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an Odense Child Cohort study

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Prediction of birth weight small for gestational age with and without preeclampsia by angiogenic markers: An Odense Child Cohort study

Short title: Angiogenic markers and SGA

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Abstract

Objective: To investigate the predictive performance of placental growth factor (PlGF) and soluble FMS-like kinase 1 (sFlt-1) on birth weight and small for gestational age (SGA), in a large, population-based cohort.

Methods: Women enrolled in the population-based, prospective Odense Child Cohort Study with early (GA<20 weeks) and/or late (≥20 weeks) pregnancy blood samples (n=1937) were included. The association between log-transformed values of the biomarkers and birth weight Z-score was studied using multivariate regression models. The prediction of SGA overall, and in women developing preeclampsia, by biomarkers was evaluated using receiver operating characteristic analyses.

Results: No substantial associations between early pregnancy biomarkers and SGA were seen. PlGF measured in late pregnancy demonstrated the strongest association with birth weight Z-score (adjusted β-coefficient=0.43 [95%CI=0.35; 0.50]). The area under curve (AUC) for predicting SGA was higher for sFlt-1/PlGF compared to sFlt-1 (0.74 vs. 0.63, p=0.006) and reached excellent prediction for SGA after preeclampsia (AUC 0.94). Optimal sFlt-1/PlGF ratio cut-offs had higher negative predictive value (NPV) and positive predictive value (PPV) for SGA (cut-off>5.0; NPV=99.1%, PPV=5.4%) compared to each marker individually.

Conclusion: The sFlt-1/PlGF ratio is a potential predictor of SGA in population-based screening, particularly in pregnancies with preeclampsia.

What is new: The sFlt-1/PlGF ratio is a potential predictor of SGA on a population level with higher predictive values compared to sFlt-1 or PlGF, but with low positive predictive value when used alone.

Keywords: preeclampsia; fetal growth restriction; angiogenesis; pregnancy; preterm birth

Introduction

A major cause of morbidity and mortality in children worldwide is being born with a birth weight small for gestational age (SGA) (1-3). SGA is especially frequent in children who are born preterm and/or to women with preeclampsia (4-7).

Infants born as SGA either result from intrauterine growth restriction (IUGR), or from low genetic growth potential, indicated by a growth trajectory which differs from the statistically expected (8, 9). Infants born both SGA and preterm often have pathological growth restriction and suffer higher complication rates in early life (10).

SGA is usually defined by population-based standard deviations. However, a customized redefinition of SGA adjusted for maternal height and weight, parity, fetal gender and gestational age may more accurately identify infants at risk of stillbirth and perinatal death (11-14). Infants identified as SGA by this redefinition were more than twice as likely to be born to a mother with preeclampsia (15).

A common origin of IUGR, SGA and preeclampsia is thought to be abnormal formation of maternal spiral arteries, due to insufficient placental development and growth (16). Formation of blood vessels, or angiogenesis, in the placenta is in part controlled by placental growth factor (PlGF), a marker of angiogenesis. PlGF potentiates the effects of vascular endothelial growth factor (VEGF) and is significantly affected by maternal age, method of conception, gestational age, racial origin and smoking status (16, 17). The angiogenic marker sFlt-1 is a potent antagonist of PlGF and VEGF. Increased concentrations of sFlt-1 lead to reduced free VEGF and hence potentially impaired placental development (3, 18). Both these markers demonstrate altered concentrations in pregnancies complicated by preeclampsia, IUGR and SGA birth weight (3, 16, 19-22).

Preeclampsia is a condition with high risk of preterm delivery (15-67%) and SGA (10-25%) (4). Novel biomarkers, particularly the sFlt-1/PlGF ratio, have been found useful in

83 ruling out or predicting especially severe and/or early-onset preeclampsia, emphasizing their
84 properties as screening tools (3, 19-26).

