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Article

Amniotic Fluid and Maternal Serum Laeverin Levels and Their Correlations with Fetal Size and Placental Volume in the Second Trimester of Pregnancy—A Prospective Cross-Sectional Study

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Abstract: Introduction: Laeverin is an extravillous trophoblast marker playing a significant role in the trophoblast migration. The purpose of this study was to evaluate the association between the amniotic and serum laeverin concentrations at 16-22 weeks of gestation and the fetal and placental ultrasound measurements in healthy, unaffected pregnancies. **Materials and methods:** This was a prospective cross-sectional study. Pregnant women with singleton pregnancies undergoing amniocentesis were recruited consecutively. Fetal structural malformations and/or aneuploidy were exclusion criteria. Fetal biometric parameters and placental growth/perfusion were measured in 44 high-risk pregnancies who had no pregnancy complication and any other chronic disease. Maternal serum and amniotic levels of laeverin were assayed with sandwich enzyme-linked immunosorbent assay. **Results:** Serum laeverin levels are decreasing slightly with the maternal age in mid-gestation. Serum laeverin level correlated negatively slightly with head size of the fetus with the gestational length at delivery ($\beta=-0.38$, 95% confidence interval (CI) -0.03-0.01), while the amniotic laeverin level correlated strongly with the abdominal circumference of the fetus ($\beta=-0.64$, 95% CI: -0.03-0.01). Furthermore, laeverin level in the amnion correlated moderately and positively with the placental volume ($\beta=0.46$, 95% CI: 0.01-0.08). **Conclusions:** Laeverin levels detected in the serum and in the amniotic fluid denotes the fetoplacental growth in uncomplicated high-risk pregnancies.

Keywords: human laeverin; serum; amniotic fluid; fetus; placenta; ultrasound

1. Introduction

Laeverin, also called aminopeptidase-Q, is expressed in the extravillous trophoblast (EVT) and affects the EVT invasion into the maternal decidua and their migration into the spiral arteries. Laeverin play a regulatory role of the epithelial-to-mesenchymal transition of the EVTs, and remodeling of the spiral arteries in the uterine wall [1–3]. Laeverin is an aminopeptidase localized on the cell membrane of the EVTs interacting with the maternal immune system [4–6]. Laeverin might cleave a relatively wide range of substrates as anti-migratory oligopeptides (kisspeptin-10) and molecules contributing to angiogenesis (angiotensin III, endokinin C and dynorphin A₁₋₈, and angiotensin III). Angiogenetic molecules regulate the local blood pressure and maintain the perfusion towards the placenta and more specifically to the placental bed [7]. Laeverin has a regulatory role in the molecular network regulating the placental growth. The migratory ability of EVTs is provided by a controlled degradation and disintegration of extracellular matrix in the maternal decidua via regulation of matrix metalloproteinases and their tissue inhibitors [8]. Laeverin cooperates with the chemokine inflammatory system in the fetomaternal interface in order to maintain the balanced control of angiogenesis in the placenta [9]. Laeverin down-regulates the N-cadherin and vimentin are

downregulated, while increases the E-cadherin production that facilitates cell locomotion of the decidual EVT. Laeverin converts kallikrein to bradykinin which sustains the pro-inflammatory state of the placenta and facilitates the EVT migration [3,10,11]. However, the direct molecular evidence is omitted since the enzymatic function of laeverin has been revealed by molecular experiments using functional physiological analogy to explain the molecular properties [4]. However, the investigation of trophoblast invasion and alterations of the enzymatic function during interactions with the uterine environment studied in trophoblast assays and explant tissues has somehow a limited significance [12].

Silencing laeverin function in trophoblast migration assay analyses revealed that laeverin has a regulatory role in EVT outgrowing [4–6,13]. The potential role of laeverin in pathomechanisms of preeclampsia in placenta has been reported. An enhanced production of laeverin impedes the EVTs and decelerates the EVT migration. An overexpression of the laeverin could be observed in preeclampsia. The greater expression of laeverin in preeclampsia results in shallow placentation and impaired spiral artery remodeling. In preeclamptic cases, the maternal serum laeverin level is lower in the third trimester than in that of healthy pregnant women [4–6,14].

Laeverin is detectable in the cell surface of placental tissue as early as 4th week of gestation until term [5,6,15]. This fact suggests that laeverin might play a physiological role in placental homeostasis since EVT migration finishes by the 16th week of gestation [7]. Notably, the laeverin serum level high in the maternal circulation in the first trimester [16], but it is decreasing in the third trimester [14]. Our research group observed that laeverin can be detected in the amniotic fluid in high-risk, complicated pregnancies, but the amniotic concentrations do not prognosticate preeclampsia. The amniotic concentrations of laeverin are much higher compared to those of maternal serum levels, but it is not revealed what the function of laeverin has in fetal life or in the amniotic cavity [17].

