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Angiopoietin 1 and 2 serum concentrations in first trimester of pregnancy as biomarkers of adverse pregnancy outcomes

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Condensation: Ang-2 and the Ang-1/Ang2 ratio is associated with several adverse pregnancy outcomes but do not predict complications any better than maternal risk factors

Short title: Ang-1 and Ang-2 as biomarkers of adverse pregnancy outcomes

ABSTRACT

Objective: To assess Ang-1, Ang-2 and the Ang-1/Ang-2 ratio levels in the first trimester of pregnancy, their association with adverse pregnancy outcomes; and their predictive accuracy.

Study Design: This cohort study measured serum Ang-1 and Ang-2 levels in 4,785 women with singleton pregnancies attending first trimester screening in New South Wales, Australia. Multivariate logistic regression models were used to assess the association and predictive accuracy of serum biomarkers with subsequent adverse pregnancy outcomes (small for gestational age, preterm birth, preeclampsia, miscarriage >10 weeks and stillbirth).

Results: Median (interquartile range) levels for Ang-1, Ang-2 and the Ang-1/Ang-2 ratio for the total population were 19.6 ng/ml (13.6-26.4), 15.5 ng/ml (10.3-22.7) and 1.21 (0.83-1.73), respectively. Maternal age, weight, country of birth and socio-economic status significantly affected Ang-1, Ang-2 and the Ang-1/Ang-2 ratio levels. After adjusting for maternal and clinical risk factors, women with low Ang-2 levels ($<10^{th}$ centile) and high Ang-1/Ang-2 ratio ($>90^{th}$ centile) had increased risk of developing most adverse pregnancy outcomes. Compared to the Ang-1/Ang-2 ratio alone, maternal and clinical risk factors had better predictive accuracy for most adverse pregnancy outcomes. The exception was miscarriage [Ang-1/Ang-2 ratio area under ROC curve (AUC) =0.70; maternal risk factors AUC =0.58]. Overall, adding the Ang-1/Ang-2 ratio to maternal risk factors did not improve the ability of the models to predict adverse pregnancy outcomes.

Conclusions: Our findings suggest that the Ang-1/Ang-2 ratio in first trimester is associated with most adverse pregnancy outcomes, but do not predict outcomes any better than clinical and maternal risk factor information.

Keywords: Angiopoietins, Ang-1 Ang-2, first trimester, serum levels, adverse pregnancy outcomes

INTRODUCTION

Neovascularisation, or new vessel formation, is essential for placental growth throughout gestation and is driven by changes in the balance between pro- and anti-angiogenic factors present in the extracellular milieu.[1] Angiopoietin 1 (Ang-1) and angiopoietin 2 (Ang-2) are angiogenic factors that play a critical role in the development of the placental vascular system. While Ang-1 helps capillary maturation and maintains vessel integrity, Ang-2 antagonises Ang-1 and destabilizes vessels. In the presence of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) or placental growth factor (PlGF), this destabilisation results in vessel sprouting and enhanced angiogenesis.[2] More than just vessel growth, signalling from angiopoietins is a significant stimulus for trophoblast growth and remodelling during placentation. Thus, the interplay between Ang-1, Ang-2, and other angiogenic factors (such as VEGF) controls placental growth and tissue neovascularisation during pregnancy.[3] It is not therefore surprising that the amount of circulating Ang-1 and Ang-2 shifts from a dominance of Ang-1 to Ang-2 during gestation reflecting the requirement for new vessel formation.[4]

Impaired placental vascular development related to imbalances in angiogenic factors are implicated in pathological pregnancies. As such, Ang-1 and Ang-2 are potential biomarkers for adverse pregnancy outcomes as they indicate the progression of placental growth and maternal vascular health during gestation.[4] Circulating levels of Ang-1 and Ang-2 have previously been associated with poor pregnancy outcome. We have reported that women whose fetuses develop intrauterine growth restriction (IUGR) have lower serum levels of Ang-2 in first trimester, suggesting impaired placental angiogenesis may be pathogenic in the disease.[5] Others have reported lower serum Ang-1 and Ang-2 in women that had an abortion or an ectopic pregnancy, with promising predictive results.[6] Preeclampsia has been associated

with both low second trimester levels of Ang-2[7] and low ratio of Ang-1/Ang-2.[8] However, these studies included a small number of cases and did not report results for other adverse pregnancy outcomes. There are no large population-based studies assessing the association of Ang-1 and Ang-2 in early pregnancy and risk of subsequent pregnancy outcomes. It is also unknown whether these angiogenic biomarkers provide any additional value to usual maternal and clinical information identifying pregnancies at risk. If women at risk of developing adverse pregnancy outcomes can be identified early in pregnancy this would allow ample time for monitoring and implementing potential preventive strategies.

The aims of this study were three-fold; i) to evaluate serum levels of serum Ang-1, Ang-2 concentrations and Ang-1/Ang-2 ratio in first trimester of pregnancy. ii) to assess the association between maternal serum Ang-1, Ang-2 concentrations and the Ang-1/Ang-2 ratio and risk of adverse pregnancy outcomes; and iii) to determine their accuracy in predicting adverse pregnancy outcomes.

MATERIALS AND METHODS

Study population and sample testing

This cohort study was conducted on women attending first trimester Down syndrome screening between July 2006 and June 2007 in New South Wales (NSW), Australia. Serum samples were collected by the Pacific Laboratory Medicine Services (PaLMs), and then archived and stored at -80°C. During this period, this was the state's only public screening service and received samples from throughout NSW.

