per protein normalized by the theoretical number of peptides is called the protein abundance index (PAI). To determine protein abundance from the nano-LC-MS/MS experiments a modified form of PAI is used: the exponential form of PAI minus one (emPAI=10PAI-1). The emPAI value is proportional to protein abundance in a protein mixture. Resultant protein and peptide lists were saved in Excel files (Microsoft Corporation).

Statistical analysis. The obtained results were analyzed statistically. The values of the analyzed parameters measured on the nominal or ordinal scale were characterized by the number and percentage, while those measured on the interval scale by the arithmetic mean, standard deviation, median, 25 and 75th percentiles and the range of variation. Due to the skewed distribution of the measured parameters assessed using the W. Shapiro-Wilk test or the heterogeneity of variance assessed using the F-Fischer test, non-parametric tests were used to analyze the existence of differences between the studied subgroups. The U. Mann-Whitney test was used to compare two independent groups. The Spearman correlation coefficient significance test was used to assess the existence of a relationship between the analyzed measurable parameters. Statistical analyzes were performed based on the Statistica v. 10.0 software (StatSoft). P<0.05 was considered to indicate a statistically significant difference.

Table II. Human placental proteins identified by LC-ESI-MS/MS from control samples.

Accession[a]	Identified protein[b]	Score[c]	Molecular mass (Da)[d]	Matched Peptides[e]	Sequence coverage (%)[f]	Exponentially Modified Protein Abundance Index[g]
Q5QNW6	Histone H2B type 2-F OS=Homo sapiens OX=9606 GN=HIST2H2BF PE=1 SV=3	2,179	13,912	50	53.2	18.58
P01009	Alpha-1-antitrypsin OS=Homo sapiens OX=9606 GN=SERPINA1 PE=1 SV=3	3,141	46,845	81	67.7	15.21
P07355	Annexin A2 OS=Homo sapiens OX=9606 GN=ANXA2 PE=1 SV=2	1,972	38,764	42	72.3	14.05
P08758	Annexin A5 OS=Homo sapiens OX=9606 GN=ANXA5 PE=1 SV=2	1,942	35,960	41	74.1	13.66
P68032	Actin, alpha cardiac muscle 1 OS=Homo sapiens OX=9606 GN=ACTC1 PE=1 SV=1	2,591	42,268	84	57	12.27
P62736	Actin, aortic smooth muscle OS=Homo sapiens OX=9606 GN=ACTA2 PE=1 SV=1	2,456	42,304	73	57	12.27
P06576	ATP synthase subunit beta, mitochondrial OS=Homo sapiens OX=9606 GN=ATP5F1B PE=1 SV=3	3,384	56,525	63	64.8	9.88
P0DML2	Chorionic somatomammotropin hormone 1 OS=Homo sapiens OX=9606 GN=CSH1 PE=1 SV=1	1,948	25,234	43	64.1	9.19
P04083	Annexin A1 OS=Homo sapiens OX=9606 GN=ANXA1 PE=1 SV=2	2,489	38,874	44	60.7	7.7
O43707	Alpha-actinin-4 OS=Homo sapiens OX=9606 GN=ACTN4 PE=1 SV=2	3,227	105,156	85	61.7	6.78
P18206	Vinculin OS=Homo sapiens OX=9606 GN=VCL PE=1 SV=4	2,047	124,182	57	48.2	3.33
Q05707	Collagen alpha-1(XIV) chain OS=Homo sapiens OX=9606 GN=COL14A1 PE=1 SV=3	2,929	194,268	79	38.5	2.32
P35579	Myosin-9 OS=Homo sapiens OX=9606 GN=MYH9 PE=1 SV=4	2,649	227,403	62	32.9	1.79
P21333	Filamin-A OS=Homo sapiens OX=9606 GN=FLNA PE=1 SV=4	2,658	282,771	67	29.4	1.21
Q9Y490	Talin-1 OS=Homo sapiens OX=9606 GN=TLN1 PE=1 SV=3	2,191	271,347	56	30.3	1.05
Q09666	Neuroblast differentiation-associated protein AHNAK OS=Homo sapiens OX=9606 GN=AHNAK PE=1 SV=2	2,362	629,113	74	14.7	0.52

[a]Database accession numbers according to: Uniprot 2019_02 (559228 sequences; 200905869 residues) database with Homo sapiens (human) (20492 sequences) filter. [b]Identified homologous proteins. [c]Mascot Search Probability Based Mowse Score. Ions score is-10xLog(P), where P is the probability that the observed match is a random event. Protein scores >31 indicate identity or extensive homology ($P<0.05$). [d]Theoretical mass (Da) of identified proteins. The values were retrieved from the protein database. [e]Number of matched peptides with Mascot search data (www.matrixscience.com). [f]Amino acid sequence coverage for the identified proteins. [g]Exponentially modified protein abundance index of identified protein according to Mascot Search data.

Results

The results of the study were obtained from 18 placentas taken from women with fetal growth disorders and from 18 control placentas. The clinical characteristics along with anthropometric measurements of mothers and their newborns are presented in Table I.

