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Angiogenic factors measured in serum, chorionic villi and amniotic fluid during the late first and the second trimester

Ph.D. Thesis

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III. Vincze M., Sikovanyecz J. Jr., Földesi I., Surányi A., Várbíró Sz., Németh G., Kozinszky Z., Sikovanyecz J. How the soluble human leukocyte antigen-G levels in amniotic fluid and maternal serum correlate with the feto-placental growth in uncomplicated pregnancies. Bioengineering. 2024, 11(5):509. (Q3)

List of abbreviations

AC: amniocentesis

CVS: chorionic villus sampling

CTB: cytotrophoblast

STB: syncytiotrophoblast

EVT: extravillous tropholast

iEVT: interstitial extravillous trophoblast

enEVT: endovascular extravillous trophoblast

mRNA: messenger ribonucleic acid

DNA: deoxyribonucleic acid

VEGF-A: vascular endothelial growth factor-A

VEGFR1: vascular endothelial growth factor receptor-1

FLT1: fms-like tyrosin kinase receptor 1

sFlt1: soluble fms-like tyrosin kinase 1

VEGFR2: vascular endothelial growth factor receptor-1

KDR: kinase insert domain receptor

eNOS: endothelial nitric oxide synthase

nNOS: neuronal nitric oxide synthase

iNOS: inducible nitric oxide synthase

NO: nitric oxide

FGF: fibroblast growth factor

bFGF: basic fibroblast growth factor

TGF-β: transforming growth factor beta

PlGF: placental growth factor

sEng: soluble endoglin

Ang-1: angiopoietin-1

Ang-2: angiopoietin-2

HLA-G: human leukocyte antigen-G

sHLA-G: soluble human leukocyte antigen-G

KIR2DL4: immunoglobulin-like receptor 2DL4

ILT2: immunoglobulin-like transcript 2

ILT4: immunoglobulin-like transcript 4

MMP: matrix metalloproteinase

PP13: placental protein 13

CRD: carbohydrate recognition domain

VI: vascularization index

FI: flow index

VFI: vascularization flow index

ELISA: enzyme-linked immunosorbent assay

CRL: crown rump length

BMI: body mass index

GDM: gestational diabetes mellitus

SGA: small for gestational age

FGR: fetal growth restriction

ACOG: American College of Obstetricians and Gynecologists

NICE: National Institute Health and Care Excellence

ISSHP: International Society for the Study of Hypertension in Pregnancy

FMF: Fetal Medicine Foundation

FIGO: International Federation of Gynecology and Obstetrics

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Introduction

The placenta provides an immunological feto-maternal interface and secretes many immune and inflammatory factors meanwhile it protects the fetus [1-3].

The appropriate fetal development depends on the adequate vascular architecture of the placenta. The placental growth includes extensive vasculogenesis and branching angiogenesis within the placental villi controlled by multifunctional proangiogenic and antiangiogenic factors expressed by trophoblasts, immune cells, epithelial cells, endothelial cells, and secreted into the amniotic fluid and the maternal circulation [4]. Both branching (the formation of new vessels) and nonbranching (elongation of vessels) angiogenesis appear in the placenta [2], [5], [6], [7]. Vasculogenesis and branching angiogenesis last to 24 post-conceptional weeks and it is followed by non-branching angiogenesis until term [2], [5], [6], [7].

During embryonic development, the trophectoderm differentiates into highly proliferative, undifferentiated, primitive cytotrophoblast (CTB) cells. Trophoblast cells are derived from CTBs through two pathways. One direction of the differentiation is the fusion of the mononucleated CTBs into multinucleated syncytiotrophoblasts (STBs) that cover the floating villi and are closely connected with maternal blood [2].

STBs are primarily involved in pregnancy-related hormone production, and the exchange of nutrients and metabolic products on the feto-maternal barrier. In the other cell line, CTBs proliferate to form anchoring villi that attach to the uterine wall [8], [9]. Extravillous trophoblasts (EVTs) detach from villous CTBs by separating from the placental villi and migrating into the decidua. Interstitial trophoblasts (iEVTs), one of the subgroups of EVTs, migrate into the deep layer of the maternal endometrium and the inner third of the myometrium, as fetal cells evade the uterine milieu. Another subset of EVTs obtains a phenotype of endothelial-like characteristics and switches to endovascular trophoblasts (enEVT). The enEVTs penetrate the uterine spiral arteries and replace the maternal endothelial cells. This is the process, how the maternal vessels are remodeled into low-resistance, high-capacity utero-placental arteries, that afford the increased blood flow towards the placenta [10]. During placental growth, the trophoblast differentiation and infiltration into the decidua and myometrium are regulated precisely in time and space by environmental factors, such as oxygen tension within the maternal–fetal barrier, and by several hormones and growth factors. Recently,

microRNAs have also been suggested to participate in the regulation of angiogenesis [2], [5], [6], [7].

The imbalanced regulation of trophoblast activities, which can lead to failure in the invasion of EVTs ('shallow placentation') into the uterus and the remodeling of the maternal spiral arteries, is one of the key mechanisms of etiopathogenesis of preeclampsia. Preeclampsia is a pregnancy-associated, severe, potentially life-threatening disorder characterized by hypertension, proteinuria and organ failure [6], [9], [11], [12], [13], [14]. It is essential to clarify the regulatory pathways of trophoblast cell differentiation and passage to explore the pathogenesis of preeclampsia and to identify reliable biomarkers that can be utilized as predictive or therapeutic targets in the prevention or treatment of preeclampsia [2].

There are numerous angiogenic factors, which regulate vasculogenesis and angiogenesis. The most studied and well-known angiogenic agents with a central role in placentation are the members of the vascular endothelial growth factor (VEGF) family. It is well established, that the key molecule is vascular endothelial growth factor-A (VEGF-A), which binds with high affinity to vascular endothelial growth factor receptor 1 (VEGFR1)/fms-like-tyrosine-kinase receptor 1 (Flt-1) and vascular endothelial growth factor receptor 2 (VEGFR2)/kinase insert domain receptor (KDR) [15]. VEGF-A is expressed by many cell types including activated macrophages, smooth muscle cells, platelets, trophoblasts and also tumor cells. In addition, the co-receptors are neuropilin-1 and neuropilin-2 [15].

VEGF-A has an impact on all steps of vasculogenesis and angiogenesis in chorionic villi. It can activate endothelial cells to produce proteases like urokinase-type and tissue-type plasminogen activators and collagenases, furthermore, VEGF-A promotes endothelial cell proliferation and migration during placentation. Vasodilation and vascular permeability in the placenta are also regulated by VEGF-A throughout endothelial nitric oxide synthase (eNOS) and via other vasoactive agents. Hypoxia, inflammatory cytokines and several growth factors induce the upregulation of VEGF-A, for example, basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β) [4], [5], [15], [16], [17], [18], [19].

The other proangiogenic member of the VEGF family is the placental growth factor (PlGF), which is predominantly expressed in the placenta. PlGF is a competitive activator of Flt-1, but it does not bind to KDR. PlGF induces the production of VEGF-A, stimulates angiogenesis via Flt-1 and separates VEGF from Flt-1, which can allow to activate KDR [4], [5], [15], [16], [17], [18].

In the phase of initial vasculogenesis and branching angiogenesis placental expression of VEGF-A, Flt-1 and KDR are intense, but PlGF is moderate. After the switch to non-branching angiogenesis, an increased expression can be discovered of PlGF and its receptor Flt-1 [4], [5], [15], [16], [17], [18], [19].

BFGF is a member of the family of FGFs. This angiogenic factor is known to have impacts on vasculogenesis, wound healing and embryogenesis. BFGF activates FGFR1 and the bFGF/FGFR system takes part of a variety of physiological conditions, including embryonic development, tissue growth and remodeling, inflammation, and vascularization [20]. bFGF is secreted by trophoblasts and placental endothelial cells. It participates in placental angiogenesis and it has a critical impact on the trophoblast cell differentiation and proliferation [20].

Nitric oxide synthase (NOS) is an enzyme, which converts L-arginine to L-citrulline and nitric oxide (NO). NO is a small molecular weight mediator with diverse functions that include vasodilatation, inhibition of platelet aggregation, and vascular remodeling. Three isoforms are known: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). Placental STB cells, villous endothelial cells and umbilical endothelial cells express eNOS. NO promotes spiral artery transformation and has a local effect on placental vasodilatation, which functions are essential for adequate placental blood flow and normal placentation [10], [21], [22], [23].

The placenta also secretes antiangiogenic factors into the maternal serum. Soluble FLT1 is an alternative spliced variant of Flt-1 and acts as an antagonist of VEGF and PlGF [15]. In physiologic pregnancy, circulating sFlt-1 plays a pivotal role in the regulation of endothelial functions and proangiogenic influence of VEGF on the maternal circulation [4], [15]. Some studies suggest the application of the sFlt-1/PlGF ratio as a predictor of pregnancy-induced hypertensive disorders, since a higher ratio of the serum concentrations could anticipate preeclampsia from the second trimester [4], [5], [15], [16], [17], [18].

Soluble endoglin (sEng) is an antiangiogenic agent, which is formed from membrane-bound endoglin by proteolysis and functioning as a coreceptor for TGF-β. TGF-β has an angiogenic effect, like inducing endothelial proliferation and migration [15], [16]. sEng modulates the TGF-β signaling on endothelial cells and reduces eNOS activity, thereby sEng mediates vasoconstriction, induces vascular permeability and interferes with endothelial proliferation and capillary formation [15], [16]. In preeclampsia, placental expression of sEng is upregulated and the maternal serum concentration is significantly higher than in uncomplicated pregnancies [15], [16].

Angiopoietins are protein growth factors and these are required for the formation of blood vessels. Angiopoietin-1 (Ang-1) has vasoprotective features, including stabilization of endothelial cells and prevention of endothelial dysfunction. Angiopoietin-2 (Ang-2) promotes leukocyte mediating proinflammatory cytokine production and endothelial activation. Ang-1 is an agonist for an endothelial tyrosine kinase receptor, called Tie2, however, Ang-2 is an antagonist. There is an imbalance of Ang-1 and Ang-2 in the case of preeclampsia in maternal serum and placenta [4], [6].

Our knowledge on the factor milieu and precise mechanisms, which are included in the process of physiologic and pathologic placentation, is lacking [2], [4], [6], [17], [19]. According to pregnancy related pathologies, there are further investigated angiogenic and immunomodulatory molecules, like HLA family and galectins. In the literature, there are an increasing number of studies about the critical role of human leukocyte antigen-G (HLA-G) and placental protein-13 (PP13) during pregnancy [12], [24], [25], [26], [27], [28], [29], [30], [31], [32], [33].

HLA-G, one of the many HLAs, is a member of the non-classical major histocompatibility complex class 1 molecules. HLA-G was first discovered in 1982 and received its final name in 1990 [29]. It is unique in that it is physiologically expressed in the placenta, specifically in EVTs; the immune-privileged cornea; some thymic epithelial cells; and pancreatic islet cells [29], [34], [35]. Pathologically, HLA-G expression has been found in malignancies such as melanoma, where it is implicated in oncologic immune tolerance [36]. HLA-G is a key molecule in maternal-fetal immune tolerance maintaining the immune homeostasis [37]. Depending on localization, seven isoforms are identified, four membrane-bound (G1-4) and three soluble (G5-7) subtypes. The membrane bound subtypes are expressed by the extravillous trophoblast cells. The soluble subtypes are produced by various cells like extravillous and villous cytotrophoblasts, chorion membrane, STBs, decidual stromal cells, fetal endothelial cells, and immune cells [26], [29].

The target receptors of HLA-G are killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4) and immunoglobulin-like transcript 2 and 4 (ILT2, ILT4) [24], [37]. HLA-G interacts with decidual natural killer cells, decidual macrophages and regulatory T cells through their ILT2 and ILT4 receptors, thereby inhibiting cytotoxicity and enhancing the production of anti-inflammatory cytokines (IL-4, IL-10, TGFβ) [25], [37]. HLA-G, particularly the soluble HLA-G isoforms play critical roles in the trophoblast invasion and remodeling of spiral arteries by connecting to decidual natural killer cells' KIR2DL4, producing proinflammatory cytokines

(IL-6, IL-8, TNF-α) and proangiogenic factors (VEGF-A, MMP) [25], [37]. On the other hand, HLA-G can bind to ILT2 on decidual natural killer cells and activate the PI3K-ACT signal transmission path [25], [37]. This interaction stimulates the production of growth promoting factors, like pleiotrophin, osteoglycin and osteopontin, which is crucial for embryonic and fetal development [25], [37].