85 Studies on PlGF and sFlt-1 concentrations in normotensive pregnant women
86 delivering SGA infants have yielded conflicting results (3, 16-18, 27, 28), and research on the
87 predictive capacities of the sFlt-1/PlGF ratio in relation to SGA is sparse and even more
88 contradicting (29-32). Optimizing pregnancy-screening programmes is crucial, in order to
89 identify pregnancies at risk of SGA.

90 We aimed to investigate associations between sFlt-1 and PlGF concentrations in early
91 and late pregnancy with birth weight standard deviation score (Z-score) or SGA-status in a
92 large, population-based prospective cohort, with subanalyses for women with or without
93 development of preeclampsia or preterm birth.

Methods

Study population

Participating women were included from the Odense Child Cohort (OCC). This is a prospective, population-based cohort of pregnant women with residence in the Municipality of Odense, Denmark, which were recruited between January 1st 2010 and December 31st 2012 as described in detail previously (33). The recruitment base consisted of 6707 pregnant women, of whom 2874 gave consent for participation and were enrolled in the OCC, **Figure 1.**

Inclusion criteria for the present study were a minimum of one blood sample analyzed for PlGF and sFlt-1, and available data on GA at blood sample time, gestational length and offspring sex and birth weight. Exclusion criteria were lack of the above information, and in addition twin pregnancy, chronic illness of the mother (e.g. diabetes, cancer, psychiatric disorders) and stillbirth.

Detailed information about cohort participants was collected using questionnaires, electronic medical records and the Municipality of Odense database. From questionnaires data were extracted on maternal level of education (classified as high school or less, high school plus 1-3 years, or high school plus 4 years or more), assisted conception (incl. *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI), classified as yes/no), alcohol consumption during pregnancy (yes/no) and gestational weight gain. From patient electronic medical records information was collected on maternal age and height, pre-gestational weight, smoking during pregnancy, preeclampsia, spontaneous or assisted delivery, parity, gestational length, and offspring sex and birth weight. The diagnoses of preeclampsia and gestational hypertension were validated retrospectively by a read-through of the electronic patient files of all included women, as described previously by our group (34). Briefly, a woman was classified as having gestational hypertension, if she had two or more episodes of

new-onset hypertension defined as >140 mmHg systolic and/or >90 mmHg diastolic, with at least four hours in between, after GA 20+0 weeks, or significant aggravation of pre-existing hypertension. Preeclampsia was defined as gestational hypertension or pre-existing hypertension with new-onset proteinuria defined as >0.3 gram of urinary protein excretion in 24 hours, or at least +1 on sterile urine dipstick after gestational week 20+0.

The Municipality of Odense database provided ethnicity data (Danish, Western or non-Western origin).

Blood sample analysis

Pregnant women were invited to donate blood samples for angiogenic marker determination in both early and late pregnancy. Venous blood samples were collected, and isolated serum after centrifugation was stored at -20° C or lower.

BRAHMS KRYPTOR assays were used to analyze the concentrations of sFlt-1 and PlGF in the blood samples as described previously (23), using the fully automated KRYPTOR compact PLUS system (KRYPTOR PlGF and KRYPTOR sFlt-1; Thermo Fisher Scientific). The assays had a detection range of 3.6–7000 pg/mL and 22–90000 pg/mL, respectively.

Because of a noteworthy overlap in GA at “early” and “late” blood sample time, the blood sample groups where redefined as early blood sample group (GA<20+0 weeks) and late blood sample group (GA ≥20+0 weeks), hereafter described as early and late pregnancy samples. If both samples from a woman were defined as early or late according to the redefinition, the redefined sample was excluded (<20 weeks; n=0, ≥20 weeks; n=2).

Outcomes

GA was routinely calculated by ultrasound-determined due date. SGA was defined as birth weight Z-score <-2, based on the routinely used Scandinavian healthy fetal weight reference (35). Preterm birth was defined as birth<37 weeks +0 days.

Statistical analysis

Statistical analysis was carried out using STATA 13.1 (StataCorp, College Station, TX).