There were no statistically significant differences between the study groups in terms of age, height, fertility, BMI before pregnancy, body weight before pregnancy and that measured at delivery. The control group was characterized by statistically significantly higher weight gain in pregnancy compared to the study group (P=0.032) and differences in placenta weight (P=0.00000005). As a result of the ultrasound analysis of vascular flows using the color Doppler technique, a statistically significantly higher mean pulsation index (PI) in the gestational uterine arteries was found in the study group compared to the control group (P=0.025), as well as a higher PI in the artery umbilical cord of fetuses with FGR compared to eutrophic ones (P=0.0001). The cerebro-placental ratio (CPR) was statistically significantly higher in the control group compared to the study group (P=0.0005). Pregnant women in the test group gave birth statistically significantly earlier than in the control group (P=0.002). Newborns with FGR were characterized by lower birth weight (P=0.0001) and shorter body length (P=0.001) as well as lower Apgar score in the first minute of life (P=0.002) compared to neonates from the control group.

The tests performed showed the presence of 356 different proteins. From this group, those proteins that were identified in comparable amounts in both samples, both in the test and control groups, were eliminated. Only those proteins that were not present in the control group were analyzed. Proteins were ranked according to their emPAI, which is proportional to the protein content of the given sample. Tables II and III show the results of the studies for control placenta (Table II) and placenta from women with FGR (Table III).

The function of the proteins identified in the control sample is described in Table IV and those in the test sample in Table V.

Discussion

The development of the human placenta is complex and not well described. For the study of placental changes such methods as cell culture technologies, advanced imaging techniques, omics technologies, biomarkers and proteomics are used (41-43).

The results of the present study with the use of a comparative analysis of proteomes from normal and FGR placentas showed in the cells of the normal placenta the presence of proteins responsible for the regulation of gene transcription control (histon, annexin) and proteins inhibiting the activity of proteolytic enzymes (alpha 1-antitrypsin), the deficiency of which may lead to oxidative stress and damage to the placenta. This inhibitor also serves an important role in the regulation of trophoblast proliferation and angiogenesis. In the normal placenta, the present study also identified actins that serve an important role in determination of cell proliferation and invasion, (mainly F-actin), rearrangement of the action cytoskeleton, regulation membrane dynamics and inflammatory response (annexin) (44-48). In the FGR placental proteome other detected proteins are mostly involved in response to stress (peroxiredoxin-2), cellular oxidation and detoxication (catalase), apoptosis (apolipoprotein), hemostatic and catabolic processes, energy transduction (pyruvate kinase, transketolase), protein folding (calreticulin, protein disulfide-isomerase) and interactions (protein disulfide-isomerase 14-3-3 protein zeta/delta), immunity (moesin) and inflammation (hemopexin). In FGR the expression of peroxiredoxin-2 antioxidant protein was also observed, the role of which is to neutralize reactive oxygen species (ROS) and regulate multiple cellular functions such as cell proliferation, differentiation and intracellular signaling. ROS accumulation is also limited by catalase (present in FGR) which inactivates H_2O_2. The 14-3-3 protein zeta/delta present in FGR integrates and controls multiple signaling pathways. The detected triosephosphate isomerase (TIP) during gluconeogenesis, provides the two substrates required for aldolase to generate fructose-1,6-bisphosphate, which is converted to fructose-6-phosphate and glucose-6-phosphate, important precursors for cell wall components and nucleic acids. Lactate dehydrogenase enzyme, which is important in maintaining a high level of pyruvate and is highly expressed in tissues with high-energy demands, was also present in the study group (49-58).

The results of the present study showed that the highest emPAI coefficient, which proves the proportionally high protein content in the FGR samples, concerns apolipoprotein 1A (Apo1A). Apo1A is one of the proteins responsible primarily for cholesterol transport, but it also has anti-inflammatory properties by influencing lipid peroxidation and the immune system. The anti-inflammatory effect of Apo 1A is based on the inhibition of the migration in the endothelium of cells of the immune system as a result of a decrease in the expression of integrins, inhibition of the activation of monocytes and the synthesis of cytokines (49). The presence of these apolipoproteins in the FGR placentas suggested disturbed cholesterol homeostasis and inflammation processes. This will result in abnormal lipids metabolisms, vascular damage, disturbances in the function of cellular molecules and incorrect folding of proteins, which would in turn be indicative of the presence of chaperones (e.g., 14-3-3 protein zeta/delta). In the studied (FGR) group no structural proteins were found in opposition to the group of proteins from the normal placentas.

The abnormal lipid metabolism during pregnancy with intrauterine growth restriction of the SGA type was also noted by Bernard *et al* (59). Another study shows that in the antenatal period, the ratio of fatty acids in the mother's blood serum compared to its concentration in the newborn decreases in FGR, which suggests increased energy and metabolic demand of the fetus (60).

Statistically significant differences in the synthesis of cholesterol in fetuses with FGR compared to eutrophic ones were also shown, e.g., the concentration of cholesterol during pregnancy in fetuses with FGR was slightly increased (2.48 times), while in the serum of correctly developing fetuses the concentration of cholesterol increased 6.54 times (61). Also in the urine of newborns from pregnancies with FGR, an increased level of myo-inositol is found, which correlates with the negative regulation of the release of free fatty acids from adipose tissue (62). In turn, the results of Bahado-Singh *et al*

Table III. Human placental proteins identified by liquid chromatography electrospray ionization tandem mass spectrometry from the group of fetal growth restriction cases.