PP13 (placental protein 13), also called galectin-13, is a member of the galectin family with a conserved carbohydrate recognition domain (CRD) [38], [39]. The CRD can connect to b-galactosidase-containing glycoconjugates and plays a significant role in biological events such as implantation and embryogenesis [33], [40], [41].

Recently, some unspecific, classical individual receptors have been identified for PP13. Affinity chromatography and mass spectroscopy determined high affinity binding of PP13 to annexin IIa, a member of Ca2+ and phospholipid binding proteins of the extracellular matrix, and to beta/gamma actin in the cytoplasm [42], [43]. PP13 has a high affinity to sugar residues, especially to N-acetyl glucose amine, fucose, and N-acetyl galactose amine [42]. It also binds sugar residues of the B and AB antigen of the ABO blood groups [44], a binding that regulates the availability of free PP13 in the blood of pregnant women [12]

PP13 is thought to have similar functions to other galectins and may be involved in modulating cell-cell, cell-matrix, cell signaling and cell adhesion interactions as well as innate and adaptive immune responses [45]. PP13 is predominantly expressed in the placenta at the maternal-fetal interface and specifically located in the cytoplasm and at the brush border membrane of the STB [42], [46], [47]. Moreover, PP13 gene expression was detected in fetal membranes and villous capillary endothelium [33]. PP13 promotes the migration and differentiation of trophoblasts and is implicated in the vascular restructuring of spiral arteries in early pregnancy. The effect involves a chain of reactions, starting from a physiological effect that affects the endothelial layer through the eNOS and prostaglandin signaling pathways, and continuing through structural stabilization of the surrounding components of the connective tissue around the blood vessels [12], [48]. In addition, it contributes to immunoregulation by inducing apoptosis of maternal T-cells [49].

Aims

Study on angiogenic factors' concentrations in chorionic villus samples.

1. To determine the levels and alterations of VEGF-A, eNOS and bFGF from chorionic villi samples between 10+6 and 18+3 gestational weeks in uncomplicated pregnancies.
2. To explore the possible correlations between VEGF-A, eNOS and bFGF concentration in the placenta.
3. To study maternal (maternal age, body mass index (BMI)) and fetal (nuchal translucency (NT)) influencing factors on VEGF-A, eNOS and bFGF concentrations.

Study on immunological and angiogenic factors' levels in the amniotic fluid and maternal serum.

1. To determine the levels and alterations of sHLA-G and PP13 from maternal serum and amniotic fluid samples between 16+0 and 22+6 gestational weeks in healthy pregnancies and pregnancies complicated with pregnancy induced hypertension, GDM treated with diet, small for gestational age (SGA) and large for gestational age (LGA).
2. To explore the possible correlations between sHLA-G and PP13.
3. To study maternal (maternal age, BMI, parity) fetal (NT, biometric data), and placental (placental volume and indices) influencing factors on sHLA-G and PP13.
4. To examine the association of sHLA-G and PP13 with pregnancy outcomes.

Analysis of angiogenic factors levels in the chorionic villi, the amniotic fluid and maternal serum in physiological and pathophysiological placentation.

1. To summarize the importance and role of angiogenic factors in physiological placentation and pregnancy associated diseases.

Materials and methods

Study on the concentrations of the angiogenic factors in chorionic villus samples

Sample characteristics

A prospective, cross-sectional cohort study was conducted in pregnant women undergoing chorion villus sampling (CVS) at the Department of Obstetrics and Gynecology, University of Szeged, Hungary. The studied population was collected and examined between January 2019 and Dec 2020. CVS was performed between 10+6 and 18+3 weeks of gestation. All patients were dated by the sonographic measurement of the crown-rump length (CRL) below 13 + 6 weeks. At our center, NT assessment and an anatomic scan are provided for all pregnancies between 11 + 0 and 13 + 6 weeks.

The indications of CVS in the 131 cases were increased NT (>2MoM for gestational age) (n = 18), chromosome aberration or gene disorder in previous pregnancy (n = 65), and advanced maternal age (n = 48). Fetuses with aneuploidies (n = 41) or any major structural abnormalities (n = 14), and in utero fetal demise (n = 1) were excluded from the analysis. As a result, 75 non-pathologic pregnancies were included in the study.

Chorionic villus sampling procedure

The patients were informed about the procedure and possible complications, before a consent form was signed prior to the procedure. All procedures were performed by the same expert operator at the outpatient unit, who followed the standard protocol.

For transabdominal CVS we used a steel needle with 1.1 mm outer diameter under ultrasonographic guidance applying the freehand technique. If the sample did not contain chorionic villi, then a second puncture was performed within a 3 cm range around umbilicus and the whole thickness of placental tissue was involved in sampling. Following CVS, anti-D immunoglobulin was administered to women without anti-D antigen.

Samples and evaluation

VEGF-A, bFGF and eNOS concentrations were checked in 1-2 mg of samples of chorionic villi. Tissue samples were kept frozen at -20 C° until testing (up to 1 month). Samples were homogenized in phosphate-buffered saline by an ultrasonic disintegrator (Soniprep 150, MSE, London, United Kingdom) at 22-micron probe amplitude for 2 x 15 s. Homogenates were centrifuged at 10,000 x g and the supernatants were collected for analysis. The preparatory process was carried out at 4 C°. The protein contents of samples were determined with the help of Bradford method, using bovine serum albumin (Sigma, Budapest, Hungary) as standard.

The angiogenic factor levels were evaluated by ELISA. The laboratory staff who performed the assays were blinded to the pregnancy outcome, and the clinician recruiting the women did not participate in analyzing the samples.

VEGF-A was quantified by enzyme immunoassay (Human VEGF Quantikine ELISA Kit, R&D Systems, Wiesbaden-Nordenstadt, Germany). The kit was calibrated with a highly purified Sf21-expressed recombinant human VEGF165 as stated by the manufacturer. The sensitivity of detection was 5 pg/ mL. The intra- and interassay variabilities were characterized by 6.5 and 8.5 coefficients of variation (CV%), respectively.

The bFGF kit (Quantikine HS ELISA Kit, R&D Systems, Wiesbaden-Norden- stadt, Germany) was calibrated using recombinant FGF. The sensitivity of FGF assay was 0.03 pg/mL. At a concentration of 5 pg/mL, the intra- and interassay coefficients of variation were 4.3 and 4.7 CV%, respectively.

eNOS /NOS3 was determined by ELISA (Cloud-Clone Corporation, Houston, TX, USA). The kit is validated to measure eNOS/NOS3 from tissue homogenates. The sensitivity of eNOS assay was <0.58 ng/mL. The intra- and interassay coefficients of variation were <10 and <12 CV%, respectively. The measurement units were expressed as pg VEGF/mg protein, pg b-FGF/mg protein and pg eNOS/mg protein.

Data and statistical analysis

Data in respect of demographic details, medical history of pregnancy, maternal height and weight at booking, sonographic findings concerning NT between the 11 + 0 and 13 + 6 weeks

of gestation, crown-rump length (CRL) or BPD (biparietal diameter) at the time of NT measurement as well as CVS was recorded. Data on BMI at booking was extracted from medical records. The number of attempts during the procedure was also registered. The gestational age at the time of CVS and the data regarding the pregnancy outcome (birth weight of the infant, gestational age at delivery) were acquired prospectively. Data were evaluated using version 22 of SPSS statistical software (Armonk, New York, IBM). The non-parametric design of continuous variables was verified by Shapiro–Wilk test. The relationship between the level of angiogenic factors (VEGF-A, bFGF and eNOS) and other continuous variables was assessed using Spearman's rank correlation coefficients and regression analyses. For further statistical analyses, all dependent variables were transformed logarithmically (log10(x)) to be fitted for the Gaussian distribution and the regression model. Multiple linear regression was adjusted for maternal age, BMI at booking in pregnancy care, primiparity and gestational age at the time of CVS as these factors determine the actual placental volume and fetal weight. All constituted confounding factors. Correlation coefficients (B) were calculated for both the univariate and multiple linear regression, whereas standardized coefficients (ß) were given for univariate analyses and semipartial correlations (r) for multivariable regression. The significance level in two-tailed tests was set at 5%. The signed informed consent was obtained in all cases and the aseptic technique was applied. Our work was carried out in accordance with the Declaration of Helsinki and our study has been approved by the institutional research ethics committee (Reference:111/2009).

Study on amniocentesis ('unselected group')

Sample characteristics

A prospective, cross-sectional cohort study was conducted in pregnant women undergoing amniocentesis at the Department of Obstetrics and Gynecology, University of Szeged, Hungary between January 2019 and December 2020. During the study period, all singleton pregnancies with increased risk of chromosomal abnormality, where amniocentesis (AC) was performed between 16+0 and 22+6 weeks of gestation, were recruited into our study. The indications for AC were increased NT at first trimester scan (≥2MoM for gestational age) (n=20), chromosome aberration or gene disorder concerning the previous pregnancy (n=11), and advanced maternal age (n=40).

Exclusion criteria of the study were identified as follows: multiple pregnancies, fetal or neonatal structural or genetic anomaly, improper localization of the placenta for sonography (placenta praevia, posterior placenta), pathological placentation (placenta accreta spectrum), self-reported drugs, alcohol, caffeine or nicotine abuse, or exposure to circulatory medication (oxerutins, calcium dobesilate), systemic disease (e. g., any type of pregestational diabetes mellitus, autoimmune disease, vasculitis, hemophilia, thrombophilia, HIV infection).

Women with complications during late pregnancy (GDM treated with diet (n=12), hypertensive related diseases (n=11), small for gestational age (SGA) at delivery (n=4), and large for gestational age at delivery (n=10)) were not excluded from the study.

Amniocentesis procedure

The patients were informed about the procedure and possible complications, before a consent form was signed prior to the procedure. All procedures were performed by the same expert operator at the outpatient unit, who followed the standard protocol. A local antiseptic was applied to the skin. A 22-gauge spinal needle was inserted under continuous ultrasound guidance and needle insertion through the placenta was avoided. Amniotic fluid (8–10 mL) was aspirated and the first 2 mL of each sample was discarded to prevent contamination with maternal cells. Blood-contaminated amniotic fluid was not utilized. Fetal heart rate was evaluated after the procedure and no stillbirth or premature rupture was observed. Following amniocentesis, anti-D immunoglobulin was administered, when it was necessary.

Samples and evaluation

Amniotic fluid and maternal venous blood were collected from each patient at the time of amniocentesis. Blood samples were centrifuged at 3400RPM for 15 minutes. Serum and amniotic fluid samples were stored at -80 °C until assayed.

PP13 and sHLA-G levels were evaluated by ELISA. The laboratory staff who performed the assays were blinded to the pregnancy outcome, and the clinician recruiting the women did not participate in analyzing the samples.

The concentration of sHLA-G was measured by kits from Elabscience Biotechnology Corporation (Houston, TX, USA). The sensitivity of the assay was 0.38 ng/ml. The intra- and inter-assay coefficients of variation were <10% according to the manufacturer. The measurements are conveyed in Table 6.

Human PP13 levels were determined by Cusabio kits (Wuhan Huamei Biotec Co. LTD. Wuhan, China). The sensitivity of the assay was <3.9 pg/ml. The intra- (<8%) and inter- (<10%) assay coefficient of variation according to the manufacturer. The results are presented in Table 6.

Data and statistical analysis

Statistical analyses were performed using SPSS version 23 (IBM). Continuous variables were expressed as mean ± standard deviation (SD) and categorical variables as numbers and percentages. The relationship between the level of angiogenic factors (sHLA-G and PP13) and other continuous variables was assessed using Pearson's correlation coefficients and regression analyses. Multiple linear regression was adjusted for maternal age, BMI at the time of genetic consultation, number of previous pregnancies and gestational age at the time of amniocentesis, as these factors determine the actual placental volume and fetal weight. Correlation coefficients (B) were calculated for both univariate and multiple linear regression, whereas standardized coefficients (ß) were given for univariate analyses and semi-partial correlations (r) for multivariable regression. Independent samples t-tests were used to determine, whether the angiogenic factor levels in body fluid were different in complicated pregnancies versus healthy controls. Paired samples t-test was applied to analyze the differences between the serum and amniotic levels of the analytes. The two-tailed statistical significance level was set at 5%, and p-values were adjusted using a Holm–Bonferroni correction for multiple comparisons.