Serum samples for this study were thawed and serum levels of Ang-1 and Ang-2 were measured by a semi-automated ELISA immunoassay (R & D Systems, Minneapolis, MN,

USA). Intra-assay and inter-assay coefficient of variation were <9.5% and the reported analytic sensitivity of the immunoassay was 0.06 – 84.3 ng/ml for Ang-1 and 0.05 – 108.9 ng/ml for Ang-2.

Data sources

Maternal information for archived serum samples was derived from the laboratory database and corresponding pregnancy and birth outcomes were ascertained via record linkage to the Perinatal Data Collection (PDC) and Admitted Patient Data Collection (APDC). The PDC is a statutory surveillance system of all births in NSW of at least 400 grams birth weight, or at least 20 weeks' gestation and includes demographic, medical and obstetric information on the mother, labour, delivery and birth outcome. The APDC is a census of all patient hospital admissions from NSW public and private hospitals, with records for both mothers and liveborn infants. It includes demographic, clinical and health services information for each admission and relevant diagnoses and procedures are recorded for each hospital admission. These are coded according to the International Classification of Diseases version 10 – Australian Modification (ICD10-AM) and Australian Classification of Healthcare Interventions, respectively. Validation studies of the PDC and the APDC show excellent level of agreement with the medical record and low rates of missing data.[9, 10] Reporting in both datasets have high specificity (> 99%) indicating few false positive reports. Only factors and outcomes accurately reported in birth or hospital data were included in analyses.[11] The NSW Centre for Health Record Linkage conducted the record linkage and identifying information was removed prior to the release of data for analysis. The CHeReL assesses the linkage quality for each study, and for this study reported <5/1000 missed links and <2/1000 false positive links. The study was approved by the NSW Population and Health Services Research Ethics Committee.

Study outcomes and explanatory factors assessed included: small for gestational age (SGA), preterm birth, preeclampsia, gestational diabetes, miscarriage and stillbirth. SGA was defined as birthweight less than the 10th centile and less than the 3rd centile (severe SGA) of the distribution for gestational age and infant sex.[12] Gestational age is reported in the birth data in completed weeks of gestation and determined by the best clinical estimate including early ultrasound (>97%) and last menstrual period. Preterm birth was defined as delivery at less than 37 weeks and very preterm birth less than 34 weeks gestation. Information on preeclampsia was obtained from both the APDC and PDC data, to maximize ascertainment.[13, 14] Preeclampsia (regardless of severity) was determined either by the box being checked in the PDC record, or if any APDC record had a diagnosis in any of the 55 fields of gestational hypertension (ICD10-AM: O13 and O16), preeclampsia (O11 and O14) or eclampsia (O15).[14, 15] Early onset preeclampsia was defined as women with preeclampsia requiring delivery at ≤34 weeks gestation. Miscarriage was defined as a spontaneous pregnancy loss between 10-20 weeks gestation and identified from APDC data, while stillbirth was defined as a spontaneous pregnancy loss after 20 weeks gestation and was identified from PDC data. To replicate an earlier study of ours, we defined a proxy measure of IUGR using combined criteria of SGA<10th centile and preterm birth <37weeks.

The key explanatory variables were Ang-1, Ang-2 and the Ang-1/Ang-2 ratio levels and covariates used in this analysis included maternal age and weight (kilograms) ascertained at the time of first trimester screening, parity (nulliparous/multiparous), smoking during pregnancy, previous diagnosed hypertension, previous miscarriage, country of birth and socio-economic disadvantage quintile. Socio-economic disadvantage was defined according to the Socio-Economic Indexes for Areas (SEIFA) relative disadvantage scores developed by the Australian Bureau of Statistics (ABS).[16] Maternal weight was missing in 831 (16.3%) of

the records. Multiple imputation was used to account for the missing maternal weight, a method that predicts missing values using existing values from other variables.[17] Other missing data were uncommon and were excluded from the analyses: smoking was missing in 55 (1.2%) and country of birth in 3 (0.1%) of the records.

Statistical analysis

Comparison of Ang-1, Ang-2 and the Ang-1/Ang-2 ratio by maternal characteristics for women with and without each clinical outcome was performed using contingency tables and student's t-test analysis for categorical and normaly distributed variables, respectively. Spearman coefficient was used to determine the correlation between Ang-1 and Ang-2. To account for differences in Ang-1 and Ang-2 values attributable to gestational week of the test, maternal age and weight, we standardized Ang-1 and Ang-2 levels using Multiple of the Medians (MoM). Kruskall-Wallis test was used to compare concentrations of Ang-1, Ang-2 and the Ang-1/Ang-2 ratio between women that subsequently had adverse pregnancy outcomes with women with unaffected pregnancies. If MoM levels were either decreased or elevated, they were dichotomized by the $<10^{th}$ or the $>90^{th}$ centile, respectively. Multivariate logistic regression was then used to assess the association between serum biomarkers with adverse pregnancy outcomes, taking into account maternal and clinical risk factors. A backward elimination method retaining only significant explanatory variables was used to fit models for each outcome.