Normally distributed data were described with means and standard deviations (SD), non-normally distributed data with medians and interquartile range (IQR), and binary data with amount and percent distribution. Two-sided t-test was used to test for differences in means between groups for normally distributed data and Wilcoxon Mann-Whitney test for non-normally distributed data. Likelihood-ratio chi-squared test was used to test for differences in distribution of the categorical characteristics. A two-sided p-value <0.05 was considered significant, p-values 0.05-0.10 were considered trends.

Drop-out analyses were performed to test whether women providing blood samples differed from cohort participants not providing blood sample with respect to age, pre-pregnancy body mass index (BMI), parity, smoking, preeclampsia and gestational length.

In association analyses, PlGF, sFlt-1 and the sFlt-1/PlGF ratio were chosen as exposures, and the primary endpoint was birth weight Z-score. The secondary endpoint was SGA as a binary outcome, with outcome stratification for preeclampsia (preeclamptic SGA/non-preeclamptic SGA) and preterm birth (preterm SGA/term SGA). Multivariate regression models were adjusted for maternal covariates (maternal age, pre-gestational BMI, smoking, parity, IVF/ICSI pregnancy, ethnicity, level of education and alcohol consumption), if these showed significant associations in crude analysis to the exposures, or changed the regression coefficient by more than 10%.

Separate analyses were performed for each exposure at early or late pregnancy. Additionally, each model was substratified for preeclampsia and for preterm/term delivery.

To test the predictive values of the angiogenic markers, area under the curve (AUC) was calculated from receiver operating characteristic (ROC) curves. Cut-points resulting in the highest possible sensitivity and specificity at the same time were chosen to calculate positive and negative predictive values (PPV and NPV) for the total and stratified populations. The diagnostic performance was classified as fail (AUC 0.50-0.60), poor (AUC 0.60-0.70), fair (AUC 0.70-0.80), good (AUC 0.80-0.90) or excellent (AUC>0.90).

Ethics

All included women gave informed consent for participation. The study was carried out according to the 2nd Helsinki Declaration and approved by the Regional Scientific Ethical Committee for Southern Denmark (no. S–20090130). Furthermore, our study was approved by the Danish Data Protection Agency.

Results

The study included 1937 women. Of these, 1433 women had an early blood sample (median 12, IQR 4.6 weeks) and 1491 women had a late blood sample (median 28.9, IQR 1 weeks); 987 women participated with blood samples in both early and late pregnancy.

Maternal characteristics are shown in **S1 in Supplemental materials**. Preterm birth occurred in 76 women (3.9%), preeclampsia in 138 women (7.1%) and 44 neonates (2.3%) were classified as SGA. As only three neonates were preterm and SGA, analyses on the preterm SGA group were not performed. Women developing preeclampsia and women with preterm delivery were more likely to be nulliparous (73.2% vs. 55%, p=0.001 and 76.3% vs. 55.5%, p=0.006) and to undergo caesarean section (34.1% vs. 21.3%, p=0.001 and 40.8% vs.

21.5%, p<0.001). Women developing preeclampsia had significantly higher pre-pregnancy BMI 25.7 vs. 23.3, p<0.0001).

Drop-out analyses showed that participants not providing a blood sample were more likely to be smokers (7.4 vs. 4.3%, p=0.003) and had a trend towards higher parity status (2.9 vs. 1.7%, p=0.051). No difference was seen in the proportions of preeclampsia, gestational length, maternal BMI or age.

In early pregnancy, median sFlt-1, PlGF and sFlt-1/PlGF ratio values were 1044.5 pg/mL, 31.4 pg/mL and 32.0, respectively, see **S2 in Supplemental materials** for table of early pregnancy outcomes. No differences were seen for women developing preeclampsia or delivering preterm.