The amniotic laeverin levels has not been determined in uncomplicated pregnancies. We wanted to explore the interrelations between the amniotic and serum laeverin levels and their correlations to the fetal weight and placental volume and perfusion assessed by ultrasound.

2. Materials and Methods

2.1. Study Design and Population

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 September 2022 and December 2023. During the study period, all single pregnancies with increased risk of chromosomal abnormality, where amniocentesis was performed between 16 + 0 and 22 + 6 weeks of gestation, were consecutively recruited into our study. Exclusion criteria were as follows: multiple pregnancies; fetal or neonatal structural and/or genetic anomaly; pathological placentation (placenta accreta spectrum, placenta previa) or improper localization of the placenta for sonographic evaluation of volume and perfusion (posterior placenta); self-reported drug, alcohol, caffeine, or nicotine abuse; and systemic disease (e.g., any type of pregestational diabetes mellitus, autoimmune disease, vasculitis, hemophilia, thrombophilia, chronic infection). Women with complications during late pregnancy (gestational diabetes mellitus treated with diet/insulin (n = 11), hypertension-related diseases (n = 10), fetal growth restriction (FGR) at delivery (n = 4), premature ruptures of the membranes and premature birth (n=6) and large for gestational age at delivery (n = 9)) were excluded from the study. Clinical follow up and outcome data were collected from patients' medical records.

2.2. Ethics Statements

A verbal and written explanation of the study was given to all participants and written informed consent from all those who agreed to participate. The study protocol was approved by the Clinical Research Ethics Committee of the University of Szeged (the reference number: 09/2017 and the date of approval: 10 February 2017). The study was carried out according to the Principles of the Declaration of Helsinki and its later amendments or comparable ethical standards.

2.3. Conventional 2-Dimensional (2-D) Sonographic Examinations

All pregnancies were dated by using the measurement of crown–rump length (CRL) at nuchal screening. NT and anatomic assessment between 11 + 0 and 13 + 6 weeks were performed by utilizing conventional methods. Ultrasound examination took place before measuring amniocentesis to determine fetal biometry, fetal anomalies, placental location, and the amount of amniotic fluid. Fetal weight was estimated according to the method of Hadlock et al. [18]. Estimated fetal weight percentile was calculated according to the local standards [19]. The ultrasound investigations were conducted by J. S. and A. S. Volume Acquisition The images used for the determination of placental volume and 3-dimensional power Doppler (3-DPD) indices were acquired at the time of the visit. All 3D scans were performed by A. S. Voluson 730 Expert ultrasound machine (GE Medical Systems, Kretztechnik GmbH & Co OHG, Tiefenbach, Austria) equipped with a convex, transabdominal, multifrequency probe (2–5 MHz), which was used to acquire all images. Each sample was examined using 3D rendering mode, in which the color and gray value information was processed and combined to give a 3D image (mode cent; smooth: 4/5; FRQ: low; quality: 16; density: 6; enhance: 16; balance: 150; filter: 2; actual power: 2 dB; pulse repetition frequency: 0.9). We used fast, low-resolution acquisition to avoid any kinds of artifacts. The 3D static volume box was placed over the highest villous vascular density zone at the umbilical cord insertion [20]. Each image was recovered from the disc in succession for processing. We recorded one sample from each patient during gestation.

2.5. Determination of Power Doppler Indices

The stored volumes were further analyzed using the virtual organ computer-aided analysis (VOCAL) program pertaining to computer software 4DView (GE Medical Systems, Zipf, Austria, version 10.4) by the same expert in 3D analysis (A.S.). The image used for recovering from the hard disc was captured and processed using the multiplanar system. The spherical sample volume was consistently 28 mL at umbilical cord insertion. The VOCAL program automatically calculated the gray- and color-scale values from the acquired spherical sample volume in a histogram in all cases. The combined use of power Doppler with three-dimensional ultrasound provides the possibility of quantifying blood in motion within a volume in-vivo. Three indices were calculated, namely the 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 gray and color voxels, a product of the number of color voxels as a percentage and the relative amplitude of these voxels [21,22]. The intra-observer errors were evaluated by repeated measurements of 3-DPD indices in 30 patients at initiation of the study. The intra-class correlation coefficients for all Doppler indices were excellent (0.99) in case of all indices.