The study protocol was approved by the Clinical Research Ethics Committee of the University of Szeged (Ref. no.:09/2017). The study was carried out according to the Principles of the Declaration of Helsinki. We obtained written informed consent from all participants.

Study on amniocentesis (selected group)

Sample characteristics

A prospective, cross-sectional cohort study was conducted on pregnant women undergoing amniocentesis at the Department of Obstetrics and Gynecology, University of Szeged, Hungary between January 2021 and May 2021. The demography of the study group was homogenous (Caucasian female subjects). During the study period, all singleton pregnancies with increased risk of chromosomal abnormality, where amniocentesis (AC) was performed between 16+0 and 22+0 weeks of gestation, were recruited into our study. The indications for AC were increased nuchal translucency (NT) at the first trimester scan (≥2 MoM for gestational age (GA)) (n=10), chromosome aberration or gene disorder concerning the previous pregnancy (n=2), and advanced maternal age (n=29).

Exclusion criteria of the study were identified as follows: multiple pregnancies; fetal or neonatal structural or genetic anomaly, improper localization of the placenta for sonography (placenta praevia, posterior placenta), pathological placentation (placenta accreta spectrum), self-reported drug, alcohol, caffeine or nicotine abuse or exposure to circulatory medication (oxerutins, calcium dobesilate) and systemic disease (e.g. essential hypertension, any type of pregestational diabetes mellitus, autoimmune disease, vasculitis, hemophilia, thrombophilia, HIV infection).

In addition, women with complications during late pregnancy (gestational diabetes mellitus treated with diet, hypertension-related diseases, small for gestational age at delivery, large for gestational age at delivery) were excluded from the study. Therefore 41 healthy pregnancies were included in our study.

Amniocentesis procedure

The patients were informed about the procedure and possible complications before a consent form was signed prior to the procedure. All procedures were performed by the same expert operator at the outpatient unit, who followed the standard protocol. A local antiseptic was applied to the skin. A 22-gauge spinal needle was inserted under continuous ultrasound guidance and needle insertion through the placenta was avoided. Amniotic fluid (8–10 mL) was aspirated and the first 2 mL of each sample was discarded to prevent contamination with maternal cells. Blood-contaminated amniotic fluid was not utilized. Fetal heart rate was

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Data and statistical analysis

Statistical analyses were performed using SPSS version 23 (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation (SD) and categorical variables as numbers and percentages. The relationship between the factors levels (sHLA-G and PP13), and the level of sHLA-G and other continuous variables was assessed using univariate and multivariate regression analyses characterized by correlation coefficient (ß) and 95% confidence interval (CI). Multiple linear regression was adjusted for well-known confounders such as maternal age, body mass index (BMI) at the time of amniocentesis, number of previous pregnancies and gestational age at the time of amniocentesis, as these factors determine the actual placental volume and fetal weight. Paired samples t-test was applied to analyze the differences between serum and amniotic levels of the analytes. The two-tailed

statistical significance level was set at 5% and p-values were adjusted using Holm–Bonferroni correction for multiple comparisons.

The study protocol was approved by the Clinical Research Ethics Committee of the University of Szeged (date of approval: 10 February 2017, reference number: SZTE 09/2017). The study was conducted according to the Principles of the Declaration of Helsinki. We obtained written informed consent from all participants.

Conventional 2-dimensional (2-D) sonographic examinations

All patients were categorized by the measurement of the crown rump length (CRL). NT and anatomic assessment between 11+0 and 13+6 weeks were performed by conventional methods. Ultrasound examination took place before measuring AC to determine the number of fetuses, fetal biometry, fetal anomalies, placental location and the amount of amniotic fluid. Fetal weight was estimated according to the method of Hadlock et al. [50] after measuring the necessary sonographic parameters (biparietal diameter, head circumference, abdominal circumference and femur length). Estimated fetal weight percentile was calculated according to the local standards [51]. The ultrasound investigations were conducted by experienced and well-trained sonographers.

Volume acquisition

Acquisition of the images used for the determination of placental volume and 3-dimensional Power Doppler (3-DPD) indices were obtained at the time of the visit. All 3-D scans were performed by A.S. Voluson 730 Expert ultrasound machine (GE Medical Systems, Kretztechnik GmbH & Co OHG, Austria) equipped with a multifrequency probe (2–5MHz) that was used to acquire all images. Each sample was examined using the 3-D rendering mode, in which the color and grey value information were processed and combined to give a 3-D image (mode cent; smooth: 4/5; FRQ: low; quality: 16; density: 6; enhance: 16; balance: 150; filter: 2; actual power: 2dB; pulse repetition frequency: 0.9). We used fast low-resolution acquisition to avoid any kind of artefacts. The 3-D static volume box was placed over the highest villous vascular density zone at the umbilical cord insertion [52]. Each image was

recovered from the disc in succession for processing. During gestation, we recorded one sample from each patient.

Determination of Power Doppler Indices

The stored volumes were further analyzed using the Virtual Organ Computer-aided Analysis (VOCAL) program pertaining to the computer software 4DView (GE Medical Systems, Austria, version 10.4) by the same expert in 3-D analysis. The image used for recovering from the hard disc was captured and processed using the multiplanar system. The spherical sample volume was constantly 28 ml. The VOCAL program calculated automatically the grey and color scale values in a histogram from the acquired spherical sample volume in all cases. The combined use of power Doppler with three-dimensional ultrasound provides the possibility of quantifying blood in motion within a volume of interest. Three indices were calculated, namely vascularization index (VI), flow index (FI) and vascularization flow index (VFI) as estimates of the percentage of the volume filled with detectably moving blood. VI (expressed as a percentage) is the proportion of color voxels in the studied volume, representing the proportion of blood vessels within the tissue. FI (expressed as a scale of 0–100) is the average value of all color voxels, representing the average power Doppler amplitude within blood vessels. VFI (expressed as a scale of 0–100) is the average color value of all grey and color voxels, a product of the number of color voxels as a percentage and the relative amplitude of these voxels [53], [54].

The intraobserver errors were evaluated by repeated measurements of the 3-DPD indices at the initiation of the study. The intra-class correlation coefficients for all Doppler indices were excellent (0.99) for all indices.

Results

VEGF-A, bFGF and eNOS concentrations in chorionic villi samples

During the study period, we examined the samples obtained from 75 pregnant women. The median age of participants was 32 years, the median BMI at booking was 23.91 kg/m², the median nuchal translucency at first trimester genetic sonography was 3.4 mm and the median gestational age at chorion villous sampling was 13.29 weeks (Table 1).

Table 1. Clinical and sonographic data (N=75)

Maternal age (years)	32 [28-36]
Number of nulliparous women in the study (%)	24 (32.0)
BMI at booking (kg/m^2)	23.91 [21.5-26.7]
CRL at the time of measurement of NT (mm)	57 [49.8-63.0]
NT between 11+0 and 13+6 weeks of gestation (mm)	3.4 [1.7-4.3]
CRL at the time of CVS (mm)	72.8 [11.3]
GA at the time of CVS (weeks)	13.29 [12.7-13.8]
Number of insertions	1 [1-1]
Had only 1 insertion (%)	68 (90.7)

Data represent median and [interquartile range] or n (%).

SD=standard deviation, NT=nuchal translucency, BMI=body mass index, CRL=crown-rump length, CVS=chorionic villus sampling, GA= gestational age

We measured the median concentrations of the factors at the time of CVS. The VEGF-A concentration was 11.1 pg/ml, the bFGF concentration was 1019.0 pg/ml and the eNOS concentration was 116.8 pg/ml (Table 2).

Table 2. Levels of angiogenic factors in samples of placental tissues (N=75)

VEGF-A concentration in chorionic villi (pg/mg)	11.1 [5.8-25.2]
bFGF concentration in chorionic villi (pg/mg)	1019.0 [749.8-1251.1]
eNOS concentration in chorionic villi (pg/mg)	116.8 [69.0-192.6]

Data are displayed as median and [interquartile range].

VEGF-A=vascular endothelial growth factor-A, bFGF=basic fibroblast growth factor, eNOS= endothelial nitric oxide synthase, SD=standard deviation.

Statistical analysis had shown significantly negative correlation between the gestational age and VEGF-A-, and also bFGF-levels (Figure 1 and 2, Table 3).

Figure 1. VEGF-A concentrations in the placenta regarding gestational age

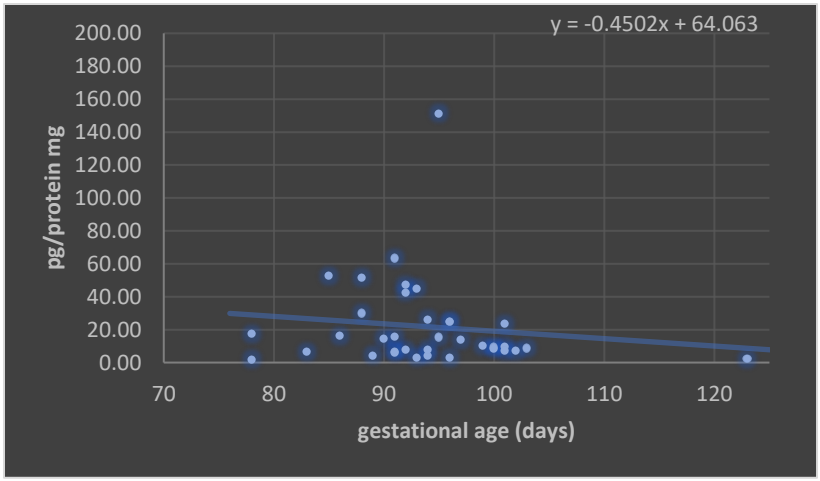
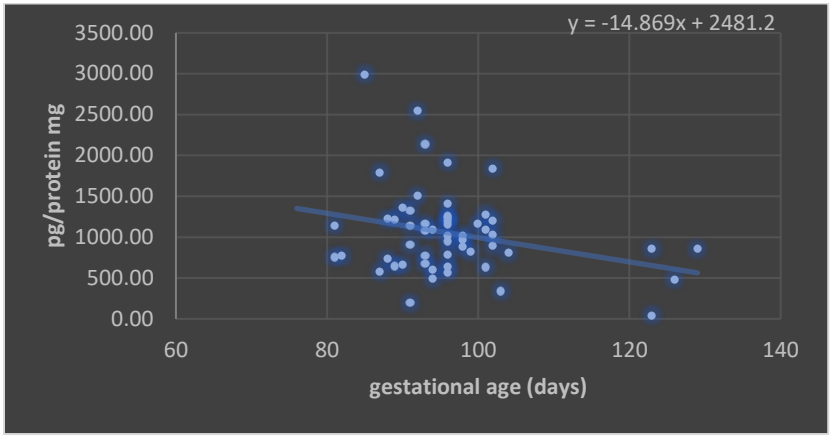


Figure 2. bFGF concentrations in the placenta regarding gestational age



We performed the statistical analysis to identify the relations of the angiogenic factors and maternal clinical parameters and nuchal translucency. We found that the VEGF-A concentration correlated negatively, whereas the bFGF concentration related positively with the pregestational maternal BMI. The bFGF concentration exerted an inverse relationship and the eNOS levels had a linear relationship with nuchal translucency. The eNOS concentrations had an adverse correlation with the VEGF-A and the bFGF concentrations. The VEGF-A concentration was significantly positively associated with the bFGF concentration (Table 3).

Table 3. Correlation between maternal as well as sonographic data and the levels of angiogenic factors in chorionic villi (N=75)

	VEGF-A concentration Spearman's rho	bFGF concentration Spearman's rho	eNOS concentration Spearman's rho
Maternal age	-0.09	0.11	0.01
Pre-pregnancy BMI	-0.27*	0.31*	-0.09
CRL at the time of measurement of NT	-0.02	-0.03	-0.14
NT between 11+0 and 13+6 weeks of gestation	-0.04	-0.36*	0.225*
GA at the time of CVS	-0.30*	-0.29*	-0.05
VEGF-A concentration		0.62*	-0.52*
bFGF concentration			-0.24*

* P < 0.05; ** P < 0.01

VEGF-A=vascular endothelial growth factor-A, bFGF=basic fibroblast growth factor, eNOS= endothelial nitric oxide synthase, BMI=body mass index, GA=gestational age, NT=nuchal translucency, CVS=chorionic villus sampling.