Late pregnancy sFlt-1 and sFlt-1/PlGF-ratio were significantly higher and PlGF was significantly lower in women developing preeclampsia vs. no preeclampsia. Similar differences were seen for preterm vs. term birth, **S3 in Supplemental materials**.

Mean birth weight Z-score was significantly lower for the infants born to women developing preeclampsia vs. no preeclampsia and for infants born preterm vs. at term. Women developing preeclampsia were significantly more likely to have an SGA neonate, and had significantly shorter gestational length, but no increased prevalence of preterm birth compared to non-preeclamptic women.

In multivariate regression models there was a lack of pronounced correlation between angiogenic marker concentrations and outcomes, and only sporadically significant results in early pregnancy, and due to this, no further analyses were carried out (data not shown).

In the multivariate regression models for biomarker concentrations in late pregnancy, log sFlt-1 and the log sFlt-1/PlGF ratio were negatively associated to birth weight Z-score (β-coefficient -0.11 (95%CI: -0.21; -0.021) and -0.079 (-0.11; -0.048), respectively), while log PlGF was positively associated to birth weight Z-score (β 0.40 (0.33; 0.48), **Table 1**. After

adjustment for maternal age, pre-gestational BMI, smoking and parity, only the associations between birth weight Z-score and PlGF (β 0.43 (0.35; 0.50) and sFlt-1/PlGF (β -0.06 (-0.095; -0.34) remained significant.

For women developing preeclampsia, crude and adjusted associations for sFlt-1, PlGF and sFlt-1/PlGF-ratio were further pronounced (β -0.65 (-0.98; -0.32), 0.83 (0.59; 1.08) and -0.19 (-030; -0.074) vs. no-preeclampsia β 0.099 (-0.0011; 0.20), 0.38 (0.30; 0.46) and -0.046 (-0.078; -0.014), respectively). For women delivering preterm, only PlGF and sFlt-1/PlGF were further pronounced (β 0.60 (0.31; 0.89) and -0.23 (-0.38; -0.078) vs. term β 0.41 (0.33; 0.49) and -0.052 (-0.083; -0.021)).

To evaluate the performance of late pregnancy angiogenic markers in predicting SGA, ROC curve AUCs were calculated. The AUCs for SGA were 0.63 (sFlt-1), 0.74 (PlGF) and 0.75 (sFlt-1/PlGF), expressing fair predictive performance of PlGF and sFlt-1/PlGF. The stratified analysis showed good to excellent performance in predicting preeclamptic SGA (AUC 0.82 (sFlt-1), 0.93 (PlGF) and 0.94 (sFlt-1/PlGF)), **Table 2**. For overall SGA, non-preeclamptic SGA and term SGA, the diagnostic performance was significantly higher for sFlt-1/PlGF ratio compared to sFlt-1 ($p \leq 0.02$), while PlGF and sFlt-1/PlGF performed similarly; the same trend was seen for preeclamptic SGA ($p=0.05$), see **S4 in Supplemental materials** to view all AUCs with confidence intervals and p-values for difference.

We chose cut-off values which had high sensitivity and specificity simultaneously in predicting SGA as follows: Late pregnancy sFlt-1 >1277 pg/mL; PlGF <244 pg/mL and sFlt-1/PlGF ratio >5.0. These cut-offs, however, had poor to moderate predictability for SGA. Yet, given an SGA prevalence of 2.2%, the NPV was excellent; PPV and NPV for sFlt-1 were 3.5% and 98.5%; PlGF 4.6% and 98.8%; sFlt-1/PlGF 5.4% and 99.1%.

Optimal cut-offs in predicting preeclampsia simultaneously occurring with SGA showed excellent performance, especially PlGF and sFlt-1/PlGF. While PlGF cut-off <154

mg/mL had the highest specificity (89.1%), an sFlt-1/PlGF cut-off >8 had the highest sensitivity (100%). The PPVs were low, but the NPV of all three predictors were between 99.8-100%.