Accession[a]	Identified protein[b]	Score[c]	Molecular mass (Da)[d]	Matched Peptides[e]	Sequence coverage (%)[f]	Exponentially Modified Protein Abundance Index[g]
P02647	Apolipoprotein A-I OS=Homo sapiens OX=9606 GN=APOA1 PE=1 SV=1	486	30,759	14	33.3	3.48
P32119	Peroxiredoxin-2 OS=Homo sapiens OX=9606 GN=PRDX2 PE=1 SV=5	300	22,016	7	25.8	2.12
P63104	14-3-3 protein zeta/delta OS=Homo sapiens OX=9606 GN=YWHAZ PE=1 SV=1	237	27,866	7	25.3	1.86
P01009	Alpha-1-antitrypsin OS=Homo sapiens OX=9606 GN=SERPINA1 PE=1 SV=3	497	46,845	12	23.9	1.46
P60174	Triosephosphate isomerase OS=Homo sapiens OX=9606 GN=TPI1 PE=1 SV=3	363	31,002	9	20.6	1.25
P07195	L-lactate dehydrogenase B chain OS=Homo sapiens OX=9606 GN=LDHB PE=1 SV=2	244	36,845	5	15.3	0.77
P04040	Catalase OS=Homo sapiens OX=9606 GN=CAT PE=1 SV=3	468	59,903	10	16.9	0.76
P02790	Hemopexin OS=Homo sapiens OX=9606 GN=HPX PE=1 SV=2	354	52,241	15	11.3	0.76
P27797	Calreticulin OS=Homo sapiens OX=9606 GN=CALR PE=1 SV=1	401	48,250	9	20.4	0.69
P07237	Protein disulfide-isomerase OS=Homo sapiens OX=9606 GN=P4HB PE=1 SV=3	316	57,403	7	16.9	0.67
P26038	Moesin OS=Homo sapiens OX=9606 GN=MSN PE=1 SV=3	487	67,870	11	10.7	0.64
P14618	Pyruvate kinase PKM OS=Homo sapiens OX=9606 GN=PKM PE=1 SV=4	248	58,360	7	8.9	0.43
P29401	Transketolase OS=Homo sapiens OX=9606 GN=TKT PE=1 SV=3	272	68,387	7	7.7	0.36
O43707	Alpha-actinin-4 OS=Homo sapiens OX=9606 GN=ACTN4 PE=1 SV=2	253	105,156	8	7.2	0.33
P00450	Ceruloplasmin OS=Homo sapiens OX=9606 GN=CP PE=1 SV=1	344	122,817	7	6.7	0.19
Q9BVA1	Tubulin beta-2B chain OS=Homo sapiens OX=9606 GN=TUBB2B PE=1 SV=1	238	50,289	4	7.2	0.18

[a]Database accession numbers according to: Uniprot 2019_02 (559228 sequences; 200905869 residues) database with Homo sapiens (human) (20492 sequences) filter. [b]Identified homologous proteins. [c]Mascot Search Probability Based Mowse Score. Ions score is-10xLog(P), where P is the probability that the observed match is a random event. Protein scores >31 indicate identity or extensive homology ($P<0.05$). [d]Theoretical mass (Da) of identified proteins. The values were retrieved from the protein database. [e]Number of matched peptides with Mascot search data (www.matrixscience.com). [f]Amino acid sequence coverage for the identified proteins. [g]Exponentially modified protein abundance index of identified protein according to Mascot Search data.

Table IV. Functions of proteins identified in control placenta.

First author, year	Proteins	Functions	(Refs.)
Arimura Y, 2018	Histone H2B type 2-F	Core component of nucleosome serves a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability	(44)
Pater D, 2021	Alpha-1-antitrypsin	*α1-antitrypsin* is a protein belonging to the serpin superfamily	(45)
Xi Y, 2020	Annexin A2	Placental anticoagulant protein IV	(46)
Monceau V, 2004	Annexin A5	Is a cellular protein with presumed function of plasma membrane repair and hemostasis	(123)
Li A, 2021	Actin, alpha cardiac muscle 1	The alpha actins are found in muscle tissues and are a major constituent of the contractile apparatus.	(124)
Yuan SM, 2018	Actin, aortic smooth muscle	α-2 *actin* is found in *smooth muscle* cells, a family of globular multi-functional proteins that form microfilaments	(125)
Jonckheere A, 2012	ATP synthase subunit beta, mitochondrial	*Mitochondrial* membrane *ATP synthase* [F(1)F(0) *ATP synthase* or Complex V] produces *ATP* from ADP in the presence of a proton gradient across the membrane	(126)
Männik J, 2012	Chorionic somatomammotropin hormone 1	Produced only during pregnancy and is involved in stimulating lactation, fetal growth and metabolism.	(127)
D'Acquisto F, 2008	Annexin A1	Plays important roles in the innate immune response as effector of glucocorticoid-mediated responses and regulator of the inflammatory process.	(47)
Peng W, 2021	Alpha-actinin-4	Alpha actinin is an actin-binding protein with multiple roles in different cell types.	(48)
Bays JL, 2017	Vinculin	*Vinculin* is a cytoplasmic actin-binding protein enriched in focal adhesions and adherens junctions that is essential for embryonic development.	(128)
Patino MG, 2002	Collagen alpha-1(XIV) chain	It likely serves a role in *collagen* binding and cell-cell adhesion.	(129)
Sudo H, 2013	Neuroblast differentiation-associated protein	Protein may play a role in such diverse processes as blood-brain barrier formation, cell structure and migration, cardiac calcium channel regulation and tumor metastasis	(130)
Sun H, 2020	Myosin-9	Serves a role in cytokinesis, cell shape and specialized functions such as secretion and capping. During cell spreading, serves an important role in cytoskeleton reorganization, focal contact formation	(131)
Su W, 2012	Filamin A	Filamins play multiple cellular roles, serving as organizers of cell structure (e.g., cytoskeleton) and function, regulating cell signaling, transcription, cell adhesion, focal adhesion assembly, cell apoptosis and organ development.	(132)
Burrudge K, 2018	Talin	Probably involved in connections of major cytoskeletal structures to the plasma membrane.	(133)