Predictive accuracy was assessed examining the area under the Receiver Operating Characteristics (ROC) curves (AUC), derived from logistic regression analysis and using log transformed levels to achieve Gaussian distribution. AUC results were examined to determine whether models performed better than chance (0.5). Models for serum biomarkers

alone, those including maternal and clinical risk factors only and with serum biomarkers and risk factors combined were compared. This approach was applied to assess whether serum levels of Ang-1 and Ang-2 provided any additional information to maternal and clinical risk factors in predicting severe adverse pregnancy outcomes by evaluating the maximum likelihood estimates using the likelihood ratio (X^2) test. Finally, estimates of predictive accuracy at a fixed 5% false positive rate were calculated including sensitivity, specificity, positive (PPV), negative predictive values (NPV) and positive likelihood ratio (LR) with exact binominal confidence intervals. A P-value of <0.05 was considered statistically significant and analyses performed using SAS software 9.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

A total of 5,183 samples were tested with health information relevant to the pregnancy available for 4,785 (92.3%) samples. We excluded 164 women whose blood sample was taken before 10 or after 14 weeks gestation; had a medical abortion, had a twin pregnancy or had an infant with a major congenital anomaly. Ang-1 and Ang-2 were undetectable in 111 and 52 samples, respectively, and these women were assigned a value equal to half the detection limit. A total of 4,621 women were included in the analysis.

Figure 1 presents a scatter plot between Ang-1 and Ang-2 serum levels, illustrating a positive correlation between Ang-1 and Ang-2 (r =0.26, P<0.001). Table 1 presents the median (IQR) serum levels of Ang-1, Ang-2 and the Ang-1/Ang-2 ratio by maternal characteristics. The mean (SD) maternal age and weight were 32.9 (4.7) years and 66.6 (14.3) kilograms, respectively. Almost half (n=2,108; 46.4%) of women were nulliparous, 279 (6.1%) smoked during pregnancy and 187 (4.1%) had previous diagnosed hypertension. The median serum

levels of Ang-1, Ang-2 and the Ang-1/Ang-2 ratio for total population were 19.7 ng/ml (IQR: 13.9 - 26.7), 16.3 ng/ml (IQR: 10.7 - 23.9) and 1.2 (IQR: 0.8 - 1.7), respectively. Serum Ang-1, Ang-2 levels and the Ang-1/Ang-2 ratio showed significant variation by maternal weight, country of birth and socio-economic disadvantage (Table 1), and there was a positive correlation between maternal weight and Ang-1/Ang-2 ratio ($r = 0.07$, $P < 0.001$). While Ang-1 and the Ang-1/Ang-2 ratio levels decreased with gestational week at sampling, Ang-2 levels did not change. There was also an increase in serum Ang-1 and Ang-2 with maternal age (Table 1).

The median (IQR) serum levels, both raw and MoM adjusted for Ang-1, Ang-2 and the Ang-1/Ang-2 ratio in pregnancies affected by adverse outcomes are presented in Table 2. Compared with unaffected pregnancies (1.02; IQR: 0.69 – 1.48), median serum levels of Ang-2 MoM were decreased for SGA <10th centile (0.90; IQR: 0.60 – 1.26), SGA <3rd centile (0.90; IQR: 0.61 – 1.24), preterm birth <37 weeks (0.89; IQR: 0.62 – 1.34), preeclampsia (0.92; IQR: 0.60 – 1.36) and miscarriage (0.59; IQR: 0.31 – 1.18). The Ang-1/Ang-2 ratio was also increased for SGA <10th centile, SGA <3rd centile, preterm birth <37 weeks, preterm birth <34 weeks, preeclampsia, early onset preeclampsia and miscarriage due mostly to the decreased Ang-2 levels. There was no difference in Ang-1 levels between women with unaffected pregnancies with women who had adverse pregnancy outcomes (Table2).

The association of low Ang-2 MoM (<10th centile) and high Ang-1/Ang-2 ratio MoM (>90th centile) with adverse pregnancy outcomes is presented in Table 3. Ang-2 <10th centile (Ang-2 <0.44 MoM) was associated with 40% increased risk of SGA<10th centile and preterm birth (<37 weeks) and a threefold increased risk of miscarriage. The Ang-1/Ang-2 ratio MoM

>90th centile (ratio >1.73 MoM) was associated with a near twofold increased risk of SGA, preterm birth, preeclampsia and an almost sevenfold increased risk of miscarriage. When our proxy of IUGR was considered (N=30), the adjusted odds ratios were 4.01 (95% confidence interval (CI) 1.76, 9.13) and 4.24 (95%CI 1.98, 9.10) for Ang-2 <10th centile and the Ang-1/Ang-2 ratio MoM >90th centile, respectively.

Figure 2 present the accuracy for the Ang-1/Ang-2 ratio in predicting adverse pregnancy outcomes. The AUC for models of the Ang-1/Ang-2 ratio alone models revealed a negligible discriminative ability in predicting most of the adverse pregnancy outcomes (AUC<0.6), compared with maternal risk factors (best accuracy for early-onset preeclampsia: AUC=0.71; LR=6.17). Adding information on Ang-1/Ang-2 ratio to maternal risk factors did not improve the ability of the models to predict adverse pregnancy outcomes, except in the case of miscarriage (Ang-1/Ang-2 AUC=0.70; P>0.001; LR=5.89). Sensitivity was 27.8% for miscarriage but less than 10% for the rest of the outcomes. The NPV was over 93% for all pregnancy outcomes, but particularly high for severe cases (>97%), indicating that women that do not have high Ang-1/Ang-2 ratio in early pregnancy were unlikely to have subsequent SGA<3rd infant, early-onset preeclampsia or miscarriage.