2.6. Amniocentesis Procedure

The patients were informed about the amniocentesis procedure and possible complications before a consent form was signed prior to the procedure. All procedures were performed by the same operator expert (J.S.) 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 were 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. Pregnant women had rest for 4–6 hours at the department.

2.7. Samples

Amniotic fluid (approximately 5ml) and maternal venous blood (approximately ml) were collected from each patient at the time of amniocentesis. Blood samples were centrifuged at 3400 rpm for 15 min. Serum and amniotic fluid samples were stored at -80 °C until assay.

2.8. Enzyme-Linked Immunosorbent Assay (ELISA)

Human laeverin in maternal serum and amniotic fluid were determined by sandwich ELISA-kit. The laboratory staff members who performed the assays were blinded to the pregnancy outcomes, and the clinician recruiting women did not participate in analyzing the samples.

The concentration of Laeverin was measured using the kits from Mybiosource (San Diego, CA, USA; Cat. No.: MBS2882930). The sensitivity of the assay was 0.23 ng/mL. The intra-assay coefficient was ≤4.7%, and the inter-assay coefficient was ≤6.3%, according to the manufacturer.

2.9. 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 were expressed as numbers and percentages. The relationship between the level of angiogenic factor and other continuous variables was assessed using Pearson’s correlation and regression analyses. The relationship between the level of laeverin and other continuous variables was assessed using univariate and multivariate regression analyses interpreted by correlation coefficient (β) and (95% CI). Multivariate linear regression was adjusted for well-known confounders such as for maternal age, BMI at amniocentesis, number of previous pregnancies and gestational age at amniocentesis, as these factors determine the actual placental volume and fetal weight. Independent sample t-tests were used to determine whether the angiogenic factor levels in body fluid were different in complicated pregnancies vs. healthy subjects. The 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 a Holm–Bonferroni correction for multiple comparisons.

3. Results

The most common indications for amniocentesis were advanced maternal age (Table 1). Previous pregnancy was affected for aneuploidy in the 15% of all cases. The maternal characteristics and perinatal outcome are displayed in Table 2. Most pregnant women who were presented for amniocentesis were at advanced age and approximately one third of them was primiparous. The gestational age at amniocenteses was between 16+0 and 22+0 weeks. Neonatal weight and gestational age at birth were without extremities. The fetal sonographic parameters are in consistence with the national figures [19], whereas the placental volume and perfusion measurements were similar to the that of healthy controls published previously by our study group (Table 3) [23]. Laeverin concentration was considerably increased in the amniotic fluid as compared to that in the maternal serum (Table 4).

Table 1. Indication of amniocentesis.

Cause for amniocentesis	Number of cases
increased nuchal translucency (NT) at first trimester scan (≥2 MoM for gestational age)	20
chromosome aberration or genetic disorder concerning the previous pregnancy	11
advanced maternal age (>37y)	40

Table 2. Clinical and obstetrical data of women with amniocentesis (N=44).

Maternal age (years)*	33.59 ± 6.49
Number of nulliparous women in the study**	13 (29.5)
BMI at the time of genetical consultation (kg/m²)*	26.42 ± 5.80
Gestational age at the time of amniocentesis (weeks)*	18.30 ± 1.44
Birthweight (grams)*	3519.55 ± 440.76
Gestational age at delivery (weeks)*	38.99 ± 0.99

*Continuous variables displayed as mean ± standard deviation (SD). ** Categorical variables are presented in number and %.

Table 3. Ultrasound data at amniocentesis (N=44)*.

Fetal biometry	
Head circumference (mm)	152.79 ± 16.08
Head circumference (percentile)	53.09 ± 30.32
Abdominal circumference (mm)	130.51 ± 16.30
Abdominal circumference (percentile)	49.94 ± 29.83
Femur length (mm)	27.53 ± 4.83
Femur length (percentile)	58.75 ± 30.15
Estimated Birthweight (grams)	251.48 ± 75.68
Estimated Birthweight (percentile)	52.20 ± 25.87
Placental sonography	
Placental volume (mm³)	231.58 ± 95.01
VI	14.57 ± 5.19
FI	48.69 ± 27.29
VFI	8.08 ± 3.70

VI: Vascularization Index, FI: Flow Index, VFI: Vascularization Flow Index. *Continuous variables displayed as mean ± standard deviation (SD).

Table 4. Levels of angiogenic factors in samples of amniotic fluid and serum (N=44)*.

Laeverin in amniotic fluid (ng/ml)	15.64 ± 9.84
Laeverin concentration in serum (ng/ml)	0.91 ± 0.73

*Continuous variables displayed as mean ± standard deviation (SD).Laeverin showed a significant negative correlation with the maternal age (p<0.05, β=- 0.38, 95% CI=- 0.07-0.01).