in addition to identifying potential FGR biomarkers, provides information on the dysregulation of placental biochemistry in FGR (41). The univariate analysis of metabolites showed a global decrease in phosphatidylcholine in FGR. The decrease in lipid metabolites can be explained by the decreased level of placental energy substrates as a result of hypoxia leading to significant changes in lipid metabolism, as pointed out by Raff *et al* (63).

Paules *et al* analyzed gene ontology and revealed the pathways and biological processes involved in late-onset FGR which were mostly related to the efflux of cholesterol and phospholipids (33). The lipoproteins Apolipoprotein C2, Apolipoprotein C3 and Apolipoprotein E with parallel pathway of LXR/RXR activation, are fundamental in the balance of cholesterol levels and known protective function against dysregulated fetoplacental lipid homeostasis. Those

Table V. Functions of proteins identified in fetal growth restriction.

First author, year	Proteins	Functions	(Refs.)
Mangaraj M, 2016	Apolipoprotein A-I	Component and a major structural protein of high-density lipoprotein, serves a vital role in reverse cholesterol transport and cellular cholesterol homeostasis. Its multifunctional role in immunity, inflammation, apoptosis, viral, bacterial infection	(49)
Duan T, 2016	Peroxiredoxin-2	Thiol-specific peroxidase that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively.	(134)
Pennington KL, 2018	14-3-3 protein zeta/delta	Adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathways. Binds to a large number of partners, usually by recognition of a phosphoserine or phosphothreonine motif. Binding generally results in the modulation of the activity of the binding partner.	(50)
Stockley RA, 2015	Alpha-1-antitrypsin	Protein produced in the liver that protects the body's tissues from being damaged by infection-fighting agents released by its immune system	(135)
Wierenga RK, 2010	Triosephosphate isomerase (TIP)	Enzyme which very fast interconverts dihydroxyacetone phosphate and D:-glyceraldehyde-3-phosphate.	(51)
Chen Y, 2019	L-lactate dehydrogenase B chain	LDH catalyzes the conversion of lactate to pyruvate and back, as it converts NAD^+ to NADH and back; transfers hydride from one molecule to another.	(88)
Fu W, 2014	Catalase	Catalyzes the reaction by which hydrogen peroxide is decomposed to water and oxygen.	(52)
Poillerat V, 2020	Hemopexin	Is the plasma protein with the highest binding affinity to heme. It is mainly expressed in liver and belongs to acute phase reactants, the synthesis of which is induced after inflammation.	(53)
Varricchio L, 2017	Calreticulin	Is a chaperone protein which resides primarily in the endoplasmic reticulum and is involved in a variety of cellular processes, among them, cell adhesion. Additionally, it functions in protein folding quality control and calcium homeostasis. Calreticulin is also found in the nucleus, suggesting that it may have a role in transcription regulation.	(54)
Khan HA, 2014	Protein disulfide-isomerase	Is a prototypic thiol isomerase that catalyzes the formation and cleavage of thiol-disulfide bonds during protein folding in the endoplasmic reticulum (ER. PDI is induced during endoplasmic reticulum (ER) stress and it serves as a vital cellular defense against general protein misfolding via its chaperone activity. It is also responsible for the isomerization, formation and rearrangement of protein disulfide bonds	(55)
Karvar S, 2020	Moesin	Is particularly important in immunity acting on both T and B-cells homeostasis and self-tolerance, regulating lymphocyte egress from lymphoid organs. Ezrin-radixin-moesin (ERM) family protein that connects the actin cytoskeleton to the plasma membrane and thereby regulates the structure and function of specific domains of the cell cortex.	(56)
Sizemore ST, 2018	Pyruvate kinase PKM	Catalyse the conversion of phosphoenol-*pyruvate* (PEP) to *pyruvate* at the final step of glycolysis.	(57)

Table V. Continued.

First author, year	Proteins	Functions	(Refs.)
Wilkinson HC, 2020	Transketolase	Is an important enzyme in the non-oxidative branch of the pentose phosphate pathway (PPP), a pathway responsible for generating reducing equivalents, which is essential for energy transduction and for generating ribose for nucleic acid synthesis. Transketolase also links the PPP to glycolysis, allowing a cell to adapt to a variety of energy needs, depending on its environment	(58)
Feng D, 2018	Alpha-actinin-4	Is a widely expressed homodimeric protein that bundles and cross-links filamentous actin.	(136)
Zanardi A, 2018	Ceruloplasmin	Binds to copper and carries it throughout your body. If you have low ceruloplasmin, it can point to a genetic condition called Wilson disease, a copper deficiency or other medical conditions	(137)
Lehmann SG, 2017	Tubulin beta-2B chain	Is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain (By similarity). TUBB2B is implicated in neuronal migration.	(138)

lipoproteins are also involved in atherosclerosis and IL-12 signaling in inflammation, lipid dysregulation and endothelial cell dysfunction. The existing oxidative stress, inflammation and placental thrombosis interrupt the placental ability to transfer nutrients and oxygen to the fetus and therefore implicate the normal fetal growth.