COMMENT

We have conducted the largest population-based study to examine maternal Ang-1 and Ang-2 levels of women in first trimester, and to assess the association with adverse pregnancy outcomes. Our study highlights that Ang-1 and Ang-2 are positively correlated and there is significant variation in Ang-1 and Ang-2 levels, and the Ang-1/Ang-2 ratio by maternal characteristics and week of sampling. Women that developed adverse pregnancy outcomes had lower serum levels of Ang-2 and higher levels of the Ang-1/Ang-2 ratios, compared with

women with unaffected pregnancies. Results suggest that the interaction of both biomarkers, proposed as a ratio, strengthens the association with adverse pregnancy outcomes compared with Ang-2 alone. However, with the exception of miscarriage, the accuracy of the Ang-1/Ang-2 ratio in predicting most adverse pregnancy outcomes was poor and did not add predictive information to routinely collected maternal and clinical risk factors.

Previous studies have evaluated the association of first trimester serum Ang-1 and/or Ang-2 levels with adverse pregnancy outcomes and their characteristics are summarized in Table 4. Three studies reported Ang-1 levels ranging from 0.96 and 27.8ng/ml in unaffected women.[6-8] Nine studies assessed Ang-2 levels and reported to vary widely (median Ang-2 ranging between 1.4 and 33.2 ng/ml).[5-8, 18] Although all studies utilized the same assay for analysis (R&D systems), differences in population characteristics and sampling time may account for variations in serum levels of Ang-2. Studies assessing the association of serum Ang-1 and Ang-2 levels with subsequent adverse pregnancy outcomes have reported conflicting results. Reduced serum Ang-2 at 10 -13 weeks was found among women that gave birth to an infant diagnosed with intra uterine growth restriction (IUGR) [5] and low Ang-1 and Ang-2 levels were found at 6-8 weeks among women with subsequent missed abortion/ectopic pregnancy.[6] In contrast, three studies reported no association between Ang-2 levels with preeclampsia,[7, 8, 18] while two of them found that women with preeclampsia had higher serum Ang-2 and lower Ang-1/Ang-2 ratio in second trimester, compared to unaffected women.[7, 8] These results represent a converse association compared with ours and other studies that assessed serum Ang-2 at term.[19, 20] Although, these studies may be limited to small sample size, inclusion of Caucasian women only and exclusion women with pre-existing disease, the precise explanation for these inconsistent results remains unknown.

The exact mechanism of how Ang-2 is regulated in unaffected versus preeclamptic pregnancies is still unidentified perhaps due to the multi factorial origin of preeclampsia. Our recent data indicated that while the placenta is a key source of Ang-2 during uncomplicated pregnancies it is the decidua that is likely to be the primary source.[21] Release of Ang-2 from the decidua is from both active secretion and new synthesis of Ang-2 protein.[21] We also noted an effect of gestational age on the levels of Ang-2 produced by decidual cells with serum from first trimester inducing greater release than that from third trimester. Thus, circulating factors present in early pregnancy, and declining throughout gestation, appear to control Ang-2 release. Normal placental development occurs in a hypoxic environment,[22] stimulating angiogenesis through the transcriptional activator hypoxia-inducible factor-1α (HIF-1α). Two of the most prominent HIF-1α targets are the pro-angiogenic factor genes such as VEGF and Ang-2.[23] Thus, this might explain the reported up-regulation of Ang-2 early in pregnancy and why it decreases after first trimester.[21] However, preeclampsia is associated with chronic hypoxia, either intermittent or persistent,[24] yet Ang-2 mRNA levels are reduced in preeclamptic placentas.[4] Thus, the raised Ang-1/Ang-2 ratio observed in preeclampsia here may reflect additional aspects of the disease, such as inflammation, which are known to be regulated by Ang-2 levels. Moreover, if the association of Ang-1/Ang-2 ratio with inflammation was stronger in these diseases then that may explain why elevated ratios are associated with pregnancy outcomes that are known to have inflammation as part of their pathogenesis, such as preterm birth, preeclampsia and miscarriage.[25-27]

Our results suggest that the Ang-1/Ang-2 ratio may be a promising biomarker for miscarriage. Especially as women with recurrent miscarriage have reduced blood vessel expression of Ang-2 related to premature advanced vessel maturation.[28] To date, one case-control study has also reported a strong association and good predictive accuracy of Ang-1

and Ang-2 for missed abortion and ectopic pregnancy.[6] However, these were measured at 6-8 weeks gestation. Our results for miscarriage must be interpreted with caution as miscarriage is under ascertained. First, women had to reach 10 weeks to be eligible for screening and second, only miscarriages resulting in admission to hospital can be identified. Further research exploring and replicating this association is warranted.

Strengths of the study were the evaluation of a large sample from population-based cohort of pregnant women attending first trimester screening. Ascertainment and follow-up of pregnancy outcomes was possible for 92% of the samples using record linkage of laboratory to birth and hospital data with only minimal missing information. Missing health and pregnancy information was mostly attributable to women giving birth in hospitals out of state. Yet, women with missing information had similar characteristics compared with those included in the study.

One of the limitations of the study was maternal weight missing in 16% of the women, which was addressed by applying multiple imputation, a technique shown to be robust and valid for dealing with missing data.[17] We did not have access to BMI information which has been found to have a significant interaction with angiogenic gene polymorphisms when predicting pre-term birth.[29, 30] BMI information may have allowed us to refine our models to increase the predictive accuracy of angiopoietins. Another limitation of the study was the differences between our cohort compared with the state total maternity population during the same period, which may be due to a healthier and more affluent population. We tackled this limitation by weighting our analyses to ensure generalizability of the results.

