Ultrasound Obstet Gynecol 2021; **58**: 716–721
Published online in Wiley Online Library (wileyonlinelibrary.com). **DOI:** 10.1002/uog.23605.

Maternal plasma soluble neuropilin-1 is downregulated in fetal growth restriction complicated by abnormal umbilical artery Doppler: a pilot study

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KEYWORDS: fetal growth restriction; neuropilin-1; placental angiogenesis; soluble neuropilin-1; umbilical artery Doppler

CONTRIBUTION

What are the novel findings of this work?
Soluble neuropilin-1, a member of the vascular endothelial growth factor family involved in branching angiogenesis, is present in maternal plasma and is significantly downregulated in pregnancies with fetal growth restriction (estimated fetal weight $< 10^{th}$ percentile), but only in those with abnormal umbilical artery Doppler.

What are the clinical implications of this work?
Maternal plasma soluble neuropilin-1 could serve as a novel biomarker for fetal growth restriction and for distinguishing constitutionally small fetuses from those with true growth compromise.

ABSTRACT

Objectives *Placental expression of neuropilin-1 (NRP1), a proangiogenic member of the vascular endothelial growth factor receptor family involved in sprouting angiogenesis, was recently discovered to be downregulated in pregnancies with fetal growth restriction (FGR) and abnormal umbilical artery (UA) Doppler. Soluble NRP1 (sNRP1) is an antagonist to NRP1; however, little is known about its role in normal and FGR pregnancies. This study tested the hypotheses that, first, sNRP1 would be detectable in maternal circulation and, second, its concentration would be upregulated in FGR pregnancies compared to those with normal fetal growth and this would correlate with the severity of the disease as assessed by UA Doppler.*

Methods *This was a prospective case–control pilot study of 40 singleton pregnancies (20 FGR cases and 20 uncomplicated controls) between 24 + 0 and 40 + 0 weeks' gestation followed in an academic perinatal center from January 2015 to May 2017. FGR was defined as an ultrasound-estimated fetal weight $< 10^{th}$ percentile for gestational age. The control group was matched to the FGR group for maternal age and gestational age at assessment. Fetal ultrasound biometry and UA Doppler were performed using standard protocols. Maternal plasma sNRP1 measurements were performed using a commercially available ELISA.*

Results *Contrary to the study hypothesis, maternal plasma sNRP1 levels were significantly decreased in FGR pregnancies as compared to those with normal fetal growth (137.4 ± 44.8 pg/mL* vs *166.7 ± 36.9 pg/mL;* $P = 0.03$*). However, there was no significant difference in sNRP1 concentration between the control group and FGR pregnancies that had normal UA Doppler. Plasma sNRP1 was downregulated in FGR pregnancies with elevated UA systolic/diastolic ratio (*$P = 0.023$*) and those with UA absent or reversed end-diastolic flow (*$P = 0.005$*) in comparison to FGR pregnancies with normal UA Doppler. This suggests that biometrically small fetuses without hemodynamic compromise are small-for-gestational age rather than FGR.*

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Accepted: 20 January 2021

Conclusions *This study demonstrated a significant decrease in maternal plasma sNRP1 concentration in growth-restricted pregnancies with fetoplacental circulatory compromise. These findings suggest a possible role of sNRP1 in modulating fetal growth and its potential as a biomarker for FGR.* © *2021 The Authors. Ultrasound in Obstetrics & Gynecology published by John Wiley & Sons Ltd on behalf of International Society of Ultrasound in Obstetrics and Gynecology.*

INTRODUCTION

Fetal growth restriction (FGR), defined as failure of a fetus to achieve its genetically determined growth potential, constitutes a major complication of pregnancy and is associated with adverse outcomes extending from fetal to adult life, including increased risks of perinatal mortality and morbidity[1–7]. Despite extensive research in this area in recent years, prenatal identification of FGR remains challenging. Ultrasound fetal biometry, the main modality for prenatal diagnosis of FGR, cannot discriminate effectively between a truly growth-restricted fetus and a constitutionally small-for-gestational-age (SGA) fetus[8,9]. Fetal Doppler provides insights into the fetal circulatory response to *in-utero* growth deprivation and has been utilized for fetal surveillance in high-risk pregnancies[10]; however, its effectiveness as a screening tool in low-risk pregnancy remains limited[11].