In contrast to the present study, Bahado-Singh *et al* (41) describe 3-hydroxybutyric acid as the most important metabolite for distinguishing between FGR and control placental tissue. 3-hydroxybutyric acid is synthesized by the liver and is a source of energy for the brain when glucose levels are low. Moreover, it is the end product of fatty acid oxidation by ketogenesis and a substrate for lipid synthesis in the biological cascade of ketolysis. Placental trihydroxybutyric acid deficiency could potentially explain the low lipid levels.

Research to date suggests that both the placenta and vascular endothelium of a pregnant woman are tissues that use oxygen to produce energy through mitochondrial-mediated oxidative phosphorylation. This highly energetic process is also supported by the formation of ROS that regulate intracellular signaling and tissue adaptation (64,65). ROS are more and more often recognized as signaling molecules that regulate physiological processes, while oxidative stress is a state that disrupts signaling pathways in the cell (65,66).

The present study implied that there was a general disruption of fetal energy substrates and metabolism in the FGR. This was evidenced by the high result obtained for peroxiredoxin-2 (Prxs). It belongs to the family of antioxidant proteins involved in the fight against free radicals. This protein causes the production of inflammatory cytokines. In addition, it performs several other functions related to the regulation of cell proliferation, differentiation and protection against oxidative stress (67,68). During inflammation, high levels of peroxides are produced by phagocytes and the cytoprotective antioxidant role of Prxs in inflammation cannot be overestimated (67,68). Peroxides also serve to regulate inflammatory signaling pathways and Prxs are known to be a critical modulator of signaling peroxides (69). Hemopexin is also associated with antioxidant stress. Hemopexin is an intracellular glycoprotein responsible for maintaining blood homeostasis by regulating free heme, which eliminates the harmful pro-oxidative and pro-infusion potential of heme (70). Hpx serves a neuroprotective role after ischemic injuries. According to Li *et al* (71) local expression of Hpx by neurons contributes to protection against free heme through induction of HO isoenzyme, or HO1. The upregulation of HO1 in ischemic astrocytes and fibroblasts has a protective effect against ischemia by reducing oxidation, stress and apoptosis. In $Hpx^{-/-}$ mice following ischemic stroke, there is evidence of increased oxidative damage, infarct volume and general neurological deficiency (71,72).

Calreticulin (CARL) in physiological, normal cells acts as a chaperone to help protein fold properly in the endoplasmic reticulum. CALR supports Ca^{2+} dependent processes such as adhesion and signaling integrin and ensures correct antigen presentation on MHC class I molecules as well as participation in Ca^{2+} transport, an essential component for placental and fetal development (54). During physiological pregnancy, 30 g Ca^{2+} migrates from mother to fetus across the placenta to facilitate the development of the fetal skeletal system (73). It has been shown that CALR, as a molecular chaperone of the placenta, is necessary for the proper development of the trophoblast and placenta (74-76). However, extracellular CALR release is unusual and, since CALR is a stress response protein, stress may be involved in extracellular CALR release (77). Studies have shown an increase in CALR mRNA and protein levels in maternal blood and placenta of patients with pre-eclampsia (78,79). Iwahashi *et al* (80) provide evidence that induction of stress in the endoplasmic reticulum leads

to the extracellular release of CALR, which may contribute to placental dysfunction by inhibiting cytotrophoblast syncytialysis. In addition, tumor cells undergo immunogenic cell death (ICD) by exposing CALRs on their surface, which promotes the uptake of tumor cells by phagocytes and ultimately supports the initiation of anti-tumor immunity. In this way, loss of function CALR mutations promotes oncogenesis not only because they disrupt cell homeostasis in healthy cells, but also because they threaten natural and therapy-controlled immune surveillance (80).

Protein disulfide-isomerase (PDIA3) is a chaperone that modulates the folding of newly synthesized glycoproteins, exhibits isomerase and redox activity and is involved in the pathogenesis of numerous diseases (81). However, the role of PDIA3 in pregnancy-related diseases remains to be elucidated. Mo *et al* (81) reveal a key role of PDIA3 in the biology of placental trophoblasts in women with PE. Immunohistochemistry and western blot analysis showed that PDIA3 expression was decreased in villi trophoblasts from women with PE compared to pregnancies with normal blood pressure. Furthermore, using the Cell Counting Kit-8 assay, flow cytometry and 5-ethynyl-2'-deoxyuridine (EdU) staining, it was found that siRNA-mediated PDIA3 knockdown significantly promotes apoptosis and inhibits proliferation in the HTR8/SVneo cell line, while overexpression PDIA3 reversed these effects. In addition, RNA sequencing and western blot analysis showed that PDIA3 knockdown inhibited MDM2 protein expression in HTR8 cells, concomitantly with a marked increase in p53 and p21 expression. Conversely, PDIA3 overexpression had the opposite effect. Moreover, immunohistochemistry and western blotting showed that MDM2 protein expression was decreased and p21 was increased in the trophoblasts of women with PE compared to women with pregnancies with normal blood pressure. PDIA3 expression is decreased in the trophoblasts of women with PE and decreased PDIA3 induces trophoblast apoptosis and inhibits trophoblast proliferation by regulating the MDM2/p53/p21 pathway (81).