In conclusion, our findings suggest that the Ang-1/Ang-2 ratio in first trimester is associated with most adverse pregnancy outcomes, but do not predict outcomes any better than clinical

and maternal risk factor information. Our results indicate a potential role of the Ang-1/Ang-2 ratio in first trimester as a predictive marker for subsequent miscarriage, but this requires further investigation.

Contributors

NN, CA, JM and CR conceived the study. NN, CA, JM and CR obtained the funding. NN, VT, JM and CR acquired the data. CG preformed the laboratory analysis. FS and NN conducted the statistical analysis. FS and NN drafted the manuscript which was approved by all authors. All authors critically reviewed the manuscript for important intellectual content. FS takes responsibility for the integrity of the data and the accuracy of the data analyses.

Conflict of Interest Disclosures

The authors report no conflict of interest.

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Table 1: First trimester An-1, Ang-2 and the Ang-1/Ang-2 ratio serum levels by maternal characteristics

Maternal characteristics	n (%)	Ang-1 (ng/ml) Median (IQR)	Ang-2 (ng/ml) Median (IQR)	Ang-1/Ang-2 ratio Median (IQR)
Total	4621	19.6 (13.6, 26.4)	15.5 (10.3, 22.7)	1.21 (0.83, 1.73)
Matenal age				
<25	267 (5.8)	18.9 (12.7, 24.4)	13.9 (10.4, 21)	1.27 (0.81, 1.65)
25 - 29	892 (19.3)	19.2 (12.8, 25.7)	14.9 (9.2, 22.7)	1.20 (0.85, 1.73)
30 - 34	1862 (40.3)	19.9 (13.9, 27.2)	16.0 (10.6, 23.4)	1.21 (0.82, 1.76)
35 - 39	1368 (29.6)	20.9 (14.9, 28.2)	16.8 (11.4, 24.4)	1.20 (0.83, 1.73)
40+	232 (5.0)	20.1 (15.1, 27.3)	16.8 (10.7, 25.6)	1.11 (0.79, 1.74)
		P=0.001[1]	P=0.001	P=0.8
Parity				
Nulliparous	2108 (46.4)	19.4 (14.0, 26.2)	15.9 (10.6, 23.3)	1.17 (0.80, 1.64)
Parous	2436 (53.6)	19.8 (13.0, 26.6)	15.2 (10.3, 22.5)	1.24 (0.84, 1.75)
		P=0.6	P=0.6	P=0.02
Smoking during pregnancy				
Yes	279 (6.1)	19.7 (13.0, 24.9)	14.3 (10.3, 21.3)	1.28 (0.88, 1.63)
No	4287 (93.9)	19.6 (13.6, 26.8)	15.7 (10.4, 23.0)	1.20 (0.82, 1.73)
		P=0.1	P=0.04	P=0.3
Maternal weight (kg)				
<55	658 (17.3)	19.7 (14.1, 26.1)	19.1 (13.2, 27.1)	0.98 (0.67, 1.32)
55 - 64	846 (22.3)	18.6 (13.0, 25.3)	17.2 (11.4, 24.5)	1.08 (0.72, 1.55)
65 - 74	736 (19.4)	19.1 (12.2, 25.0)	15.8 (9.9, 22.8)	1.16 (0.78, 1.63)
75 - 84	790 (20.8)	18.8 (13.5, 24.5)	14.0 (10.3, 22.0)	1.24 (0.85, 1.75)
85+	766 (20.2)	21.7 (14.5, 29.1)	13.0 (8.8, 18.4)	1.60 (1.10, 2.32)
		P<.001	P<.001	P<.001
Gestational week at sampling				
10	520 (11.3)	22.7 (16.2, 29.5)	14.3 (9.9, 22.1)	1.47 (1.00, 2.21)
11	1651 (35.7)	20.3 (14.1, 26.9)	15.8 (10.3, 22.9)	1.21 (0.88, 1.73)
12	1772 (38.3)	19.1 (13.0, 25.8)	15.6 (10.3, 23.0)	1.19 (0.79, 1.66)