Numerous potential biomarkers for predicting FGR have been investigated, but their accuracy and utility remain limited[12]. As fetoplacental vascular development and, specifically, branching angiogenesis are known to be compromised in FGR pregnancy[13,14], several investigators have explored the angiogenic pathways to identify the placental mechanisms of FGR[15–17]. However, the effect of specific angiogenic mediators on the placenta and fetal growth is still unclear[18,19]. Several studies have suggested that maternal plasma factors may serve as potential biomarkers to identify abnormal fetal growth[20–22].

A recent study showed that neuropilin-1 (NRP1) is downregulated in the placenta of FGR pregnancies complicated by absent end-diastolic flow in the umbilical artery (UA)[23]. This is relevant, as NRP1, a proangiogenic transmembrane glycoprotein belonging to the vascular endothelial growth factor (VEGF) family[24,25], is involved in sprouting angiogenesis[26,27]. The soluble form of NRP1 (sNRP1) is an angiogenesis inhibitor that acts as an antagonist to NRP1[27]. Little is known about the roles of NRP1 and sNRP1 in regulating placental angiogenesis in normal and FGR pregnancies. This information is of translational significance, as presence of sNRP1 in maternal circulation could potentially serve as a biomarker of compromised placental vascular development and fetal growth.

Therefore, the objective of this prospective proof-of-concept study was to test the hypothesis that sNRP1 would be detectable in maternal circulation and its concentration would be upregulated in pregnancies with FGR, consistent with its antiangiogenic function. We further hypothesized that the upregulation of sNRP1 might correlate with the severity of FGR, as assessed by UA Doppler.

METHODS

This was a prospective case–control study involving 40 pregnancies between 24 + 0 and 40 + 0 weeks' gestation followed in an academic perinatal center from January 2015 to May 2017. The study group comprised pregnancies with FGR, defined as an ultrasound-estimated fetal weight $< 10^{th}$ percentile for gestational age, according to the guidelines of the American College of Obstetricians and Gynecologists (ACOG), which constitutes the standard of practice in the USA[2]. The control group included uncomplicated pregnancies with estimated fetal weight between the 20^{th} and 90^{th} percentiles at the time of enrolment and was matched to the study group in a 1:1 ratio for maternal age (± 5 years) and gestational age (± 5 weeks). Pregnant mothers who had an ultrasound examination for fetal weight assessment were approached for consent and participation in the study. The inclusion criteria for the study were: maternal age of 18–45 years; singleton pregnancy without an anomaly; and gestational age ascertained according to the ACOG guidelines[28]. The exclusion criteria included: fetal abnormality, including malformation and aneuploidy; multiple gestation; maternal inflammatory condition, including systemic lupus erythematosus, chronic obstructive pulmonary disease, Crohn's disease, ulcerative colitis and rheumatoid arthritis; maternal infection, including hepatitis B or C and human immunodeficiency virus; obstetric complication, including chorioamnionitis and prelabor rupture of the membranes; and maternal disease, including pregestational and gestational diabetes. The exclusion process was completed at the time of enrolment to minimize bias.

Maternal demographic and neonatal data were extracted from medical records, and the birth-weight percentile was determined using standard reference values[29]. The study was approved by the institutional review boards of Truman Medical Center/University of Missouri–Kansas City School of Medicine. Informed consent was obtained from each patient according to the institutional review board protocol. Control subjects were counseled and provided consent prior to their ultrasound examination, and were advised that UA Doppler studies would be conducted for research purposes.