Among the sonographic parameters, the fetal head circumference exhibited a slightly negative correlation with the laeverin level (p<0.05, β=- 0.38, 95% CI=- 0.03-0.01). An inverse correlation was observed between the abdominal circumference both in absolute values (p<0.05, β=- 1.40, 95% CI=- 1.15-0.02) and expressed in percentile (p<0.05, β=-0.64, 95% CI=- 0.31-0.06) and amniotic laeverin level. A higher placental volume was interrelated to a higher laeverin level (p<0.05, β=0.46, 95% CI= 0.01-0.08) (Table 5).

Table 5. Correlation between maternal as well as sonographic data and the levels of laeverin in the maternal serum and amniotic fluid (N = 44).

Variables	Laeverin level in the serum				Laeverin level in the amniotic fluid			
	Simple linear regression		Multivariate linear regression		Simple linear regression		Multivariate linear regression	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Maternal characteristics								
Maternal age	-0.38*	-0.07-0.01*	-0.23	-0.08-0.03	-0.06	-0.10	-0.08	-0.90-0.67
Previous parity	-0.04	-0.33-0.27	0.09	-0.28-0.42	-0.02	-0.16	0.01	-4.83-4.87

BMI at the time of genetical consultation (kg/m²)	-0.17	-0.07-0.03	-0.08	-0.06-0.04	-0.08	-0.13	-0.07	-0.85-0.61
Birthweight (grams)	0.10	0.01-0.01	0.20	0.01-0.01	-0.03	-0.01	-0.02	-0.01-0.01
Birthweight (percentile)	-0.05	-0.01-0.01	0.04	-0.01-0.01	-0.11	-0.04	-0.10	-0.21-0.14
GA at delivery	0.36	-0.01-0.08	0.41	0.01-0.08	0.11	0.15	0.11	-0.46-0.77
GA at the time of amniocentesi s (weeks)	0.21	-0.01-0.04	0.10	-0.03-0.04	-0.02	-0.02	-0.08	-0.52-0.38
Ultrasound characteristics								
Head circumferenc e (mm)	-0.38*	-0.03-0.01*	-0.48	-0.04- -0.03	-0.09	-0.30-0.19	-0.16	-0.39-0.20
Head circumferenc e (percentile)	-0.20	-0.02-0.01	-0.25	-0.02-0.01	0.03	-0.13-0.15	0.12	-0.14-0.22
Abdominal circumferenc e (mm)	-0.01	-0.03-0.03	-0.84	-0.14-0.06	0.23	-0.17-0.35	-1.40*	-1.15-0.02*
Abdominal circumferenc e (percentile)	-0.31	-0.03-0.01	-0.29	-0.04-0.02	-0.74**	-0.34- -0.09**	-0.64*	-0.31- 0.06*
Femur length (mm)	0.17	-0.04-0.09	0.19	-0.27-0.32	0.19	-0.37-0.83	-0.87	-3.20-1.06
Femur length (percentile)	-0.04	-0.01-0.01	0.09	-0.02-0.02	0.20	-0.07-0.16	-0.13	-0.17-0.11
Estimated Birthweight (grams)	0.05	-0.01-0.01	0.75	-0.04-0.06	0.38	-0.02-0.09	-2.94	-0.53-0.03
Estimated Birthweight (percentile)	-0.30	-0.03-0.01	0.06	-0.04-0.05	-0.32	-0.30-0.10	-0.68	-0.48-0.05
Placental volume (mm³)	0.01	0.01	0.15	-0.01-0.01	0.46*	0.01-0.08*	0.57	0.02-0.09
VI	0.02	0.12	0.21	-0.03-0.08	-0.18	-1.00-0.36	-0.19	-1.12-0.46
FI	-0.01	-0.15	-0.12	-0.01-0.01	-0.11	-0.15-0.08	-0.11	-0.17-0.10
VFI	0.02	0.11	0.23	-0.04-0.13	-0.19	-1.54-0.51	-0.20	-1.71-0.67

BMI: body mass index, GA: Gestational age, VI: Vascularization Index, FI: Flow Index, VFI: Vascularization Flow Index. * P < 0.05; ** P < 0.001.

4. Discussion

Laeverin could be detected in the amniotic fluid and this finding is in coherence with our previous study [17]. There is still no molecular evidence that the amniotic epithelial cell membrane produces laeverin. The laeverin levels in the amniotic fluid appear to be interrelated to the placental

development, volume and fetal growth. This could be explained by the fact that an increasing chorionic mass including both extra- and intravillous trophoblasts as gestation advances produces an increasing amount of laeverin. However, laeverin is a glycosylated protein enzyme and cannot diffuse through the subamniotic layers (mesenchymal stroma and basement membrane) [24].