13	678 (14.7)	18.5 (13.3, 24.7)	15.7 (10.4, 22.2)	1.17 (0.81, 1.61)
		P<.001	P=0.2	P<.001
Country of birth				
Australia & New Zealand	3032 (67.0)	19.7 (13.4, 26.2)	15.0 (10.1, 22.2)	1.27 (0.87, 1.76)
Pacific Islands	45 (1.0)	19.5 (10.5, 26.7)	17.4 (11.6, 19.8)	1.35 (0.69, 2.50)
Europe, NA & SA	530 (11.7)	20.5 (14.0, 26.7)	16.9 (11.8, 25.4)	1.07 (0.75, 1.52)
Middle east	96 (2.1)	18.7 (12.8, 24.7)	15.9 (10.9, 21.9)	1.06 (0.72, 1.60)
South east Asia	254 (5.6)	22.1 (15.1, 28.5)	18.2 (11.3, 25.7)	1.17 (0.82, 1.74)
China, Hong Kong & Taiwan	216 (4.8)	18.9 (14.0, 26.7)	19.5 (13.6, 28.8)	0.97 (0.64, 1.38)
Japan and Koreas	126 (2.8)	19.2 (13.9, 24.9)	16.8 (13.0, 26.2)	1.00 (0.68, 1.33)
India & surroundings	154 (3.4)	19.0 (12.2, 28.7)	12.8 (7.3, 21.6)	1.26 (0.80, 2.01)
Central and South America	51 (1.1)	19.5 (16.6, 26.8)	12.7 (10.9, 23.2)	1.38 (0.89, 1.70)
Africa and Caribbean	22 (0.5)	14.1 (10.7, 22.5)	12.6 (6.4, 20.3)	1.20 (0.85, 1.39)
		P=0.01	P<.001	P<.001
SEIFA Socio-economic disadvantage quintile				
1 (most disadvantage)	365 (7.9)	20.9 (14.8, 29.1)	15.5 (10.8, 22.2)	1.28 (0.89, 1.79)
2	580 (12.6)	19.9 (13.5, 26.0)	14.6 (9.8, 21.9)	1.28 (0.86, 1.78)
3	779 (16.9)	18.7 (13.2, 25.0)	14.9 (10.1, 22.5)	1.19 (0.79, 1.75)
4	669 (14.5)	17.5 (12.2, 24.7)	14.8 (9.5, 21.4)	1.16 (0.83, 1.64)
5 (least disadvantage)	2205 (48.0)	20.0 (14.1, 26.7)	17.0 (11.1, 24.5)	1.13 (0.79, 1.61)
		P<.001	P<.001	P<.001

[1] P-values for Kruskall-Wallis test; NA: North America; SA: South Africa; SEIFA: Socio-Economic Indexes for Areas

Table 2. Demographic characteristics and serum levels of Ang-1 and Ang-2 of the study population by pregnancy outcome

Maternal characteristics	Median Ang-1 ng/ml (IQR)	Median Ang-1 MoM (IQR)	Median Ang-2 ng/ml (IQR)	Median Ang-2 MoM (IQR)	Median Ang-1/Ang-2 ratio (IQR)	Median Ang-1/Ang-2 ratio MoM (IQR)
Unaffected (n=3730)	19.6 (13.8, 26.4)	0.98 (0.69, 1.32)	16.6 (11.0, 24.4)	1.02 (0.69, 1.48)	1.14 (0.79, 1.62)	0.93 (0.65, 1.30)
SGA<10th centile (n=445)	18.7 (13.7, 25.8)	0.94 (0.71, 1.30)	14.8 (10.0, 22.3)*	0.90 (0.60, 1.26)*	1.25 (0.84, 1.75)	1.05 (0.74, 1.50)*
SGA<3rd centile (n=109)	17.7 (13.5, 26.3)	0.91 (0.71, 1.30)	14.1 (10.8, 21.7)	0.90 (0.61, 1.24)*	1.19 (0.77, 1.77)	1.09 (0.70, 1.54)*
Preterm birth <37 weeks (n=310)	19.5 (13.8, 29.1)	0.97 (0.70, 1.41)	14.3 (9.4, 22.4)*	0.89 (0.62, 1.34)*	1.37 (0.89, 1.97)*	1.06 (0.72, 1.51)*
Preterm birth <34 weeks (n=84)	18.3 (15.2, 25.1)	0.93 (0.76, 1.33)	14.4 (9.9, 21.1)	0.90 (0.65, 1.21)	1.38 (0.90, 1.97)*	1.07 (0.73, 1.53)
All preeclampsia (n=163)	20.8 (15.4, 28.3)	1.05 (0.80, 1.39)	14.7 (9.2, 20.2)*	0.92 (0.60, 1.36)	1.44 (0.98, 1.96)*	1.12 (0.75, 1.65)*
Early-onset preeclampsia (n=14)	18.1 (16.1, 24.8)	0.87 (0.80, 1.32)	14.6 (7.8, 17.8)	0.81 (0.50, 1.36)	1.73 (1.20, 2.47)*	1.42 (0.82, 1.86)
Miscarriage (n=39)	18.3 (15.2, 27.3)	0.97 (0.81, 1.45)	9.9 (5.4, 19.7)**	0.59 (0.31, 1.18)*	1.85 (0.97, 3.25)*	1.56 (0.78, 2.55)*
Stillbirth (n=23)	19.0 (15.1, 26.6)	0.93 (0.77, 1.37)	17.2 (10.9, 28.6)	1.04 (0.71, 1.63)	1.10 (0.70, 1.48)	0.84 (0.56, 1.12)

* P<0.05; **P<0.001 for difference by using Kruskall-Wallis test; SGA: Small for gestational age; SD: Standard deviation; IQR: Interquartile range; MoM: Multiple of the media

Table 3. Logistic regression results of Ang-2 and the Ang-1/Ang-2 ratio on adverse pregnancy outcomes