In FGR, the 14-3-3 zeta/delta proteins were also identified described as specific proteins of the brain tissue; their first described function was to activate the synthesis of neurotransmitters (82).

Currently, ~200 different cellular proteins have been identified as binding partners for 14-3-3 proteins and they are involved in almost every cellular process, including signal transduction, cell cycle control, apoptosis, transcription regulation, cytoskeleton rearrangements, cell adhesion, chromosome maintenance, protein localization, protein transport, protein degradation, exocytosis, endocytosis, development and stress response (83,84). The 14-3-3 proteins play a key role in subcellular localization. Injured central nervous system (CNS) neurons, unfortunately, have a poor ability to regenerate spontaneously, resulting in permanent functional deficits following hypoxic injury (84). Kaplan *et al* (85) show that the 14-3-3 adapters are central proteins that are attractive targets for manipulating cell signaling. Researchers demonstrate a positive role for 14-3-3s in axon growth and regulation of phosphorylation and function of 14-3-3s. They showed that fusicoccin-A (FC-A), a small molecule stabilizer of protein-protein interactions 14-3-3, stimulates axon growth *in vitro* and regeneration *in vivo* (85).

Moesin (MSN) is a member of the ezrin-radixin-moesin (ERM) family of proteins, which binds plasma membrane proteins to actin fibers in the cell cortex and is essential for vascular endothelial function. They are found in cell surface structures such as microvilli, filopodia, beauty, wrinkle membranes, retraction fibers and cell adhesion sites where actin fibers are associated with plasma membranes. Ezrin-radixin-binding protein-50-kDa (EBP50) is a protein that serves an important role in cancer development. Embryo and tumor growth are similar. Embryo implantation is a key process for a successful pregnancy although the mechanism of embryo implantation is not fully understood. Lipopolysaccharides can stimulate endothelial cells to secrete MSN (86). Additionally, MSN is required for induced endothelial cell hyperpermeability and inflammatory responses and high levels of MSN in the blood are detected in mice and human patients with sepsis (87). Hence, MSN is involved in the pathogenesis of sepsis and MSN may be a potential biomarker for assessing the severity of endothelial damage during sepsis (88). MSN deficiency in mice significantly affects lymphocyte homeostasis; the number of NK cells in peripheral blood and bone marrow decreases, but it increases in the spleen. MSN-deficient NK cells show increased cell death and impaired signaling in response to IL-15, suggesting that MSN regulates NK cell survival through IL-15-mediated signaling. It can therefore be that MSN can be regarded as a regulator of NK cell homeostasis *in vivo* (89).

Noteworthy is the presence of such proteins in FGR as lactate dehydrogenase (LDH). LDH intracellular enzyme is important in energy production in nearly all cells in the body. Its highest concentrations are in the heart, liver, muscles, kidneys and lungs. The total concentration of lactate dehydrogenase is made up of five different enzyme variants (isoenzymes), which are produced by different tissues. Only a small fraction of LDH can be found in the blood because the enzyme is released into it when cells die or damage. Therefore, serum lactate dehydrogenase is a nonspecific marker of tissue damage in the body, while an increase in placenta with FGR is indicative of placental insufficiency (88).

Pyruvate kinase (PKM) serves a key role in regulating cellular metabolism. The conversion of phosphoenolpyruvate (PEP) to pyruvate, which is catalyzed by pyruvate kinase, is the final rate-limiting step in glycolysis. There are four isomeric, tissue-specific forms of pyruvate kinase found in mammals: PKL, PKR, PKM1 and PKM2. PKM1a and PKM2 are formed by a single mRNA transcript of the PKM gene by alternative inclusion. The PKM2 dimer regulates the rate of the glycolysis step that shifts glucose metabolism from the normal respiratory chain to lactate production in cancer cells. In addition to being a regulator of metabolism, it also acts as a protein kinase that contributes to oncogenesis. It is mainly described in neoplastic processes (57). Hasan *et al* (90 demonstrate the expression of PKM2 in normoxic states (20% O_2) and hypoxic states (0.1% O_2) in two prostate cancer cell lines, PC3 and LNCaP. The authors show that hypoxia significantly increases the expression of PKM2 mRNA in both cell lines (46). This suggests that under hypoxic conditions, PKM2 expression is further promoted by HIF-1α activation (90). Tumor angiogenesis is initiated by PKM2 dimer in the blood, thereby increasing endothelial cell proliferation, migration and cell-ECM

adhesion, leading to tumor growth (91,92). Most important is the activation of IGF-IR, a PKM2-mediated tumor angiogenesis event, by disrupting the NF-κB/miR-148a/152t feedback loop, promoting tumor growth and angiogenesis (93). Under hypoxia, IGF-1/IGF-IR mediates the interaction of HIF-1α with the NF-κB p65/RelA subunit and the PKM2 promoter and PKM2 expression is also enhanced by the repression of miR-148a and miR-152 (94). The binding leads to nuclear translocation of PKM2, where it acts as a protein kinase and interacts with other molecules to control the expression of VEGF, thus promoting tumor angiogenesis.