HLA-G and PP13 concentrations in maternal serum and amniotic fluid gained at amniocentesis

Considering the exclusion criteria, we selected 71 pregnant women in the study initially, and after regrouping, we created a subgroup of 41 cases without any pregnancy or maternal complication. Both groups had similar obstetric and clinical parameters without any significant differences, which are shown in Table 4.

Table 4. Clinical and obstetrical data of women with amniocentesis

	Unselected (N=71)	Selected (N=41)
Maternal age (years)*	34.52 ± 5.78	33.63 ± 6.51
Number of nulliparous women in the study**	23 (32.39)	12 (29.3)
BMI at the time of genetical consultation (kg/m^2)*	26.98 ± 5.90	26.35 ± 6.19
Gestational age at the time of amniocentesis (weeks)*	18.25±1.42	18.11±1.65
Fetal weight at delivery (grams)*	3424.08 ± 538.63	3351.22 ± 370.10
Gestational age at delivery (weeks)*	39.09 ± 1.38	39.01 ± 1.32

*Continuous variables displayed as mean ± standard deviation (SD).

** Categorical variables are presented in number and %.

At the time of amniocentesis, we performed fetal biometry and placental volume measurements, as well as the placental perfusion 3-DPD indices were determined. The means and standard deviations of the results are shown in Table 5.

Table 5. Ultrasound data at amniocentesis

	Unselected (N=71)*	Selected (N=41)*
Fetal biometry		
Head circumference (mm)	151.54 ± 16.18	152.72 ± 15.91
Head circumference (percentile)	53.50 ± 30.79	53.74 ± 30.28
Abdominal circumference (mm)	129.18 ± 17.07	132.46 ± 17.62
Abdominal circumference (percentile)	51.63 ± 27.88	52.32 ± 28.94
Femur length (mm)	27.04 ± 4.73	27.65 ± 4.81
Femur length (percentile)	55.91 ± 30.52	58.09 ± 29.28
Estimated fetal weight (grams)	243.20 ± 74.12	257.00 ± 79.78
Estimated fetal weight (percentile)	52.92 ± 26.81	52.79 ± 25.36
Placental sonography		
Placental volume (mm^3)	227.36 ± 93.21	215.20 ± 92.20
VI	14.11 ± 5.14	14.41 ± 5.48
FI	44.97 ± 22.64	43.33 ± 8.55
VFI	8.21 ± 3.63	8.52 ± 4.05

VI: Vascularization Index, FI: Flow Index, VFI: Vascularization Flow Index.

*Continuous variables displayed as mean ± standard deviation (SD).

The primary aim of the study was to determine the angiogenic factors' concentrations and the links between these molecules in the body fluids. The mean concentrations of PP13 and HLA-G in maternal serum and amniotic fluid are indicated in Table 6.

Table 6. Levels of angiogenic factors in samples of amniotic fluid and serum

	Unselected (N=71)*	Selected (N=41)*
PP13 concentration in amniotic fluid (pg/ml)	8.68 ± 9.85	9.91 ± 12.05
PP13 concentration in serum (pg/ml)	204.23 ± 171.34	221.96 ± 207.88
sHLA-G concentration in amniotic fluid (ng/ml)	55.89 ± 19.51	53.39 ± 19.00
sHLA-G concentration in serum (ng/ml)	55.84 ± 30.50	51.05 ± 26.99

PP13: Placental Protein-13, sHLA-G: soluble Human Leukocyte Antigen-G.

*Continuous variables displayed as mean ± standard deviation (SD).

There was a strong positive correlation between the sHLA-G in the serum and the amniotic fluid in both groups ($p<0.01$). The sHLA-G and PP13 serum concentrations exhibited a negative correlation in both groups, but the corresponding figure was more pronounced in the case of the unselected group (Table 7).

Table 7. Correlation between the levels of angiogenic factors in the maternal serum and amniotic fluid

Unselected group (N=71)				
	sHLA-G serum		sHLA-G amniotic fluid	
	β	95% CI	β	95% CI
PP13 serum	-0.37**	-3.66; -0.54**	-0.28	-5.21; 0.05
PP13 amniotic fluid	-0.18	-0.15; 0.03	0.09	-0.11; 0.20
sHLA-G amniotic fluid	0.54**	0.21; 0.51**	-	-
	PP13 serum			
PP13 amniotic fluid	0.04	-5.26; 6.92	-	-
Selected group (N=41)				
	sHLA-G serum		sHLA-G amniotic fluid	
PP13 serum	-0.43*	-5.76; -0.63*	-0.32	-7.66; 0.56
PP13 amniotic fluid	-0.19	-1.05; 0.30	0.14	-0.15; 0.33
sHLA-G amniotic fluid	0.29**	0.30; 0.72**	-	-
	PP13 serum			
PP13 amniotic fluid	0.03	-6.98; 8.15	-	-

PP13: Placental Protein-13, sHLA-G: soluble Human Leukocyte Antigen-G.

* $P < 0.05$; ** $P < 0.01$

Our study's secondary outcomes were the interrelations between the levels of the angiogenic factors, the maternal and sonographic data, the time of delivery, and the birthweight. The alteration of sHLA-G and PP13 concentrations was not significant during the studied period in body fluids. It was an interesting finding that in the case of uncomplicated pregnancies, there was an inverse relation between the first trimester nuchal translucency and the second trimester amniotic fluid sHLA-G concentration. Significant interactions were found between the serum sHLA-G and the sonographic abdominal percentile, and also the sonographic estimated fetal weight percentile in the selected population. Comparing the groups, other interesting findings were noticed regarding the serum and amniotic fluid sHLA-G concentrations and placental sonographic measurements. The serum sHLA-G concentration appeared to be increased with a larger placental volume in the unselected group (β=0.28, 95% CI: 0.01; 0.17), but not in the selected group (Table 8). The maternal age of the unselected group interrelated positively with placental volume (p=0.02) (3. Figure), so we divided the maternal age (<36 years, >35years) and the placental volume (<201 mm³, >200 mm³) into two groups, and we investigated the relationship with serum sHLA-G. As it is seen on 4. Figure, the sHLA-G concentrations were not different in the group of placental volume under 201 mm³. In contrast, in the group with placental volume above 200 mm³, pregnant women with age above 35 years showed markedly higher serum sHLA-G concentrations (Figure 4).

Figure 3. Placental volume and maternal age in unselected group (p=0.02)

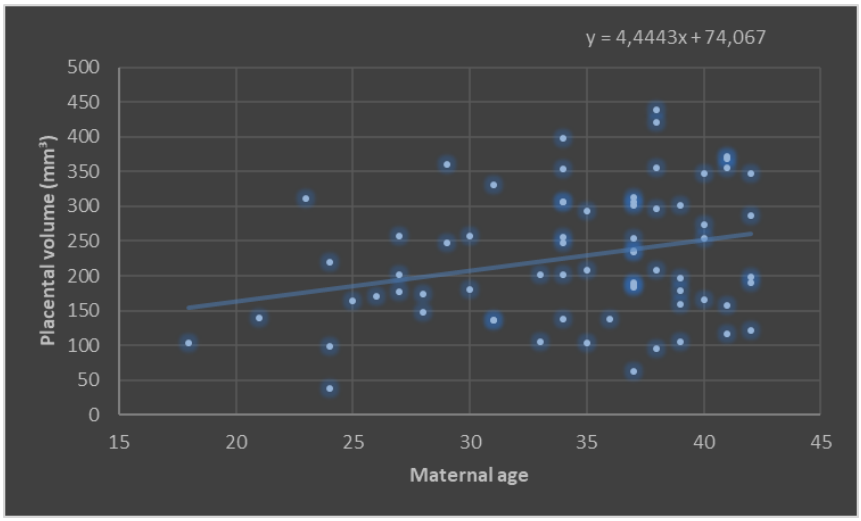
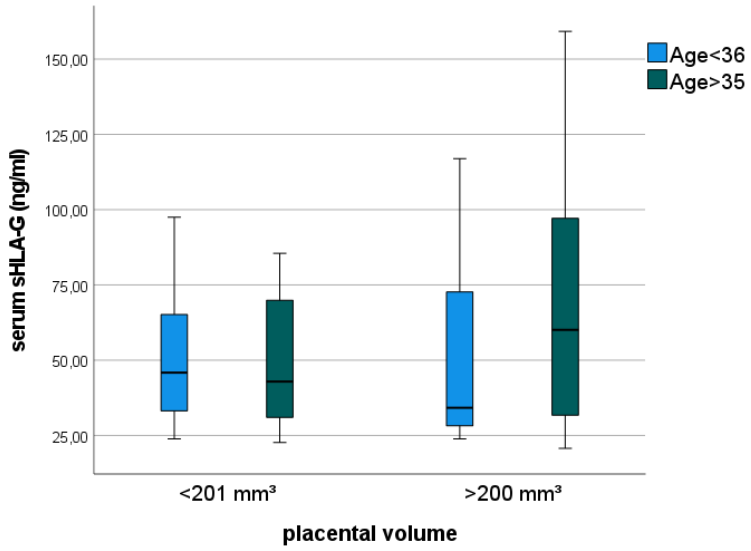


Figure 4. Serum sHLA-G concentrations in divided groups on placental volume and maternal age in unselected group



There was an inverse significant correlation between the serum sHLA-G and the VFI in the selected group (β= -0.34, 95% CI: -3.58; 0.46), but not in the unselected group. The amniotic fluid sHLA-G had inverse correlations with the VFI in the unselected (β= -0.37, 95% CI: -3.17; -0.62) and selected group too (β= -0.44, 95% CI: -3.28; 0.63). Furthermore, the amniotic fluid sHLA-G is correlated negatively with the VI in the selected group (β= -0.34, 95% CI: -2.13; 0.06), but not in the unselected group (Table 8).

In the selected group the maternal age correlated positively with VFI (Figure 5). Therefore, we divided VFI (<9, >9) into two groups and statistically analyzed the coherency with amniotic fluid sHLA-G depending on maternal age. Pregnant women above 35 years old have higher amniotic fluid sHLA-G concentrations in the lower range VFI group and higher range VFI group as well (Figure 6).

Figure 5. VFI and maternal age in the selected group

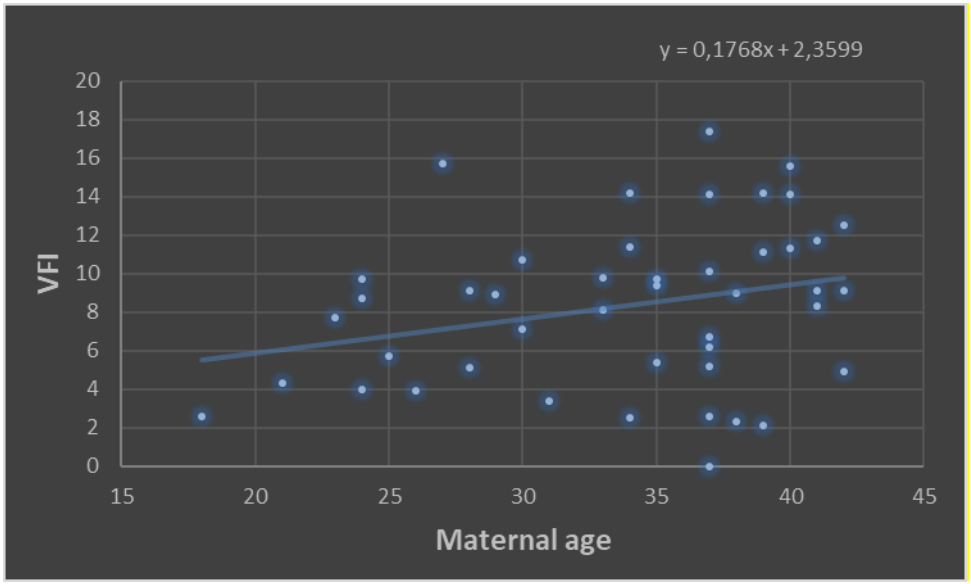
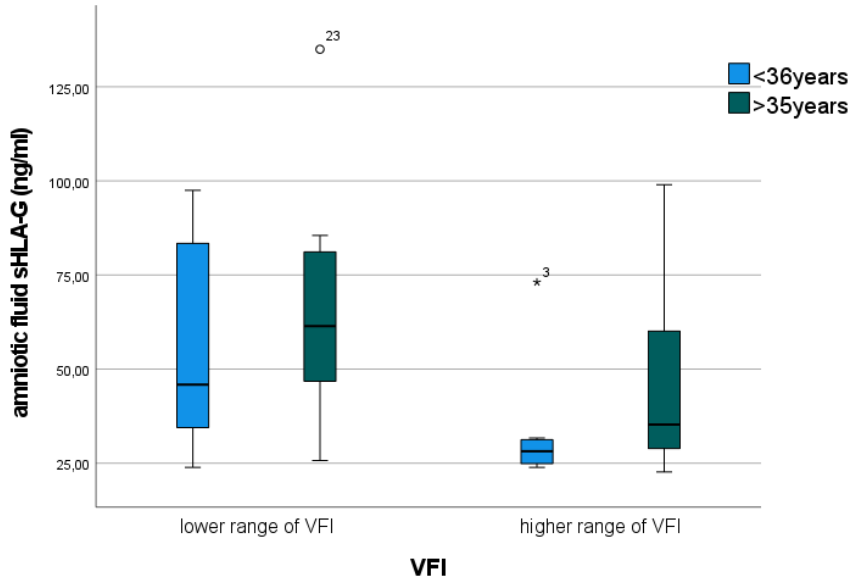


Figure 6. Amniotic fluid sHLA-G concentrations in divided groups on VFI and maternal age in the selected group



We analyzed the sHLA-G levels' correlations between uncomplicated pregnancies and pregnancies complicated with pregnancy induced hypertension, GDM treated with diet, SGA and large for gestational age (LGA) fetuses. Serum sHLA-G concentrations were detected to be higher in pregnancies with LGA (p=0.044). We found no altered concentrations of any factor regarding pregnancy induced hypertension, GDM treated with diet and SGA.