Pregnancy outcome	Ang-2 (MoM) <10th centile (<0.44)			Ang-1 / Ang-2 ratio >90th centile (>1.73)		
	Affected women (n)	Univariate OR (95% CI)	Adjusted OR[1] (95% CI)	Affected women (n)	Univariate OR (95% CI)	Adjusted OR[1] (95% CI)
SGA<10th centile (n=445)	61	1.40 (1.05, 1.86)	1.41 (1.05, 1.90)	75	1.70 (1.30, 2.23)	1.82 (1.38, 2.39)
SGA<3rd centile (n=109)	11	0.96 (0.51, 1.80)	0.99 (0.53, 1.88)	19	1.70 (1.03, 2.81)	1.76 (1.05, 2.94)
Preterm birth <37 weeks (n=311)	43	1.39 (1.00, 1.94)	1.41 (1.00, 1.96)	50	1.60 (1.17, 2.19)	1.63 (1.19, 2.25)
Preterm birth <34 weeks (n=85)	11	1.27 (0.67, 2.42)	1.28 (0.68, 2.43)	15	1.76 (1.00, 3.10)	1.77 (1.00, 3.13)
All preeclampsia (n=163)	20	1.16 (0.72, 1.87)	1.22 (0.75, 1.98)	32	1.99 (1.34, 2.96)	2.09 (1.39, 3.16)
Early-onset preeclampsia (n=14)	3	2.27 (0.63, 8.01)	2.18 (0.61, 7.84)	3	2.24 (0.61, 8.14)	2.42 (0.66, 8.87)
Miscarriage (n=39)	12	3.86 (1.94, 7.70)	3.28 (1.63, 6.60)	17	6.83 (3.53, 13.23)	6.67 (3.44, 12.92)
Stillbirth (n=23)	1	NA	NA	2	0.69 (0.14, 3.32)	0.71 (0.17, 3.02)

[1] Odds ratio using multivariate logistic regression adjusted for maternal age, maternal weight, smoking during pregnancy, previous diagnosed hypertension, previous miscarriage, country of birth or socio economic disadvantage; OR: Odds ratio; SGA: Small for gestational age; SD: Standard deviation; IQR: Interquartile range; MoM: Multiple of the median; NA: not applicable

Table 4: Characteristics of studies assessing Ang-2 during pregnancy

Reference	Country	Study design	Gestational week at sampling	Study outcome	n	Median Ang-2 (ng/ml)		P- value
						Cases	Controls	
Wang, 2007[5]	Australia	Case-control	10 - 13	SGA & IUGR	IUGR=13 SGA=8 Controls=23	IUGR=14.1 SGA=29.2	26.6	<0.01 0.53
Akolekar, 2009[18]	UK	Case-control	11 - 13	Preeclampsia	Preeclampsia=35 Gestational hypertension=88 Controls=214	Preeclampsia=6.3 Gestational hypertension=6.9	6.8	NS
Bolin, 2009[8]	Sweden	Longitudinal cohort	10	Preeclampsia	Preeclampsia=19 Controls=43	13.8	10.2	NS
Leinonen, 2010[7]	Finland	Case-control	12 - 15	Preeclampsia & IUGR	Preeclampsia=49I IUGR=16 Controls=59	Preeclampsia=38.8 IUGR=30	33.2	NS
Daponte, 2012[6]	Greece	Case-control	6 - 8	Missed abortion & ectopic pregnancy	Missed abortion=30 Ectopic pregnancy=30	Missed abortion=0.4 Ectopic pregnancy=0.3	1.4	<0.001 <0.001

					Controls=33			
Schneuer, 2013	Australia	Case-cohort	10 - 14	Various adverse pregnancy outcomes	Preeclampsia=163 SGA=445 Controls=3444	Preeclampsia=14.7 SGA=14.8	16.6	<0.05

SGA: small for gestational age; IUGR: Intra uterine growth restriction; NS: Not significant

Figure 1: Scatter plot between Ang-1 and Ang-2 levels.