Transketolase (TKT) in the non-oxidative branch of the pentose phosphate pathway (PPP); it regulates the level of ribose-5-phosphate (R5P) and de novo nucleotide biosynthesis (95). Maintaining genome integrity is essential because genomic information regulates cell proliferation, growth arrest and important metabolic processes in cells (96). Genomes are constantly exposed to endogenous and environmental DNA-damaging agents such as oxidizing agents, nitrosamines and polycyclic aromatic hydrocarbons. Altered cellular metabolism, which is intertwined with DNA damage and repair pathways, leads to genomic instability, while accumulation of genome instability results in metabolic abnormalities (97,98). A number of different metabolic pathways are involved in de novo synthesis of nucleotides (99,100). R5P, an intermediate product of PPP, is an important precursor in the biosynthesis of both DNA and RNA. PPP is one of the branches of glycolysis and serves a key role in meeting the cellular requirements of biosynthesis and antioxidant defense (101). Moreover, PPP is essential for repairing double-strand breaks after DNA damage has occurred in mammalian cells (101). TKT and transaldolase are the two major enzymes that mediate reversible reactions in non-oxidative PPP. The main purpose of PPP is the production of R5P and NADPH. R5P is the major backbone of RNA and is critical for nucleotide synthesis. NADPH is the major antioxidant that keeps the two major redox molecules, glutathione and thioredoxin, in a reduced state. Thus, NADPH counteracts ROS, allowing cells to survive oxidative stress.

Alpha-actinin (ACTN) members maintain the structures of the cytoskeleton and modulate cell mobility (102). Among the four members of the ACTN family in humans, ACTN2 and ACTN3 are specific to muscle cells while ACTN1 and ACTN4 are ubiquitous (103,104). ACTN4 is present at the leading edge of moving cells, suggesting that ACTN4 may be involved in cell migration (103,104). Moreover, ACTN4 signaling connects integrins with the actin cytoskeleton and enhances the invasion of trophoblasts in the placenta (105). ACTN4 deficiency dramatically reduces the proliferation and invasion of various neoplastic cells (106). Accumulating evidence strongly suggests that ACTN4 may be involved in trophoblast proliferation and invasion. However, the true functions of ACTN4 in trophoblast and placenta development remain to be elucidated.

Peng *et al* (48) suggest that ACTN4 expression is essential for normal trophoblast proliferation and differentiation in early pregnancy. Downregulation of ACTN4 may result in insufficient proliferation, invasion and migration of the trophoblast via the AKT/GSK3β/Snail pathway which may lead to pre-eclampsia. Proper development of the placenta and its component, pedigree in the early stages, is crucial for a successful pregnancy. Dysregulation of extravillous trophoblasts (EVT) disrupts the normal invasion of trophoblasts into the uterus, which in turn leads to incomplete remodeling of the spiral arteries and placental hypoperfusion (107). In Peng *et al* (48), ACTN4 was mainly expressed in cytotrophoblasts (CTBs) and EVT of the normal placenta but was barely detected in these cells from placental severe pre-eclampsia. Decreased ACTN4 levels reduce villi, trophoblast proliferation and ex vivo explants overgrowth. Moreover, the deficiency of ACTN4 results in significant inhibition of cell invasion and motility. Such attenuated proliferation, invasion and migration are a result of ACTN4 mediated by inactivation of the AKT/GSK3β/Snail pathway. CTBs are the so-called placental epithelial stem cells that, depending on the received signals, can maintain a balance between their differentiation into both ST and EVT. In addition, isolated CTBs without proliferation capacity may spontaneously differentiate into STBs after 24-h cultivation, suggesting that the self-renewal potential of CTBs is necessary to maintain proliferation and differentiation capacity (48).

Ceruloplazmin is an acute-phase protein, both pro-oxidative and antioxidant, synthesized by hepatocytes and involved in angiogenesis, coagulation and nitric oxide (NO) homeostasis (108,109). The main role of ceruloplasmin in the turnover of iron is the oxidation of Fe^{2+} to Fe^{3+}, a process necessary for the binding of iron to transferrin (the main iron transporting protein) and ferritin (the main iron storing protein) (109). Increased serum levels of ceruloplasmin have been associated with an increased risk of cardiovascular disorders and serve as a predictor of adverse clinical outcomes in patients with acute coronary syndromes or myocardial infarction (110,111). Significantly higher concentrations of ceruloplasmin are found in patients with pre-eclampsia in whom the placental expression of ceruloplasmin, most likely derived from syncytiotrophoblasts, is high (112). Bellos *et al* (113) show that serum ceruloplasmin may be a useful screening and control tool for assessing pregnant women with a history of developing preeclampsia.