Table 8. Correlation between maternal as well as sonographic data and levels of sHLA-G in maternal serum and amniotic fluid

	sHLA-G level in serum				sHLA-G in amniotic fluid			
	Selected population (n=41)		Unselected population (n=71)		Selected population (n=41)		Unselected population (n=71)	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Clinical and obstetric characteristics								
Maternal age	0.01	-1.31; 1.38	0.09	-0.86; 1.84	-0.20	-1.51; 0.39	-0.13	-1.31; 0.45
Previous parity	-0.12	-13.18; 6.00	-0.76	-11.57; 6.34	-0.08	-8.54; 5.33	-0.11	-8.10; 3.48
BMI at the time of genetic consultation (kg/m^2)	0.19	-0.57; 2.21	0.002	-1.31; 1.32	-0.04	-1.22; 0.93	-0.05	-1.06; 0.71
Birth weight (grams)	-0.02	-0.03; 0.02	0.179	-0.01; 0.03	0.01	-0.02; 0.02	0.12	-0.01; 0.02
Birth weight (percentile)	0.05	-0.30; 0.40	0.18	-0.08; 0.47	-0.02	-0.27; 0.24	0.08	-0.13; 0.24
NT	-0.11	-17.87; 8.79	-0.15	-19.91; 5.09	-0.30*	-17.78; 0.86*	-0.15	-12.81; 3.84
CRL at NT	0.30	-0.08; 2.52	0.12	-0.60; 1.64	0.10	-0.71; 1.32	0.16	-0.29; 1.18
GA at the time of delivery	-0.11	-8.85; 4.34	-0.10	-8.82; 3.99	0.02	-4.45; 5.08	0.06	-3.27; 5.06
GA at the time of amniocentesis (weeks)	-0.13	-8.22; 3.45	-0.14	-8.03; 2.50	0.24	-1.12; 7.10	0.36	-3.01; 3.94
Fetal sonography at the time of amniocentesis								
Head circumference (mm)	-0.12	-0.75; 0.35	-0.09	-0.67; 0.32	0.26	-0.08; 0.69	0.07	-0.25; 0.40
Head circumference (percentile)	-0.03	-0.32; 0.28	0.06	-0.21; 0.34	-0.08	-0.27; 0.17	0.02	-0.17; 0.20
Abdominal circumference (mm)	0.01	-0.71; 0.71	0.06	-0.53; 0.75	0.33	-0.08; 0.86	0.31	-0.04; 0.77
Abdominal circumference (percentile)	0.41*	-0.08; 0.75*	0.24	-0.12; 0.67	0.26	-0.12; 0.51	0.21	-0.11; 0.43

Femur length (mm)	0.07	-2.54; 1.74	-0.10	-2.73; 1.35	0.23	-0.61; 2.53	0.22	-0.35; 2.05
Femur length (percentile)	0.20	-0.18; 0.59	0.06	-0.28; 0.43	-0.00	-0.30; 0.29	-0.03	-0.24; 0.20
Estimated fetal weight (grams)	-0.04	-0.16; 0.14	-0.01	-0.14; 0.14	0.26	-0.04; 0.17	0.25	-0.03; 0.15
Estimated fetal weight (percentile)	0.41*	-0.02; 0.84*	0.17	-0.20; 0.59	0.24	-0.14; 0.52	0.14	-0.16; 0.38
Placental sonography at the time of amniocentesis								
Placental volume (mm^3)	0.02	-0.09; 0.10	0.28*	0.01; 0.17*	-0.09	-0.09; 0.05	-0.00	-0.06; 0.06
VI	-0.10	-2.00; 1.08	0.02	-1.40; 1.63	-0.34*	-2.13; 0.06*	-0.18	-1.62; 0.32
FI	0.05	-0.86; 1.14	0.08	-0.23; 0.43	-0.18	-1.12; 0.34	0.14	-0.10; 0.32
VFI	-0.34*	-3.58; 0.46*	-0.12	-3.10; 1.09	-0.44*	-3.28; 0.63*	-0.37**	-3.17; -0.62**

* P < 0.05; ** P < 0.01

VI: Vascularization Index, FI: Flow Index, VFI: Vascularization Flow Index.

In the selected sample, the serum and amniotic fluid PP13 concentrations had a significant negative correlation with the birth weight (β= -0.42, 95% CI: -0.55; 0.07/ β= -0.41, 95% CI: -0.02; 0.01). While in the unselected group, the serum PP13 concentration has no significant interaction with birth weight, while the amniotic fluid PP13 concentration shows a significant correlation in the unselected population (β= -0.26, 95% CI: -0.01; 0.00). Besides this, the amniotic fluid PP13 levels had positive correlations with the gestational age at delivery regarding both groups, and the relation was more significant in the selected group (β= -0.42, 95% CI: -0.55; 0.07/ β= -0.41, 95% CI: -0.02; 0.01). Respecting the fetal biometric data, the amniotic fluid PP13 levels correlated directly with the fetal abdominal circumference (β= 0.54, 95% CI: -7.47; 2.25/ β= 0.39, 95% CI: -4.32; 0.95). The sonographic placental parameters and PP13 were not in context (Table 9). We analyzed the PP13 levels' correlations between uncomplicated pregnancies and pregnancies complicated with pregnancy induced hypertension, GDM with diet, SGA and large for gestational age (LGA). No one pathologic pregnancy condition was a distinguishing factor for the PP13 levels.

Table 9. Correlation between maternal as well as sonographic data and levels of PP13 in maternal serum and amniotic fluid

	PP13 level in serum				PP13 in amniotic fluid			
	Selected population (n=41)		Unselected population (n=75)		Selected population (n=41)		Unselected population (n=75)	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Clinical and obstetric characteristics								
Maternal age	0.18	-5.45; 16.02	0.12	-4.27; 10.87	-0.04	-0.69; 0.56	-0.07	-0.56; 0.33
Previous parity	0.04	-72.10; 90.63	0.05	-43.09; 62.48	0.05	-4.02; 5.20	0.04	-2.60; 3.48
BMI at the time of genetic consultation (kg/m^2)	0.02	-11.61; -12.65	0.10	-4.77; 10.50	-0.13	-1.07; 0.47	-0.10	-0.65; 0.30
Birth weight (grams)	-0.42*	-0.55; 0.07*	-0.10	-0.12; 0.05	-0.41*	-0.02; 0.01*	-0.26*	-0.01; 0.00*
Birth weight (percentile)	-0.48**	-6.73; 1.37**	-0.22	-2.68; 0.27	-0.11	-0.22; 0.11	-0.11	-0.13; 0.05
NT	-0.12	-147.56; 76.71	-0.07	-91.75; 55.94	-0.10	-8.06; 4.47	-0.09	-5.67; 2.94
CRL at NT	-0.20	-16.70; 4.85	-0.08	-7.82; 4.43	0.04	-0.68; 0.84	-0.01	-0.42; 0.40
GA at the time of delivery	0.05	-10.18; 13.40	0.09	-24.85; 51.25	0.54**	-7.47; 2.25**	0.39*	-4.32; -0.95*
GA at the time of amniocentesis (weeks)	-0.14	-10.34; 4.66	-0.09	-44.96; 22.06	0.24	-0.11; 0.70	0.22	-0.33; 3.42
Fetal sonography at the time of amniocentesis								
Head circumference (mm)	-0.15	-7.97; 2.97	-0.09	-3.88; 1.99	0.15	-0.15; 0.39	0.13	-0.09; 0.25
Head circumference (percentile)	0.05	-2.37; 3.09	0.06	-1.24; 1.99	-0.26	-0.26; 0.03	-0.25	-0.18; 0.01
Abdominal circumference (mm)	-0.28	-10.94; 2.59	-0.17	-6.98; 2.62	0.42*	0.02; 0.72*	0.39*	0.04; 0.57*
Abdominal circumference (percentile)	-0.08	-4.61; 3.26	0.01	-2.72; 2.81	0.27	-0.08; 0.35	0.22	-0.06; 0.25
Femur length (mm)	-0.14	-26.11; 12.76	-0.01	-15.77; 8.50	0.13	-0.72; 1.46	0.14	-0.40; 1.04

Femur length (percentile)	0.07	-2.59; 3.60	0.12	-1.14; 2.59	-0.25	-0.27; 0.06	-0.19	-0.19; 0.04
Estimated fetal weight (grams)	-0.23	-2.11; 0.72	-0.15	-1.48; 0.63	0.31	-0.02; 0.14	0.31	-0.01; 0.12
Estimated fetal weight (percentile)	0.04	-4.21; 4.95	0.09	-2.22; 3.52	0.04	-0.24; 0.29	0.02	-0.16; 0.19
Placental sonography at the time of amniocentesis								
Placental volume (mm^3)	0.12	-0.51; 1.04	0.08	-0.34; 0.63	-0.03	-0.05; 0.04	-0.05	-0.04; 0.02
VI	0.17	-7.26; 19.51	0.12	-4.87; 12.90	-0.02	-0.82; 0.74	-0.00	-0.55; 0.54
FI	-0.11	-11.45; 6.02	-0.09	-2.45; 1.25	-0.21	-0.82; 0.19	-0.12	-0.16; 0.06
VFI	-0.02	-18.24; 20.60	0.02	-12.44; 14.80	-0.01	-1.12; 1.12	0.01	-0.79; 0.83

* $P < 0.05$; ** $P < 0.01$

VI: Vascularization Index, FI: Flow Index, VFI: Vascularization Flow Index.

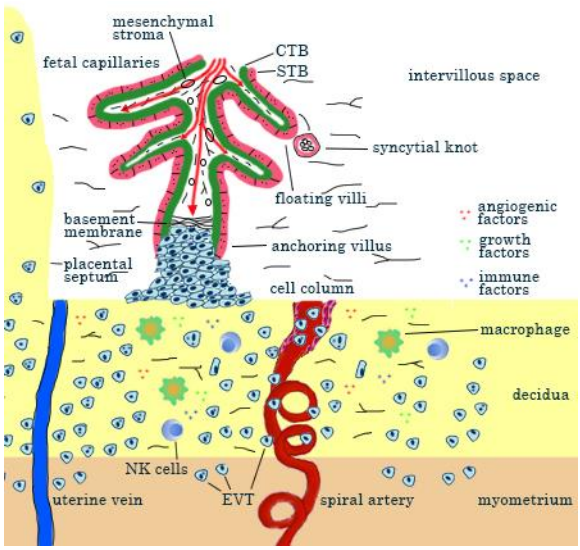
Discussion

Placentation, angiogenesis

Different trophoblast cell lineages differentiate from the trophoectoderm in the early stage of pregnancy. These trophoblast subtypes form the chorionic villi, invade the maternal decidua and initiate the remodeling of spiral arteries. These processes are strictly regulated and controlled by angiogenic factors. Angiogenic factors also have an impact on the maternal and fetal vasculatures. Physiological placentation requires materno-fetal immune tolerance, which is promoted by immunogenic molecules and specific immune cells on the feto-maternal interface [13], [14] (5. Figure).