At the time of enrolment, a maternal venous blood sample (10 mL) was collected in a purple-top EDTA tube. After collection, samples were inverted gently 8–10 times to mix and then centrifuged at 1100–1300 g for 15 min at room temperature. Plasma supernatant was then transferred to new tubes in 1-mL aliquots and stored at −80 °C, until use. Plasma sNRP1 analysis was performed using a commercially available ELISA (Quantikine DNRP10, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, individual samples were analyzed in triplicate and then averaged to calculate sNRP1 levels. Cohort

levels for FGR and control pregnancies were expressed as mean ± SD.

Study data were collected and managed using REDCap (Research Electronic Data Capture) electronic data capture tools hosted at the University of Missouri Kansas City[30]. REDCap is a secure, web-based application designed to support data capture for research studies, which provides an intuitive interface for: validated data entry; audit trails for tracking data manipulation and export procedures; automated export procedures for seamless data downloads to common statistical packages; and procedures for importing data from external sources.

Statistical analysis

The study size was determined by a sample of convenience, given the exploratory nature of the study and the lack of available data on sNRP1 levels in FGR pregnancy. Descriptive statistics are reported as *n* (%). Continuous variables are reported as mean ± SD. Normality, skewness and kurtosis of the distribution of plasma sNRP1 data were assessed using the Shapiro–Wilk test. Two-tailed Student's *t*-test was used for comparison of continuous variables and Fisher's exact test for comparison of categorical variables. Unadjusted linear regression analysis was performed to assess the association between plasma sNRP1 level and UA Doppler status (normal UA blood flow *vs* absent or reversed end-diastolic flow in the UA *vs* elevated UA systolic/diastolic (S/D) ratio). The relationship between gestational age and sNRP1 level in the control group was assessed using Pearson correlation. A *P*-value < 0.05 was considered statistically significant. Statistical analysis was performed using STATA software version 13.1 (StataCorp., College Station, TX, USA).

RESULTS

Clinical characteristics of the mothers in the control and study groups are summarized in Table 1. The matched case–control design of the study is evident from the similar maternal age and gestational age at assessment between the two groups. While tobacco use was significantly more frequent in the FGR group compared with controls, there was no difference in the prevalence of hypertensive disorders between the two groups. The amniotic fluid index was significantly lower in the FGR group compared with controls (9.2 ± 3.7 cm *vs* 11.9 ± 2.5 cm; $P = 0.011$). As expected, the estimated fetal weight (1751 ± 708 g *vs* 2419 ± 907 g; $P = 0.013$) and estimated-fetal-weight percentile (7.4 ± 5.5 *vs* 59.9 ± 17.3; $P < 0.001$) were significantly lower in the FGR group compared with the control group (Table 1). Moreover, the UA S/D ratio percentile was significantly higher in the FGR population (72.3 ± 22.2 *vs* 51.2 ± 18.7; $P = 0.004$).

The neonatal outcome data are presented in Table 2. As expected, FGR pregnancies were delivered at an earlier gestational age (35.4 ± 4.7 weeks *vs* 39.2 ± 1.7 weeks; $P = 0.005$) and had lower birth weight (1830 ± 727 g *vs* 3172 ± 558 g; $P < 0.001$) compared with control pregnancies. Although our study was not powered for these outcomes, no differences in 5-min Apgar score or umbilical cord blood gas parameters were observed between the two groups. Additionally, no significant correlation between gestational age and sNRP1 level in the control group was observed ($r = 0.14$; $P = 0.57$),

Table 1 Clinical and ultrasound characteristics, and concentration of maternal plasma soluble neuropilin-1 (sNRP1), in 20 pregnancies with fetal growth restriction (FGR) and 20 matched uncomplicated controls