Discussion

In this large population-based cohort study, PlGF concentrations measured in late pregnancy (GA ≥ 20 weeks) were highly correlated to birth weight Z-score, with even stronger correlations to birth weight in women with preeclampsia or preterm birth. The ratio of sFlt-1/PlGF measured at the same GA was excellent in predicting absence of SGA (high NPV) in the whole population, however the prediction of SGA was poor (low PPV) with a selected cut-off of >5.0. The predictive capacities were even stronger for women with preeclampsia and SGA infants. The performance of sFlt-1 and PlGF as individual biomarkers was inferior to the sFlt-1/PlGF ratio with lower PPVs and NPVs in ROC analyses.

In agreement with previous research (3, 16, 28, 32, 36, 37) we showed that PlGF concentrations were positively associated to birth weight Z-score, while sFlt-1 concentrations and the sFlt-1/PlGF-ratio were negatively associated to the same outcome. The strongest correlation between angiogenic markers and birth weight were seen in women with preeclampsia or preterm delivery. Similarly, in the predictive models, the angiogenic markers had the highest NPVs when predicting preeclamptic SGA. The improved prediction of preeclamptic SGA suggested a higher proportion of pathological SGA in this group as opposed to non-preeclamptic SGA (10, 15). We speculate that lower sFlt-1/PLGF ratio and higher PlGF values, or a healthier, pro-angiogenic profile, may be more common in constitutional SGA. Further studies of possible differential expression of angiogenic markers in pathological as opposed to constitutional SGA would be valuable. The predictive performance of angiogenic markers on SGA applying customized models, which show better

detection rates of pathological SGA(15), would also be highly interesting in regard to population-based screening.

Other large screening studies have evaluated combined screening in the absence of preeclampsia in the second and third trimester (38-41), incorporating maternal characteristics, biophysical and biochemical markers. While PlGF contributed significantly to the combined screening and sFlt-1 did not, the performance of sFlt-1/PlGF ratio was not evaluated (39, 40). We found that the NPV of sFlt-1/PlGF ratio was superior compared to PlGF, when predicting overall SGA and preeclamptic SGA. Future research should investigate the performance of the sFlt-1/PlGF ratio in a similar combined screening model.

The predictive potential of sFlt-1/PlGF for preeclampsia has already been evaluated by AUCs in a similar dataset from the Odense Child Cohort (42). However in the current study, the sFlt-1/PlGF showed notably larger AUCs, when predicting preeclamptic SGA.

Another study has also investigated the predictive properties of the sFlt-1/PlGF ratio for preeclampsia (21). Although the study found good AUCs when predicting preeclampsia, they were inferior compared to our study's AUCs for predicting preeclamptic SGA, in spite of the former study only including women with suspected preeclampsia (21). This indicates a further potential for using angiogenic markers in predicting the more severe cases of preeclampsia with fetal growth restriction.

Strengths of our study included the population cohort design with detailed background information allowing for adjusted analyses; the relatively large sample size, the use of birth weight Z-score to correct for sex and gestational length, the high precision in ultrasound-based GA, the validation of the preeclampsia diagnosis, and the use of a novel assay for sFlt-1 and PlGF analysis. This study was restricted by a small number of cases, but did not fail to prove a substantial correlation, giving reason to believe that a large cohort with a higher fraction of SGA could find an even more pronounced correlation.

Limitations included the use of self-reported data for some items, the differences between participants and drop-outs, and the low numbers with preterm SGA disallowing analyses for this subgroup. In addition, the cohort population was somewhat selected (33). Selection bias could have lowered the fraction of SGA infants both in the cohort in general and in this study.

In conclusion, late pregnancy PlGF correlated to birth weight Z-score in this population-based cohort. The sFlt-1/PlGF ratio had the highest PPV and NPV in predicting SGA. More pronounced associations were seen in preeclamptic pregnancies with SGA. Both angiogenic markers have potential in screening on population level for constitutional, but especially for pathological SGA, preferably in a combined screening method including other parameters.

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