In 2022, the work of Surekh *et al* (114) was published, assessing the effect of maternal iron deficiency anemia (IDA) in 200 pregnant women on the expression of the iron transporter, although not ceruloplasmin but cyclopen in the term placenta. Placental cyclopen expression was investigated by mRNA analysis and protein immunohistochemistry. The cyclopene mRNA and the protein expression in the placenta showed a statistically significant increase with increasing the severity of anemia. The immunohistochemical expression of the cyclopen protein showed a statistically significant increase with the increase in the severity of the anemia. Similarly, placental cyclopen mRNA expression was higher in anemic mothers compared to non-anemic mothers. Surekh *et al* (114) showed for the first time a marked increase in cyclopene expression at both protein and mRNA levels in the term placenta in maternal IDA. This study helped determine how placental iron transport proteins could be regulated in response to maternal iron status and newborns and broadened our knowledge on the relationship between the iron state in the mother and the newborn and the mechanisms of modifying the placental iron transport concerning these parameters.

A prospective cohort analysis of 107 single pregnancies who underwent amniocentesis at 16-22 weeks according to standard genetic indications showed glucose, alkaline

phosphatase (ALP), LDH, ceruloplasmin, ferritin, highly sensitive C-reactive protein and IL-6 in the mother's blood and amniotic fluid (115). The median concentration of ferritin in the amniotic fluid and IL-6 and the mean concentration of ALP in the amniotic fluid were higher in the group of premature infants, but this difference did not reach statistical significance. The maternal mean levels of ALP and LDH were slightly higher. Only the median maternal ferritin concentration in the intrauterine growth restriction group was higher than in the patients corresponding to the gestational age (P=0.03). In conclusion, low levels of glucose in the amniotic fluid are associated with the risk of preterm labor, while high levels of ferritin in the mother's blood increase the risk of FGR.

A similar study investigated the levels of zinc, copper, iron and magnesium ions and certain binding proteins in the amniotic fluid under FGR conditions (116). FGR showed a decrease in the content of zinc, iron and magnesium ions and an increase in the content of copper in the amniotic fluid in the second and third trimesters of pregnancy. In these trimesters, levels of ceruloplasmin, ferritin and Ca^{2+}, Mg^{2+} and ATPase were lower in FGR, while levels of zinc-a-2-glycoprotein were higher than during the same periods of normal pregnancy. Changes in the parameters tested in the amniotic fluid were associated with disturbances in the development of newborns.

Tubulin beta-2B chain (TUBB2B) is a cytoskeleton component that serves a key role in CNS corticogenesis, mediating mitosis and cell translocation and the formation of synaptic connections (117,118). Heterozygous *de novo* missense variants in tubulin genes are associated with a heterogeneous group of disorders characterized by cortical malformations cerebral dysplasia, is known as 'tubulinopathies'. Affected patients most often show a range of neurodevelopmental disorders, including cognitive and motor impairment, abnormal muscle tone and epilepsy (119-121).

Alpha-1 antitrypsin (AAT) is a serum protein synthesized in the liver and secreted into the blood. AAT deficiency is associated with various clinical symptoms of the neonatal period (45). The most common symptom is 'neonatal hepatitis syndrome', occasionally referred to as 'neonatal cholestasis' or 'cholestatic hepatitis'. The increase in antitrypsin-1 in FGR indicates the activation of compensatory mechanisms that protect the fetus.

Proteomic analysis in iron-treated mice compared to control mice showed 66 differentially expressed hippocampal proteins (30 upregulated and 36 downregulated) (122). Bioinformatics analysis showed that the deregulated proteins included, but was not limited to, mitochondrial-associated proteins (e.g., ADP/ATP translocator 1 and zeta/delta 14-3-3 protein) cytoskeleton proteins (TUBB2B and tubulin alpha-4A chain). Research suggests that dysregulation of synaptic, mitochondrial and cytoskeleton proteins may be involved in memory impairment induced by molecular mechanisms of iron neurotoxicity (122). In the future, it would be worthwhile to perform validation of the detected proteomic differences.

The limitations of the present study included increased risk of false positive results due to relatively small sample size and large number of tested targets that might cause random detection of statistical significance when margin for alpha error is set as <0.05).

Current screening tools used in pregnancy e.g., clinical factors, ultrasound scan and placental biomarkers are unable to identify the risk of growth impairment of fetuses. Comparative analysis of proteomes from normal and FGR placentas show significant differences. The changes detected in the FGR placenta proteome are complex and mainly concern proteins involved in the stress response, cellular oxidation and detoxification, apoptosis, catabolic processes, energy transduction and inflammation. The data from the present study about proteome and late FGR presents notable findings for understanding the disease pathophysiology. Elucidating proteomic changes in the placenta may help to uncover the underlying mechanism of the FGR and identify novel targets for therapeutics. Future work in validating these proteomic differences may also enable identification of early diagnosis for FGR. Additionally, the proteomics results can serve as a screening tool where proteins identified as significantly changed will need to be confirmed by traditional validation methods (for example western blotting).

Acknowledgements

Not applicable.

Funding

The present study was supported by Medical University of Lublin (grant no. DS 128).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TG participated in conceptualization, data curation, formal analysis and writing the draft. AS conceived and designed the study, collected data, visualization and writing the original draft. RN participated in formal analysis, investigation, methodology. AGJ performed experiments and writing the original draft. AK participated in analysis and interpretation of data, data curation, funding acquisition, supervision and original writing. WK analyzed and interpretation of data, the project administration, supervision and review and editing. TG, AS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Bioethics Committee at the Medical University of Lublin (approval no. KE-0254/87/2020). Written informed consent was obtained from all subjects included.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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