The imbalance of proangiogenic and antiangiogenic factors could lead to the failure of trophoblast invasion and impaired placentation [13], [14]. Disrupted placental development is a well-known cause of preeclampsia and fetal growth restriction. Angiogenic factors could have a role in other pregnancy-associated diseases, like gestational diabetes, gestational hypertension, placenta accreta spectrum disorders and abruption of the placenta. The other risk factor of impaired placentation could be the defect of immune tolerance or immunoreactive condition, which could lead to miscarriage.

Figure 7. Feto-maternal interface



Levels of the observed angiogenic factors in non-pathologic pregnancies

VEGF-A concentration in the chorionic villi shows a moderate, but significant decrease between the 11th and 19th weeks of gestation in our study. It is in accordance with the article by Jia et al. [55], who also presented a decreasing tendency of VEGF-A protein concentration in chorionic villi in the course of pregnancy. By contrast, Chen et al. [5] reported an increasing trend of VEGF expression in the placenta during all trimesters, which might be explained by the larger volume of amnion and chorion. Geva et al. [56] described an almost unchanged VEGF-A mRNA expression in chorionic villi through the first, second and third trimester in a small sample of pregnant women. Furthermore, Lash et al. [57] found no significant alteration of VEGF-A mRNA expression from chorionic villous samples between 9^{th} and 18^{th} gestational weeks. Only a few research presented that the maternal serum VEGF-A concentration has a peak in the first trimester and there is a decreasing trend through the second and third trimester [58], [59]. It can be explained by the shift of branching and non-branching angiogenesis around the 20^{th} gestational week [4], [58], [59]. Cord blood VEGF-A concentrations are significantly higher than maternal serum concentration in the third trimester, which may be the result of dynamic angiogenesis in the feto-placental unit [60].

There is a paucity of data in the literature on the eNOS concentration in the chorionic villi and maternal serum. Elevating eNOS expression and activity were detected in ovine placental villi during sheep pregnancy [61], [62]. Rutherford et al. [63] demonstrated a significant difference in human eNOS activity related to the first trimester and term in chorionic samples, which could indicate an upward trend in eNOS expression as pregnancy advances. Our results suggest that the eNOS protein concentration is settled in the 11^{th}-19^{th} gestational weeks. In the course of gestation, as the fetal/placental ratio increases the eNOS has to maintain the fetoplacental circulation by its increasing concentration and activity.

One study describes the high expression pattern of bFGF in the first trimester in chorion samples [64], and another study found weak immunoreactivity in term placentas [20], so the conclusion can be a decreasing trend through trimesters. These correspond to our findings, that bFGF concentration in chorionic villi has a significant decrement between the 11^{th} and 18^{th} gestational week. Regarding maternal serum concentrations, Kreicberga et al. [20] noticed a continuous decline from the first trimester onwards, but Hill et al. [65] detected a slight elevation in the second trimester and then a sharp drop in the third trimester. bFGF has a prominent role in the early stage of pregnancy by initiating de novo formation of blood vessels,

this might be the explanation of its high concentration in the first trimester, but its decreasing level in the phase of non-branching angiogenesis.

The positive correlation between the VEGF-A and bFGF in the chorion in the late first and early second trimester could be intricately linked to the end of vasculogenesis and the shift of branching/non-branching angiogenesis. This is in relation to the significant correlation of VEGF-A and bFGF in the maternal serum through the whole gestation [58]. During the second and third trimester elevating eNOS levels are associated with increasing local expression of VEGF-A and bFGF in ovine placentas [5], [66], [67]. It is in contrast with our findings between the 11^{th} and 18^{th} gestational week, VEGF-A and bFGF levels show an adverse relationship with eNOS concentration. Hypoxia, NO and other stimulatory substances could excite VEGF-A and bFGF production [5], [6], [58], [68]. During the early second trimester, the expanding chorionic villi need high eNOS levels and activity to produce enough NO. Increasing NO could trigger positive feedback on VEGF-A and bFGF expression, and negative loop on eNOS activity, respectively.

Regarding the maternal factors, pre-pregnancy BMI was found to be significantly correlated with VEGF-A and bFGF. VEGF-A concentrations are antagonistic, and bFGF concentrations are agonistic to BMI in the late first and early second trimester. On the contrary, Salvolini et al. [69] described elevated VEGF-A and eNOS concentrations in term placentas, explained as a compensatory mechanism for changes in placental blood flow associated with obesity. Sirico et al. [70] have the same results on VEGF-A concentration and pre-pregnancy BMI defining the concept of “placental diabesity”. It can be assumed, that pre-pregnancy obesity has an influence on placental VEGF-A secretion. The expanding activated adipose tissue produces multiple angiogenic factors, such as VEGF-A and bFGF, which induce neovascularization [71]. In summary, “placental obesity” in the third trimester is most likely reflected by the higher angiogenic factor production. Our results refer to normal weighted or mild overweighted women and the investigated gestational interval was different than that in the mentioned articles, which could explain the disparity of the correlations.

sHLA-G concentration did not alter significantly in the maternal serum and amniotic fluid in the mid trimester, despite the expansive increase of sHLA-G protein secreting cells. The inconsiderable changes in the longitudinal level of sHLA-G in the body fluids are in line with the results reported by others [27], [72], [73], [74], [75]. It is worth mentioning that serum sHLA-G levels have wide intraindividual variations, but in general, the concentrations are increased in the first trimester and decreasing slowly throughout the second and third trimester

[76], [77]. HLA-G 14 bp ins/del polymorphism is widely investigated due to pregnancy-associated diseases with controversial results, but there is an agreement, that heterozygous and homozygous genotypes are characterized by diminished HLA-G expression [29], [77], [78], [79], [80], [81].

Soluble HLA-G level measurements in amniotic fluid are considered as a rarity [29], [82], [83]. Amniotic fluid soluble HLA-G concentration correlates exceedingly with the maternal serum levels of sHLA-G at the time of amniocentesis. One of the possible explanations may be that mostly EVTs, but also various other cells produce sHLA-G for the same stimulus both to maternal serum and to amniotic fluid, on the other hand, there could be an individual transport mechanism for sHLA-G through the feto-placental interface.

Serum sHLA-G concentrations tend to be increased by fetal growth, so our study supports the idea, that the serum sHLA-G is able to predict the weight percentile among the fetuses with normal weight in non-complicated gestations. The potential physiological mechanism could be that sHLA-G stimulates decidual NK cells that promote fetal growth by producing growth promoting factors and controls the trophoblast invasion during the mid-pregnancy [37], [84]. Pregnancy associated diseases could change the levels of serum sHLA-G, that could be the elucidation of non-significant results in the unselected group. The relationship between placental parameters and body fluid sHLA-G concentrations presents controversial outcomes considering the complicated and non-complicated groups. Larger placental volume is associated with higher serum sHLA-G in the unselected group, however, in the selected group, the interrelation becomes insignificant. The reason could be searched in the selection criteria, and it should be investigated on a larger population. Several placental vascular indices show significant adverse correlations with sHLA-G both in maternal serum and amniotic fluid, but it should be noted, that only VFI and amniotic fluid sHLA-G are correlated regarding the non-selected group. The selection criteria in the subgroup excluded the variables, which may confound the outcomes. It can be assumed that sHLA-G expression may be responsive to reduced blood flow and the emerging vascular network in the expanding placenta as gestation advances, since sHLA-G participates in the progress of vasculogenesis [27]. On the other hand, the HLA-G specific interaction of decidual NK cells and macrophages provides the excretion of angiogenic and proinflammatory factors implicated in vascular remodeling and affects the extent of trophoblast invasion [85], which is in comprehension with the lower perfusion indices detected in the placenta [52], [86].

The course of PP13 concentration through the second trimester is non-significant and stable in the maternal serum and amniotic fluid according to our research. In maternal blood, there is a slight reduction, whereas the levels in the amniotic fluid are almost constant. Studies reported an ascending tendency of PP13 levels throughout all trimesters [46], [87], [88], [89]. Serum and amniotic fluid concentrations differ by order of magnitude in favor of amniotic fluid. Only two earlier studies have interpreted the secretion of PP13 into the amniotic fluid [30], [88]. One of them described a similar difference between the two body fluid compartments, but in the third trimester [88]. Another research comprising a small-scale study [30], that represents twice more concentration in the amniotic fluid samples between the 19^{th} and 20^{th} gestational weeks which underlies our observations.

PP13 concentrations were not correlated to the placental size and vascular indices, but amniotic fluid samples have correlated conversely with birth weight and the time of delivery in both examined groups. Only in the selected group, did serum PP13 concentration indicate a similarly negative relation to birth weight, but not the time of delivery. Body fluid PP13 concentrations in the second trimester may predict birth weight during non-pathologic gestations. This is somehow in contrast with the significant linear relationship between amniotic fluid PP13 and fetal abdominal circumference, which is an obvious indicator of fetal weight. Sahraravand et al. [90] presented that maternal serum PP13 level is associated with placental volume in the first trimester. It is unclear, why the second trimester PP13 levels prognosticate lower birth weight at delivery since it is secreted from placental trophoblasts, amniotic membrane, villous capillary endothelium and is involved in normal implantation and placentation.

An interesting and novel finding is that serum HLA-G and serum PP13 concentrations are correlated inversely during the mid-trimester, which is controversial with our recent knowledge about the comparable functions of these two angiogenic factors. Therefore, we speculate that there is a direct or indirect connection between the molecular mechanisms, for example, negative feedback mechanism, which influences their activity.

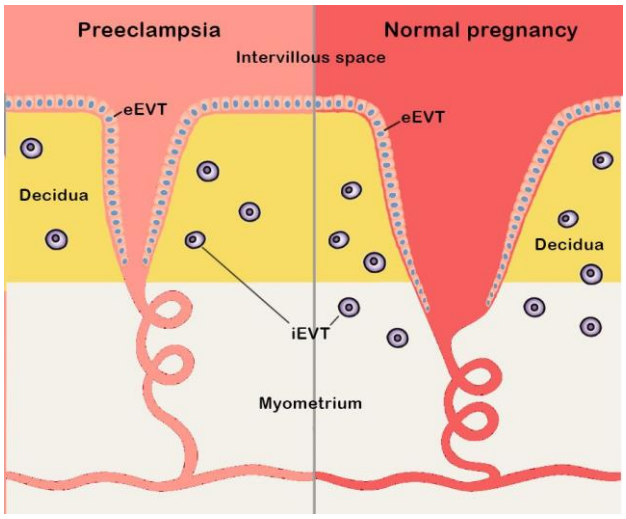
Pathologies associated with placentation and angiogenic factors

Preeclampsia

Preeclampsia is recently the leading cause of maternal and perinatal morbidity and mortality. According to the onset of clinical manifestation, it is distinguished between early and late onset preeclampsia depending on whether the diagnosis made before or after the 34th gestational week. Impaired placentation in the first trimester is often associated with early onset preeclampsia, and maternal endothelial dysfunction is usually the cause of late onset preeclampsia [91].

It is widely accepted, that the pathomechanism of early onset preeclampsia includes the reduced number and invasiveness of iEVTs, just like the fewer enEVTs in the spiral arteries, causing shallow invasion because of poor differentiation and increased apoptosis [92] (Figure 6).

Figure 8. Shallow trophoblast invasion in preeclampsia



Preeclampsia could be diagnosed if gestational hypertension (blood pressure >140/90 Hgmm) occurred 2 occasions 4 hours apart and proteinuria could be verified or most of the listed conditions befall: thrombocytopenia, renal insufficiency, elevated liver transaminase, persistent cerebral or visual symptoms, pulmonary oedema [93].

The maternal risk assessment for preeclampsia is an essential part of pregnancy care. Presently, many centers use only clinical risk factors in the selection of high-risk populations for developing preeclampsia recommended by American College of Obstetricians and Gynecologists (ACOG), National Institute Health and Care Excellence (NICE), and International Society for the Study of Hypertension in Pregnancy (ISSHP) guidelines [94], [95], [96] (Table 10).