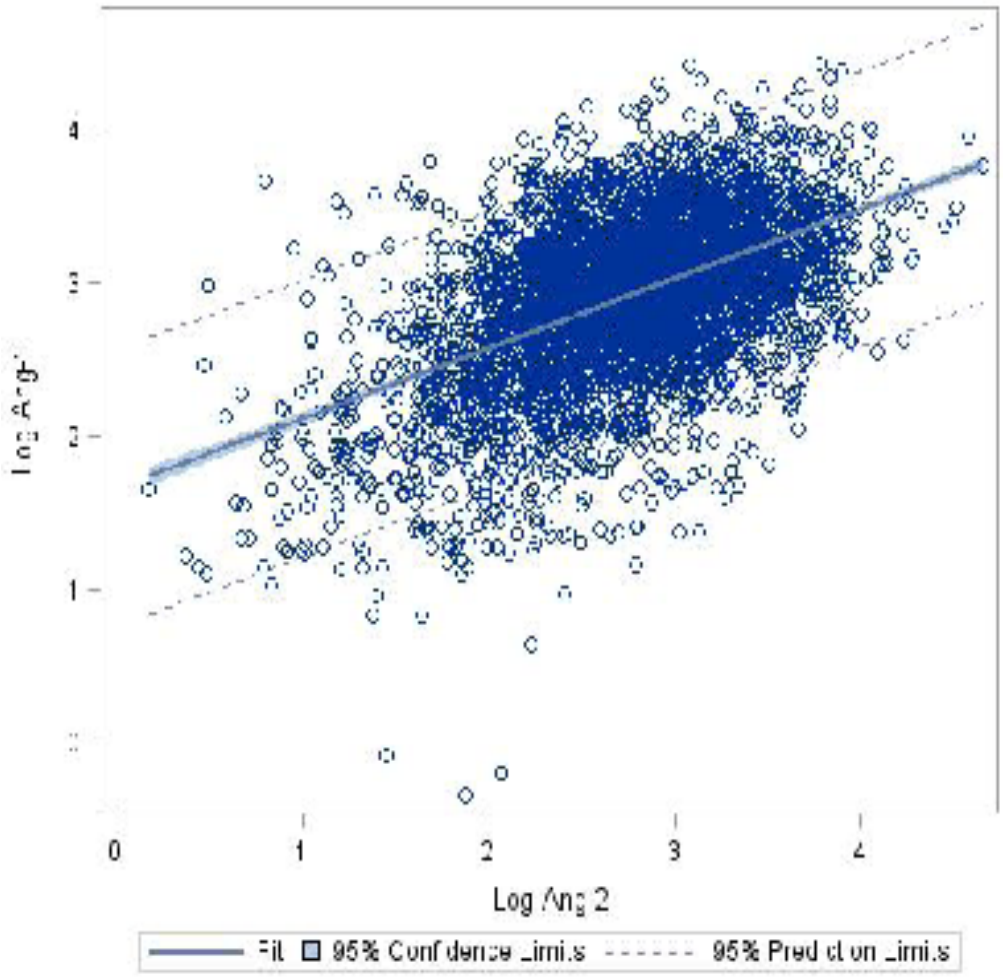

Figure 1: Circles represent each pregnant woman measurement

Figure 2: Receiver Operating Characteristic curves comparing first trimester serum Ang-1/Ang-2 ratio with maternal and clinical risk factors predicting adverse pregnancy outcomes.

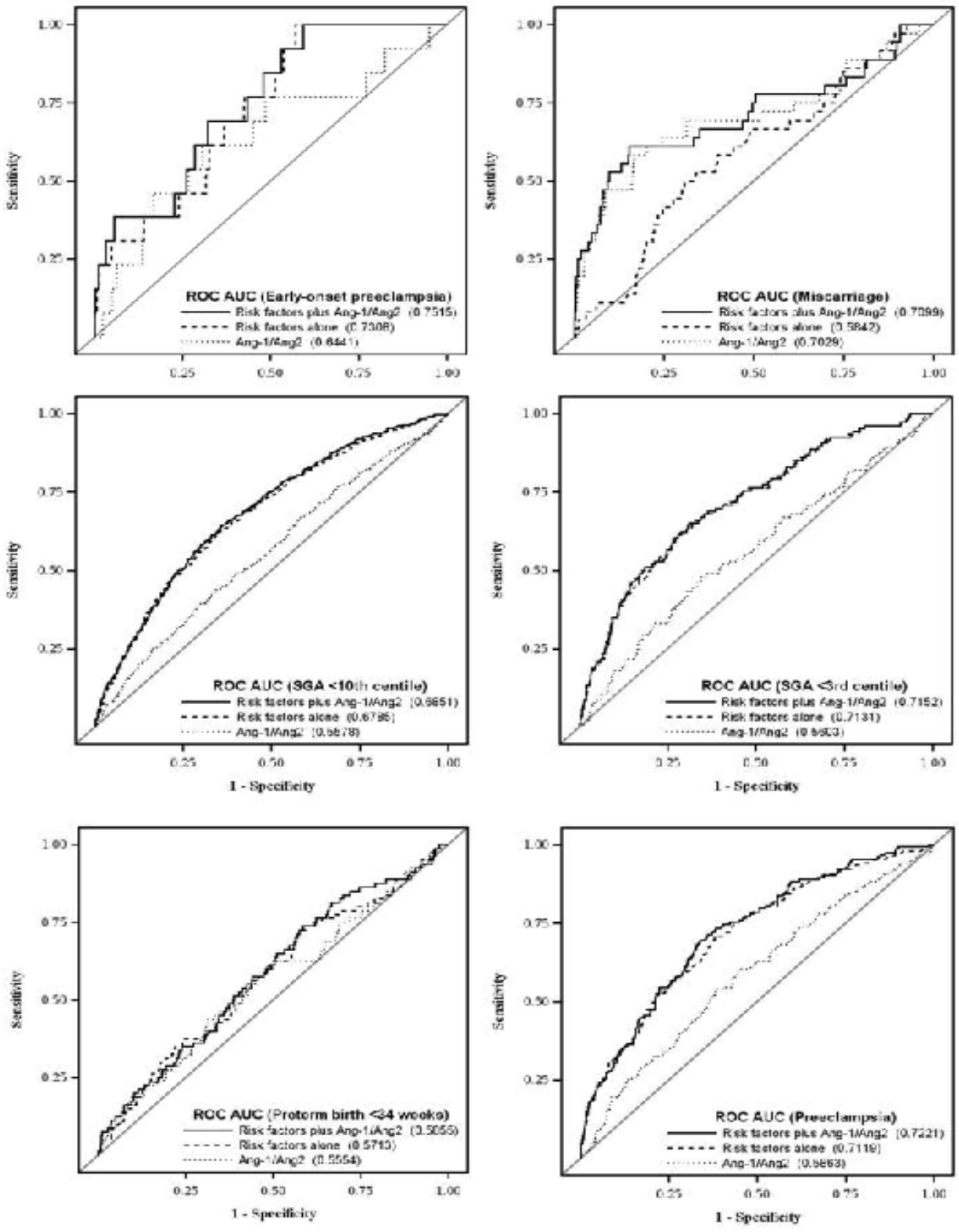

Figure 2: AUC: Area under the curve. Risk factors are: maternal age, parity, smoking during pregnancy, maternal weight, previously diagnosed hypertension, previously diagnosed diabetes, country of birth or socio-economic disadvantage