The most obvious explanation is that amniotic membrane cells should express laeverin at least in the mid-trimester. By contrast, immunostaining of amniotic membrane obtained from term pregnancy did not demonstrate laeverin production [1]. A less obvious explanation could be that the fetus is secreting the protein into the amniotic cavity since the amniotic fluid is surrounding the fetus. Although it has been reported that the serum level in the umbilical vein and artery after delivery is not significantly different suggesting that laeverin has no function identifiable in the fetus [14]. Predominantly, an increased level of laeverin in the fetal circulation could be expected in preeclampsia, because the leakage of the protein into the fetal capillaries in the chorionic villi has been described after delivery [1,5].

It is unclear why soluble laeverin has the highest level in the amniotic fluid between 16 and 22 weeks of gestation and not in the serum. Sandwich ELISA kit appeared to be a versatile test for the quantitation of soluble laeverin homodimer according to the manufacturer. However, this fact is coherent with our previous results [17], where we demonstrated that laeverin is excreted in higher concentration into the amniotic cavity in mid-gestation in high-risk pregnancies including not only unaffected pregnancies but complicated pregnancies as well.

Laeverin is a stable cell-membrane bound protein with slow turnover and has an imperative role in EVT passage into the uterine wall [2–6,13,25]. Laeverin is specifically expressed on EVT in the placenta throughout the pregnancy co-expressed with HLA-G as a marker of EVT activity [13]. The serum level of laeverin has been not significantly elevated in mid pregnancy correlating with the placental function and reflecting the fact that restructuring of the spiral arteries is finishing by that gestational period [26]. Notably, the serum laeverin levels are showing a slight increasing tendency during 8-14 weeks [16] and much higher than in the second half of the pregnancy [14]. It is contradictory, that the serum level of laeverin was in very different ranges in our study and in the other studies which can be explained by the variations of the study sampling and different commercial ELISA kit applied in the studies [14,16]. The laeverin level in the maternal blood decreases sharply after delivery [14].

Paradoxically, laeverin production in the placenta is increasing normally as gestation advances in normal pregnancies until term gestation [4,6,16], but the detected level of the protein in the maternal serum is decreasing gradually [14]. This corresponds well with the fact that laeverin might play a central role in placental homeostasis mostly in the first half of the pregnancy [15], since EVT migration finishes by the 22nd week of gestation [27]. A chorionic overexpression of laeverin in placenta could be detected in preeclampsia leading to a more pronounced difference in serum laeverin level in the early third trimester [5,6,14]. The enhanced intracellular effect of laeverin suppresses the migratory ability, which is characteristic for preeclampsia [4–6].

Serum laeverin concentration has not been associated with a discriminative serum concentration in the first trimester between preeclampsia and unaffected controls [16]. In our previous study, we could not find any correlation between serum or amniotic fluid protein level and later development of preeclampsia [28]. However, the number of participants with hypertension-related conditions were very low in both studies [16,17]. On the contrary, a lower serum laeverin level in 22-24 weeks of gestation could predict preeclampsia that occurs later [14].

Serum laeverin is decreasing with the maternal age. Taking into account that an advanced maternal age represents a risk for reduced placental volumetric measurement (Kozinszky, Fang), it is plausible that a decreased amount of EVT excretes less soluble laeverin. Interestingly enough, the amniotic laeverin levels correlates well with the fetal abdominal diameter, whereas the serum levels interfere with the head circumference. Both ultrasound parameters are the major determinants of the actual fetal weight. It is still to elucidate the reason of the interrelation of the laeverin levels in various body fluids to the fetal parameters, but apparently a larger fetus is having a larger placenta which reflects the extent of laeverin excretion. These facts are in line with the results published by our

research group that revealed a slight to moderate correlation between laeverin levels and fetal and placental size, but in complicated pregnancies. However, the fetal growth measurements correlate with the laeverin levels with the opposite sign in the present study in uncomplicated pregnancy. Further research is required to describe how the laeverin levels influence the fetal growth in complicated and non-complicated pregnancies.

5. Conclusions

The present study demonstrated that laeverin can be detected in the amniotic fluid in normal, healthy pregnancies, however its function in the amniotic cavity has not been described earlier. Both the amniotic fluid and serum laeverin levels are stable during the mid-gestation. Laeverin levels measured in different body fluid levels are in mild to moderate correlations with the estimated placental volume and fetal weight parameters.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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