Table 10. Risk assessment for preeclampsia

Maternal risk	ACOG (2018)	NICE (2019)	ISSHP (2021)
High risk	Diagnosis of preeclampsia in a previous pregnancy	Diagnosis of hypertensive disease during a previous pregnancy	Diagnosis of hypertensive disease during a previous pregnancy
	Multifetal gestation	Chronic kidney disease	BMI >30 kg/m²
	Renal disease	Autoimmune disease	Chronic hypertension
	Autoimmune disease	Chronic hypertension	Pregestational diabetes mellitus
	Diabetes mellitus (Type 1 or type 2)		Chronic kidney disease
	Chronic hypertension		Systemic lupus erythematosus/antiphospholipid syndrome
			Conception by assisted reproductive technology
Moderate risk	First pregnancy	First pregnancy	First pregnancy
	Maternal age > 35 years	Maternal age > 40 years	Maternal age > 40 years
	BMI >30 kg/m²	Interpregnancy interval > 10 years	Multifetal pregnancy
	Family history of preeclampsia	BMI >35 kg/m²	Prior pregnancy with placental abruption
	Sociodemographic characteristics	Family history of preeclampsia	Prior pregnancy with stillbirth
	Personal history factors	Multifetal pregnancy	Prior pregnancy with fetal growth restriction

In first trimester screening, it could be more accurate to combine clinical risk factors, mean arterial pressure, mean uterine artery pulsatility index and maternal angiogenic

biomarkers to determine the possibility of later developing preeclampsia. The Fetal Medicine Foundation (FMF) and International Federation of Gynecology and Obstetrics (FIGO) recommend a combined test with maternal risk factors, mean arterial pressure, placental growth factor, and uterine artery pulsatility index on first trimester screening (11^{th}-13^{th} weeks) [94]. On the second trimester scan (18^{th}-22^{th} weeks) it is emphasized to reassess maternal risk factors, mean arterial pressure and uterine artery pulsatility index regarding asymptomatic pregnant women. If the second trimester combined test or third trimester clinical examination indicates elevated risk for preeclampsia, the sFlt-1/PlGF ratio could be used to predict developing preeclampsia in the short-term. The most general cut-off values are: below 35, between 35 and 85, and above 85. Compared with PLGF, the sFlt-1/PLGF ratio has been shown to have a similar sensitivity for prediction and diagnosis of preeclampsia, but a higher specificity [94], [95], [96]. In high-risk population, it is recommended to apply low dose aspirin to prevent future preeclampsia [94], [95], [96]. FIGO proposes 150 mg/day from the first trimester scan to the 36^{th} gestational week [94].

The results are not completely consistent about VEGF-A concentrations and VEGF-A mRNA expression in maternal serum and placenta of normal third trimester pregnancies and preeclamptic pregnancies. Most of the studies reported that chorionic VEGF-A expression and serum concentration are decreased in severe preeclampsia, which is in line with the failure of trophoblast invasion, endothelial dysfunction and maladaptation of maternal systemic vasculature [4], [97], [98], [99], [100], [101], [102], [103], [104]. Keshavarzi et al. [105] found that the relative VEGF-A mRNA expression is higher in preeclamptic placentas, furthermore one of the VEGF promoter region polymorphism (-634CC genotype) can be a potent biomarker for preeclampsia. A few other studies demonstrated higher serum levels in preeclamptic pregnant women [106], [107]. Chedraui et al. [108] reported elevated VEGF-A levels in the umbilical artery in case of preeclampsia, but not in the umbilical vein compared to those of healthy pregnancies. Two studies found, that serum VEGF-A levels are increased in case of pregnancy induced hypertension without preeclampsia [58], [102]. The possible explanation could be a compensatory mechanism to restore placental blood flow in gestational hypertension, moreover in severe preeclampsia, elevated sFlt-1 bounds VEGF-A with high affinity, which could result in decreased free VEGF-A in maternal plasma.

eNOS is the most potent source of NO in the placenta during gestation. Several studies found relatively higher expression of eNOS mRNA in preeclamptic placentas [21], [109], [110]. However, serum eNOS and NO were unchanged or non-significantly decreased according to

some papers [111], [112], [113], [114]. Placental eNOS expression might react as a compensatory mechanism to maintain placental blood flow, but the eNOS protein bioactivity could be decreased, because of the elevated oxidative stress in preeclampsia, which causes eNOS uncoupling [109], [115]. Serum eNOS and NO concentration could be variable depending on red blood cell NO production, reactive oxygen species and diet. Chedraui et al. [108] investigated the umbilical cord NO levels during delivery in normal and preeclamptic pregnancies, and they found significantly higher levels in umbilical arteries and vein in preeclamptic samples explained by an adaptive response to vascular resistance and defective placental circulation.

Serum bFGF concentration in preeclampsia described no significant difference from uncomplicated controls by Lygnos et al. [58] in the third trimester. Hohlagschwandtner et al. [116] found that third trimester serum bFGF was significantly higher in mild preeclampsia, but not in chronic hypertension or severe preeclampsia, explained by the upregulation of bFGF induced by vasopressors in the case of undamaged endothelium.

Soluble HLA-G levels are significantly lower in serum obtained from pregnant women with preeclampsia in the third trimester [29], [82], [117], [118], [119], [120], [121]. Hsu et al. [122] described lower expression of HLA-G by peripheral HLA-G+ T-cells in preeclamptic serum. Tang et al. [119] observed extensive DNA methylation of the HLA-G promoter region in preeclampsia in samples extracted from chorionic villi, which causes reduced gene expression. Biyik [123] reported, that first trimester maternal serum sHLA-G doesn't predict later developing preeclampsia. It is controversial with Marozio et al. [124], who found more than twofold increased risk for preeclampsia based on lower sHLA-G concentration in early pregnancy. Our results imply, that second trimester serum and amniotic fluid sHLA-G concentrations are not correlated with later developing pregnancy induced hypertension. From placental samples, several studies found similarly diminished expression of HLA-G in preeclampsia [29], [100], [117], [119], [125], [126]. Preeclampsia is associated with oxidative stress, which could also be accompanied by placental HLA-G expression [127]. Amniotic fluid and cord blood HLA-G levels did not differ in preeclampsia and healthy pregnant controls [82].

PP13 and its capability in preeclampsia screening have a great literature in the last two decades. Most of the studies highlighted the significantly decreased maternal serum PP13 protein concentration in the first trimester as a potential biomarker for preeclampsia [30], [31], [32], [33], [47], [88], [128], [129]. The highest detection rate was found in case of early onset preeclampsia (80%), which could be 90 % combining first or second trimester determination of

uterine artery pulsatility index [12], [31], [32], [33], [130], [131], [132], [133]. Similarly, placental PP13 mRNA expression was found to be lower in gestational hypertensive disorders, than controls around the first trimester and third trimester [12], [31], [46], [88]. Our results confirm, that second trimester serum and amniotic fluid PP13 concentrations are not correlated with later developing pregnancy induced hypertension. At the time of the disease, maternal serum PP13 levels were several times higher, which is considered to be a consequence of the necrotic release of PP13 from damaged STBs [30], [31], [32], [134], [135]. Recently, three types of PP13 polymorphism were discovered which is in relation to preeclampsia. The truncated variant is a shorter PP13, because of an earlier stop codon resulting in two missing amino-acid from the CRD domain. The heterozygous form is an effective predictor of severe early preeclampsia [12], [136]. The promoter variant has an adenosine/cytosine heterozygous genotype in the -98th position, which causes reduced PP13 mRNA expression in the placenta [12], [137]. These two variants were discovered among colored population, so ethnicity could be a risk factor too for severe preeclampsia [136], [137]. The Dex-2 variant was isolated from the placenta of Israeli pregnant women affected by preeclampsia and fetal growth restriction. This mutant allele is completely absent from the second exon [30]. Sammar et al. [88] examined PP13 levels in amniotic fluid and umbilical cord. The authors reported twice fold higher PP13 levels in preeclamptic amniotic fluids, which could indicate the enhanced permeability of fetal membranes [88]. PP13 levels are negligible in fetal serum, but it is amplified in HELLP syndrome [88].

Fetal growth restriction

Small for gestational age (SGA) is defined as a birth weight of less than 10th percentile for gestational age. Two subgroups are classified: constitutionally normal infants who are SGA and infants who are SGA because of growth restriction with a birth weight lower than the expected optimal birth weight. Fetal growth restriction (FGR) often occurs alongside preeclampsia in association with placental dysfunction. The etiology is varied; it could be maternal, fetal or placental. Risk assessment includes maternal diseases (pregestational diabetes mellitus, chronic hypertension, renal insufficiency, autoimmune disease, and cyanotic cardiac disease), drug abuse, teratogenic exposure, multiple gestation, maternal and fetal infectious diseases, fetal genetic or structural anomaly and gestational hypertensive disorders with

placental malperfusion. Fetal biometry and Doppler velocity measurements (umbilical artery, cerebral media artery, and ductus venosus) are useful for screening the existence and severity of fetal growth restriction. Some articles suggest serum PlGF (below 100 pg/ml) with or without uterine artery pulsatility index measurement in the first trimester as a biomarker for later developing fetal growth restriction [95], [138], [139]. In addition, elevated sFlt1/PlGF ratio in the second and third trimester could increase the detection rate for FGR [95], [140], [141], [142].

In association with FGR, placental VEGF-A expression was found to be increased [143], [144], but maternal serum concentrations were shown to be controversial. Bartha et al. [145] described that serum VEGF-A levels are elevated in intrauterine growth restriction complicated with preeclampsia, but do not differ in those cases without preeclampsia. Pei et al. [146] presented a lower serum VEGF-A concentration in the second and third trimester in pregnancies complicated with fetal growth restriction. VEGF-A expression could be a compensatory mechanism to hypoxia and majority of serum VEGF-A concentrations could be bound to sFlt1 causing limited levels of free form. Szukiewicz et al. [143] indicated a significant influence of compromised vascular response to VEGF-A on the total placental vascular resistance in intrauterine growth restriction. A few studies examined uterine artery injection of VEGF-A on animal models to treat fetal growth restriction with significantly improved results, which could suggest VEGF-A as a possible future therapeutic tool [147], [148].

Evidence indicate, that in pregnancies complicated by FGR, eNOS expression is augmented in the placental endothelium and umbilical artery, but not in the umbilical vein [64], [149], [150]. It has to be noted, that activated eNOS is decreased in the endothelium of veins and arteries too [149]. It depends on shear stress and oxygenation. There is no difference regarding maternal serum eNOS concentrations, but higher levels of asymmetric dimethylarginine were observed by Laskowska et al. [151], which is an inhibitor of eNOS.

Notably, there are a few studies about the association of bFGF and intrauterine growth restriction. Barut et al. [64] presented higher bFGF gene expression in placentas from FGR complicated pregnancies, which could promote enhanced endothelial cell proliferation and migration. Wallner et al. [152] investigated maternal serum, umbilical artery and umbilical vein samples at the time of delivery in FGR and control groups. No differences were found in maternal serum and umbilical artery, but umbilical vein samples showed greater bFGF concentrations in the FGR group. They hypothesize that it could be caused by secretion via a

non-classical pathway, such as cell death, which appears to occur under hypoxic conditions, such as FGR [152].

Steinborn et al. [118] reported that second trimester serum sHLA-G concentrations are significantly lower in case of pregnancies complicating with FGR, so the molecule could be a prognostic tool. They defined a cut-off value of 63.3 U/ml. In the third trimester, no significant correlations were observed [27], [118], [123]. In contrast, our outcomes present no significant correlations between second trimester serum and amniotic fluid sHLA-G concentrations and FGR. Furthermore, Biyik [123] found no correlations in any trimester of pregnancy in association with preeclampsia or IUGR and serum sHLA-G levels corroborating with our study.

Maternal serum PP13 concentrations were observed in a few studies according to FGR without preeclampsia [30], [128], [153], [154], [155]. The first trimester screening potential was explored in the first line, and most of the results show a significant decrease in serum PP13 in the fetal growth restricted group [30], [128], [153], [155]. In contrast, Cowans et al. [154] represented a non-significant unsubstantial decrease in serum PP13 levels in the first trimester compared to the control group. Additionally, Burger et al. [30] found no significance in third trimester serum PP13 concentrations between the affected and control groups, which is in line with our results in second trimester serum and amniotic fluid samples.