Characteristic	*FGR (n = 20)*	*Controls (n = 20)*	P*
Maternal age (years)	25.8 ± 5.9	25.3 ± 5.4	0.781
Parity	1 (0–2)	0 (0–3)	0.567
Pregestational BMI (kg/m^2)	30.7 ± 6.8	30.8 ± 8.2	0.966
Tobacco use			0.025
Never	9 (45)	16 (80)	
Current	8 (40)	1 (5)	
Former	3 (15)	3 (15)	
Chronic hypertension	3 (15)	0 (0)	0.231
Hypertensive disorder			0.695
GH	0 (0)	1 (5)	
PE	1 (5)	0 (0)	
PE with severe features	2 (10)	1 (5)	
GA at scan (weeks)	34.5 ± 4.5	34.4 ± 4.3	0.919
EFW (g)	1751 ± 708	2419 ± 907	0.013
EFW percentile	7.4 ± 5.5	59.9 ± 17.3	< 0.001
UA Doppler			
Normal	12 (60)	20 (100)	
Elevated S/D ratio	4 (20)	0 (0)	
A/R EDF	4 (20)	0 (0)	
UA S/D ratio percentile	72.3 ± 22.2	51.2 ± 18.7	0.004
AFI (cm)	9.2 ± 3.7	11.9 ± 2.5	0.011
sNRP1 (pg/mL)	137.4 ± 44.8	166.7 ± 36.9	0.030

Data are presented as mean ± SD, median (interquartile range) or *n* (%). *Student's *t*-test or Fisher's exact test was used, as appropriate. A/R, absent or reversed; AFI, amniotic fluid index; BMI, body mass index; EDF, end-diastolic flow; EFW, estimated fetal weight; GA, gestational age; GH, gestational hypertension; PE, pre-eclampsia; S/D ratio, systolic/diastolic ratio; UA, umbilical artery.

Table 2 Neonatal characteristics of 20 pregnancies with fetal growth restriction (FGR) and 20 matched uncomplicated controls

Characteristic	*FGR (n = 20)*	*Controls (n = 20)*	P*
GA at delivery (weeks)	35.4 ± 4.7	39.2 ± 1.7	0.005
Male sex	8 (40)	11 (55)	0.527
Birth weight (g)	1830 ± 727	3172 ± 558	< 0.001
5-min Apgar score < 7	3 (15)	0 (0)	0.230
UA pH	7.26 ± 0.05	7.27 ± 0.06	0.553
UA base excess (mEq/L)	5.3 ± 2.2	6.1 ± 3.3	0.424
UV pH	7.30 ± 0.07	7.34 ± 0.07	0.151
UV base excess (mEq/L)	5.17 ± 2.6	4.52 ± 3.3	0.537

Data presented as mean ± SD or *n* (%). *Student's *t*-test or Fisher's exact test was used, as appropriate. GA, gestational age; UA, umbilical artery; UV, umbilical vein.

though this finding should be interpreted with caution, as this study was not powered to assess the effect of gestational age on sNRP1 concentration.

Regarding the primary outcome, maternal plasma sNRP1 levels were significantly lower in FGR pregnancies compared to those with normal fetal growth (137.4 ± 44.8 pg/mL *vs* 166.7 ± 36.9 pg/mL; $P = 0.03$). Figure 1 illustrates the relationship between plasma sNRP1 concentration and UA blood flow impedance as reflected by UA Doppler status. Compared with the control group, maternal plasma sNRP1 concentration was significantly downregulated in FGR pregnancies with elevated UA S/D ratio ($P = 0.007$) and those with absent or reversed end-diastolic flow in the UA ($P = 0.001$). However, there was no significant difference between the control group and the FGR group that had normal UA Doppler ($P = 0.618$). Moreover, plasma sNRP1 concentration was significantly downregulated in the FGR group with elevated UA S/D ratio ($P = 0.023$) and that with UA absent or reversed end-diastolic flow ($P = 0.005$) compared with the FGR group that had normal UA Doppler. This finding suggests that the FGR fetuses with normal UA Doppler represented SGA and not true FGR.

Given the significant difference in the rate of tobacco use between cases and controls, with a greater number of current or former tobacco users in the FGR group (Table 1; $P = 0.025$), we compared sNRP1 levels within each group based on smoking status and found no difference in either group.

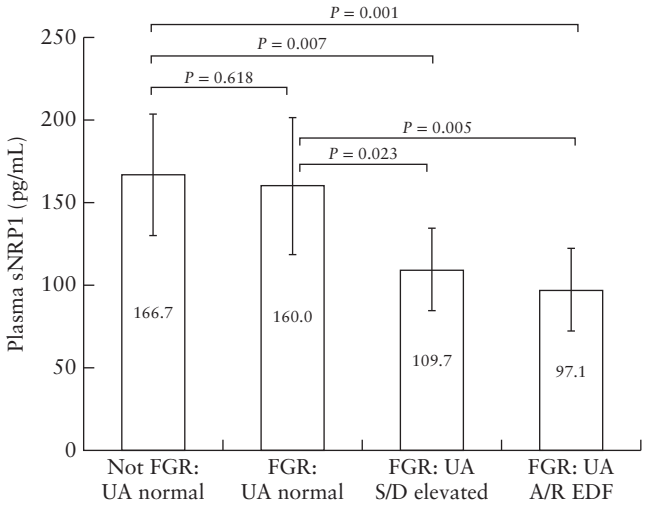

Figure 1 Maternal plasma soluble neuropilin-1 (sNRP1) concentration according to umbilical artery (UA) Doppler status, in 20 pregnancies with normal fetal growth and normal UA Doppler (controls), 12 pregnancies with fetal growth restriction (FGR) and normal UA Doppler, four pregnancies with FGR and elevated UA systolic/diastolic (S/D) ratio and four pregnancies with FGR and UA absent or reversed (A/R) end-diastolic flow (EDF). The bars represent mean sNRP1 values and the whiskers are 95% CI of the mean. Compared with the control group, maternal plasma sNRP1 concentration was significantly downregulated in FGR pregnancies with elevated UA S/D ratio or with A/R EDF in the UA. There was no significant difference in plasma sNRP1 concentration between the control group and FGR pregnancies with normal UA Doppler.

DISCUSSION

Main findings

In this study, we found that sNRP1 is present in maternal plasma and is significantly downregulated in pregnancies with true FGR, characterized by estimated fetal weight $< 10^{th}$ percentile and Doppler evidence of fetoplacental circulatory compromise. The abnormal Doppler UA flow patterns included elevated S/D ratio, absent end-diastolic flow and reversed end-diastolic flow, reflecting progressive deterioration of fetoplacental perfusion. These observations are original and expand upon our previous work that reported downregulation of placental NRP1 mRNA and protein expression in FGR pregnancies complicated by absent or reversed end-diastolic flow in the UA[23]. However, our findings are contrary to our study hypothesis, which anticipated upregulation of maternal plasma sNRP1 in pregnancies with FGR, since it is an antagonist of NRP1. It is therefore evident that the molecular mechanisms of NRP1 and sNRP1 in the pathophysiology of FGR require further elucidation. Our preliminary study provides plausibility that maternal plasma sNRP1 may be mechanistically involved in fetal growth compromise and may have a role as a biomarker for differentiating constitutional SGA from true FGR.

NRP1, sNRP1 and angiogenesis

Highly conserved in vertebrates, the role of NRP1 in axonal guidance and angiogenesis has been investigated extensively in various developmental processes and disease states[31–35]. Its structural organization includes an extracellular, a transmembrane and an intracellular region. The b1 and b2 domains of the extracellular region act as ligands for VEGF and Semaphorin 3 (SEMA3) and are essential for angiogenesis[25]. The process involves the transformation of dormant endothelial cells into tip cells in response to an angiogenic signal[26]. The adjacent endothelial cells preferentially become stalk cells and proliferate to form the stalk of the vessel branch. NRP1 along with VEGF and VEGF receptor-2 (VEGFR-2) promote tip cell formation, whereas the Notch ligands delta-like canonical Notch ligand 4 (DLL4) and Jagged1 inhibit tip cells, and their dynamic balance regulates the tip and stalk cell differentiation. There is evidence that NRP1 may modulate decidual vascular development during implantation[36]. Expression of NRP1 protein has been demonstrated in human decidual samples throughout pregnancy, with higher expression in early than in late pregnancy[37].