Gestational diabetes mellitus

GDM is impaired glucose tolerance occurring with onset or first recognition during pregnancy. The pathophysiology is thought to be similar to type 2 diabetes and involves the same risk factors, like obesity, genetic disposition and lack of physical activity [156], [157]. There are increasing levels of factors in serum (triglycerides, cholesterol, leptin, TNF-α, adipokines), which could contribute to gestational diabetes [158], [159], [160], [161]. Researches aim to detect other factors that influenced GDM, like hypoxia-inducible factor 1-alpha or peroxisome proliferator-activated receptor, which could be promising targets for pharmacological therapy [158], [162].

According to some studies, maternal serum samples, collected after oral glucose tolerance test, have shown significantly increased VEGF-A mRNA expression and protein concentration [58], [163], [164], [165]. Krishnasamy et al. [166] measured the serum antiangiogenic spliced variant VEGF165b and total VEGF-A concentrations, and they found that an elevated plasma VEGF ratio could indicate gestational diabetes in the third trimester. There are conflicting data on the placental expression of VEGF-A at term [70], [167]. The

potential molecular processes for increasing VEGF-A level in GDM might be that advanced glycation end-products accumulate and impair placental cells or bind to their receptors, which enhance VEGF production by the activation of NF-kB pathways. Another potential mechanism could be transient fetal hypoxia caused by elevated maternal hemoglobin A1c, which induces VEGF-A expression in return [164]. The possible reason for reduced placental expression could be the constantly high glucose levels, especially in poor metabolic control, which causes severe hypoxia and ischemia with inhibition of binding VEGF/VEGFR-2 and consequent reduction of capillarization in the placenta [167].

Hyperglycemia could lead to increased production of reactive oxygen species and reactive nitrogen species resulting in oxidative stress. These mechanisms are associated with the decreased availability of NO resulting from eNOS uncoupling [168]. Mordwinkin et al. [168] represented significantly increased eNOS expression in maternal blood and cord blood, but NO was decreased in maternal serum at high glucose courses.

There is a paucity of data on the involvement of bFGF in GDM pathology. Zhou et al. [169] represented, that hyperglycemia is suppressing the bFGF regulated cell migration in human umbilical vein endothelial cells. The levels of bFGF in third trimester maternal serum were insignificantly decreased in GDM compared to healthy pregnancies according to Lygnos et al. [58].

Dysfunction of immune regulation may have influence on developing gestational diabetes [170]. Early third trimester maternal serum sHLA-G concentration and term placental HLA-G expression were reported to be decreased in pregnancies associated with GDM [171], [172]. These results are supported by the evidence of the reduction of HLA-G+ regulatory T-cells in third trimester serum samples compared to controls [170]. GDM treated with diet is not influenced by second trimester serum and amniotic fluid sHLA-G concentrations based on our results.

PP13 has immune regulatory and anti-inflammatory functions, so it might be in association with the development of gestational diabetes [33], [158], [173]. Recently, only two studies dealt with PP13 expression and concentration due to developing gestational diabetes [158], [173]. The results were in agreement with significantly decreased expression in chorionic villi and significantly reduced serum concentration in both the second and third trimester in pregnancies complicated by GDM [158], [173]. Considering our results, second trimester serum and amniotic fluid PP13 concentrations do not affect development of mild GDM. The contradiction may arise from the fact, that GDM were not insulin depending and was kept in balance.

Increased nuchal translucency thickness and aneuploidy

The relation between nuchal translucency thickness and aneuploidies is largely confirmed [174], [175], [176], [177], [178]. Nuchal translucency thickness measurement is the most significant part of antenatal screening between the 11^{th} and 13^{th} +6 weeks. Increased nuchal translucency thickness is associated with trisomy 13, trisomy 18, trisomy 21, Turner syndrome, other sex chromosome abnormalities, cardiovascular structural diseases and genetic syndromes. The possible mechanisms for developing increased NT can be the different composition of extracellular matrix, cardiovascular and/or lymphatic malformation [176], [179], [180], [181], [182]. Free beta human choriogonadotropin (βhCG) and pregnancy-associated plasma protein A (PAPP-A) are incorporated in combined first trimester screening as biomarkers. PAPP-A is reduced in trisomy 13, trisomy 18, trisomy 21 and Turner syndrome as well, free βhCG is elevated in trisomy 21 but decreased in trisomy 13 and 18 [183], [184], [185], [186], [187], [188], [189]. Sonographic screening performance can be increased by nasal bone, tricuspid valve flow and ductus venosus blood flow measurements [177]. Nowadays, noninvasive prenatal tests are available, which analyze fetal cell-free DNA and can detect chromosomal abnormalities with high sensitivity and specificity [177].

As VEGF-A has an essential role in vascular differentiation and vascular permeability, and there is evidence of enhanced VEGF-A expression in lymphatic endothelial cells from first and second trimester fetal samples with nuchal edema alone or complicated with trisomy 18 or 21 [174], [175]. Our result of chorionic villi VEGF-A concentrations reflects no significant correlation with increased nuchal translucency. VEGF-A and eNOS expression was demonstrated to be decreased in amniotic fluid collected in mid-trimester related to pregnancies complicated with Down-syndrome, explained by the chorionic villi hypoplasia and abnormal placentation [190], [191]. In contrast, NO concentrations were elevated in amniotic fluid, which could be due to elevated βhCG or a compensatory mechanism to low levels of VEGF-A, producing on other pathways than eNOS [190], [191]. Our study manifests higher eNOS concentrations in chorionic villi with higher nuchal translucency without any chromosomal abnormality, which might be in relation to dilated lymphovascular inflowing.

BFGF expression and concentration are not studied according to the literature. Our results reflect an inverse correlation between chorionic villi bFGF concentrations and increased nuchal translucency without chromosomal abnormality, but the physiologic mechanism is unexplained.

We could speculate that bFGF might enhance lymphovascular endothelial cell proliferation and migration, which could make the lymphovascular system more compact against fluid accumulation.

PP13 has been investigated in association with aneuploidies. Serum PP13 concentrations are decreased in trisomy 13, trisomy 18 and Turner syndrome, but not in Down syndrome at the time of combined first trimester screening. Moreover, PP13 concentration shows significant correlations with free βhCG and PAPP-A concentrations [178], [192].

There is only one study, which determined sHLA-G concentrations in connotation with chromosomal abnormalities [193]. The authors observed no correlation between concentrations of sHLA-G in embryo culture media and aneuploidy where preimplantation genetic diagnosis was performed [193].

Spontaneous preterm labor and preterm premature rupture of membrane

Preterm labor is defined as labor between the 24th and 37th gestational weeks. The pathophysiology of spontaneous preterm labor and preterm premature rupture of membranes are multifactorial, but infection and inflammation are the most consistently recognized risk factors. Most of the cases are associated with intraamniotic bacterial colonization, but clinically intrauterine infections with chorioamnionitis and/or funisitis could also be observed [83], [194], [195]. The inflammatory process includes the excessive production of a proinflammatory cytokine cascade, which causes myometrial contractions, amniotic membrane rupture and cervical ripening.

VEGF-A expression is demonstrated in decidua and fetal membranes too. Besides various functions, the molecule has a role in amniotic membrane permeability [60], [196]. Interestingly, preterm premature membrane rupture does not influence maternal and cord blood VEGF-A concentrations [60]. Amniotic membrane and maternal decidua VEGF-A expression is enhanced in cases of preterm premature membrane rupture without infectious signs compared with cases of preterm labor with intact membrane [196]. Moreover, membrane rupture with chorioamnionitis displayed further enhancement in expression [196]. These results suggest that VEGF-A could be a common mediator for membrane impairment and subsequent rupture through activation of tPA and MMPs [196].

NO and its metabolites have an important role in the relaxation of the myometrium and cervical ripening during labor too. During labor, myometrial NO synthases are inhibited, but cervical NO production is increasing, which is enhancing proinflammatory cascade [23], [194], [197]. According to Marinoni et al. [23], NO metabolite concentrations have no significant correlations among pregnant women with preterm labor, term labor or no labor in maternal and cord blood. The authors found significantly higher concentrations in amniotic fluid during preterm or term labor compared to not in labor controls [23]. In the placenta and fetal membranes, eNOS expression did not differ among any groups [23]. Törnblom et al. [197] represented higher mRNA levels of eNOS, iNOS and nNOS in cervical tissue during preterm labor compared to term labor.

A few articles demonstrated concordant results about increased HLA-G expression and concentrations regarding spontaneous preterm labor and preterm premature membrane rupture combined with intraamniotic infections [83], [118], [195]. The conclusion is that sHLA-G and membrane bound HLA-G participate in the host response to microbial invasion of the amniotic cavity and chorion.

There is no data about bFGF expression and concentrations in association with spontaneous preterm labor. Sammar et al. [88] reported that PP13 expression and concentrations do not differ in pregnancies complicated with spontaneous preterm labor nor in the placenta, amniotic fluid, maternal serum or in cord blood.

Conclusion

Our studies explored the angiogenic factors' levels (such as VEGF-A, eNOS, bFGF, sHLA-G, PP13) and the influencing factors in uncomplicated pregnancies during the late first and second trimester from blood samples, chorionic villi samples and amniotic fluid samples.

These angiogenic factors might have a role in developing pregnancy-related diseases, like pregnancy associated hypertension, preeclampsia, FGR and gestational diabetes mellitus.

VEGF-A concentrations have a decreasing tendency in the chorionic villi through the early second trimester regarding our study, which could be due to the shift of branching and non-branching angiogenesis. The literature demonstrates controversial data about the alteration of VEGF-A expression in the placenta during pregnancy. The levels of bFGF are also decreasing through the early second trimester in the chorionic villi, which is in line with the few literature data. The concentration of placental eNOS has not changed significantly according to our study. Other research presented an upward trend of eNOS expression in the course of gestation. Maternal BMI correlates positively with bFGF levels and negatively with VEGF-A levels in chorionic villi. The median BMI was 23.91 kg/m², so the population was in the normal weight range or overweight range. Publications demonstrate higher placental VEGF-A, bFGF and eNOS expression in term regarding obese pregnant women, which could indicate that adipose tissue produces multiple angiogenic factors. The alteration of chorionic villi VEGF-A and bFGF concentrations show a direct correlation in the early second trimester, which is in line with literature data according to serum concentrations. Placental eNOS levels are related inversely to VEGF-A and bFGF concentrations. Biologically active eNOS enzyme produces NO, which could trigger VEGF-A and bFGF expression, so our findings could imply a negative feed-back mechanism on eNOS expression. We found an upward trend of chorionic villi eNOS concentration and a downward trend of chorionic villi bFGF concentrations regarding nuchal translucency. The correlations might be explained by the effect of these factors on lymphovascular dilation and cell migration, but this should be supported by further research.

The sHLA-G concentrations have wide individual variations and there is no obvious trend observed in the alteration during all trimesters. We found no significant change in the levels of the sHLA-G in mid trimester. Serum and amniotic fluid sHLA-G concentrations correlate significantly in both groups and serum sHLA-G and serum PP13 levels correlate inversely in both groups. Higher serum sHLA-G indicates a larger placental volume in the

unselected group. A subanalysis showed, that in the case of a larger placental volume (>200 mm^3), maternal age could be a relevant confounder, notedly the serum sHLA-G levels were increased related to higher maternal age (>35 years). Particular placental vascular indices represent negative correlation mostly with amniotic fluid sHLA-G concentrations, but few with serum sHLA-G concentration too. In divided groups depending on VFI, a subanalysis shows that amniotic fluid sHLA-G concentrations are higher with advanced maternal age (>35 years). Based on these results, we could declare that sHLA-G could influence the second trimester placental vascularization, which could be enhanced by advanced maternal age. The angiogenic factors concentrations were compared in the unselected and selected groups, and the results support that serum sHLA-G concentration in the second trimester is increased in the case of LGA fetuses at term.

PP13 concentrations are also not altered during the experimental period, but other studies detected an ascending tendency of PP13 levels throughout pregnancy. The amniotic fluid concentration of PP13 is multiplies than serum concentration referred to gestational age, which is in line with the literature. Second trimester serum and amniotic fluid PP13 concentrations could prognosticate the birth weight in the normal range referred to gestational age, but there was no correlation between normal birth weight and SGA or LGA fetuses. On the other hand, mid trimester amniotic fluid PP13 levels correlated positively with gestational age at delivery. These outcomes implicate, that PP13 levels in body fluids may have a prognostic relevance of third trimester fetal growth and the time of delivery.

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