sNRP1 molecules, expressing either the complete extracellular domain or parts of it, have been identified in humans, primates and mice[38–40]. Endogenous isoforms of sNRP1 act as antiangiogenic agents by binding and sequestering VEGF165[27,41]. Thus, our finding of downregulation of sNRP1 in FGR pregnancies requires further experimental elucidation. Interestingly, it has been shown in a murine model that a dimer of sNRP1 can bind

with VEGF165, phosphorylate VEGFR-2 in endothelial cells and promote sprouting angiogenesis[42].

Maternal sNRP1 as a potential biomarker for FGR

Our findings raise the possibility of using maternal plasma sNRP1 assay as a biomarker for distinguishing constitutional SGA fetuses from those who are growth restricted. Although UA Doppler can identify true FGR fetuses with hemodynamic compromise, it is limited in making that differentiation early. There are significant challenges in developing a diagnostically useful biomarker for FGR. A systematic review and meta-analysis of 53 studies, involving almost 40 000 singleton pregnancies, evaluated the predictive accuracy of 37 biomarkers for FGR and found that none of them had a high enough predictive accuracy for clinical application[43]. Although angiogenic markers showed significant promise, their predictive accuracy was low. A recent review on the challenges of developing an accurate biomarker for FGR has highlighted the need for a systematic approach to future biomarker research[12]. Given that maternal plasma sNRP1 levels correlate with the severity of uteroplacental insufficiency, as evidenced by UA Doppler investigations in this study, sNRP1 may represent a unique biomarker for FGR that warrants further exploration.

Strengths and limitations

Our study has several strengths. First, it was a prospective case–control study with a phenotypically well-defined population comprised of biometrically small fetuses, further categorized by the presence or absence of placental circulatory insufficiency. Our finding of downregulation of sNRP1 in the former group and not in the latter provides molecular evidence supporting the Delphi consensus definition of FGR as proposed by Gordijn *et al.*[44] and adopted in the recent guidelines of the International Society of Ultrasound in Obstetrics and Gynecology[9]. Second, the cases and the controls were matched individually for both maternal age and gestational age in order to minimize confounding. Third, to the best of our knowledge, this is the first study to report the presence of sNRP1 in maternal circulation. This is significant, as NRP1 is known to be involved in promoting branching angiogenesis and to be downregulated in FGR placentae with clinical evidence of increased fetoplacental flow impedance. Our findings, however, do not establish a causal relationship between sNRP1 and FGR.

We recognize several limitations of our study. First, although we followed the ACOG guideline for defining FGR as ultrasound-estimated fetal weight $< 10^{th}$ percentile, which remains the prevailing standard of clinical practice in the USA[2], the inadequacy of this definition has been recognized and is discussed in the previous section. Second, as this was an exploratory study, it was not powered to detect modest differences between groups (e.g. control *vs* FGR with normal UA Doppler) and to perform subgroup analyses. Third, our population consisted of patients attending a single university-based tertiary perinatal center, which may limit the generalizability of the findings and may have introduced selection bias. Fourth, this study was not designed to, and therefore could not, address other pertinent issues, such as the effect of gestational age, other obstetric or medical complications, and racial diversity on maternal sNRP1 levels.

Conclusions

We demonstrated a significant decrease in maternal plasma sNRP1 concentration in FGR pregnancies with compromised fetoplacental circulation. These findings suggest that sNRP1 along with NRP1 may be involved in modulating fetal growth. Furthermore, sNRP1 in maternal circulation may have the potential to differentiate constitutional SGA fetuses from those who suffer from true FGR. Realization of this potential, however, will require future systematic investigation of the clinical efficacy of maternal plasma sNRP1 measurements at different gestational ages in large and diverse populations. Future mechanistic studies should also address the biological role of NRP1 in modulating fetal placental vascular development in normal pregnancies and those with compromised fetal growth.

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